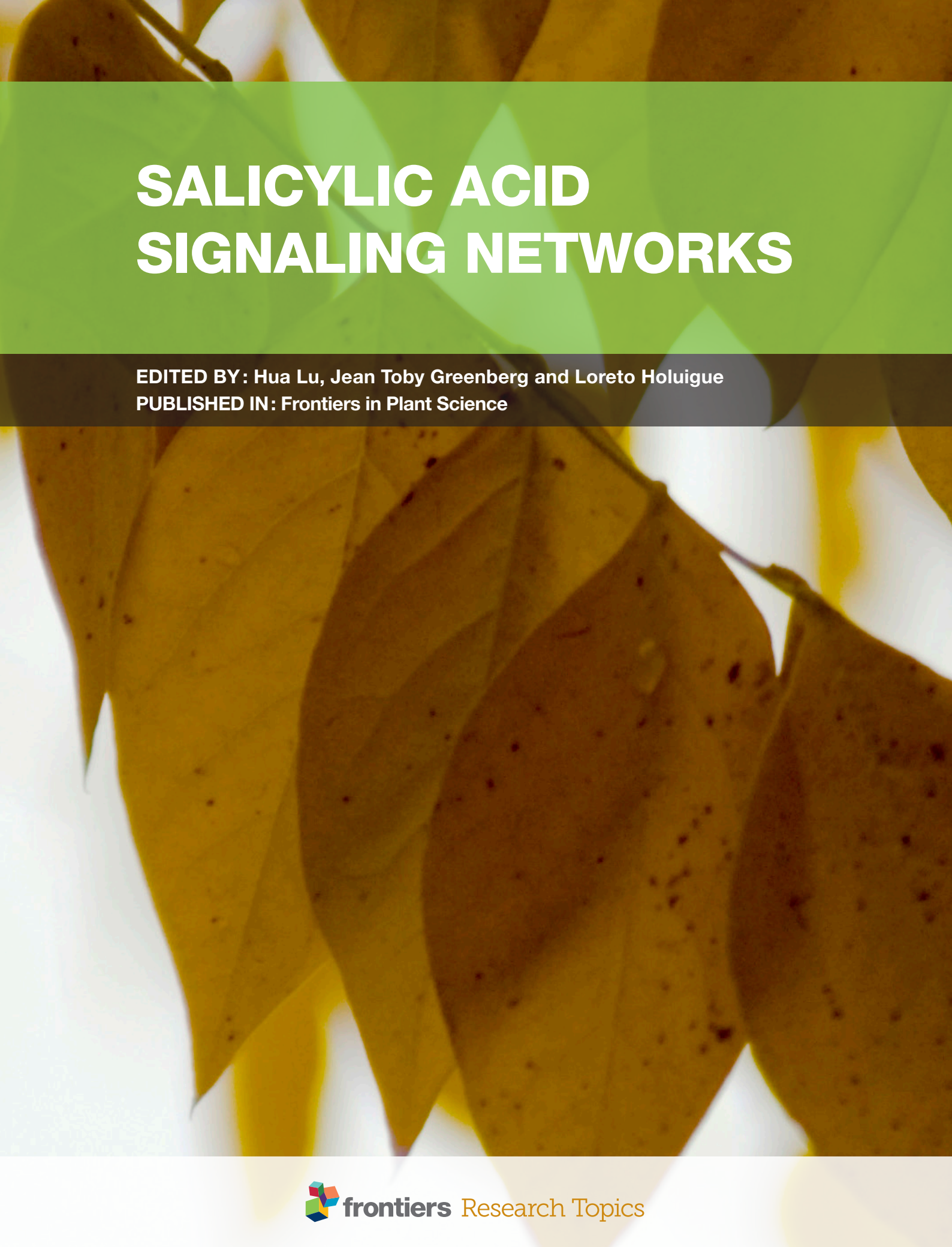


SALICYLIC ACID SIGNALING NETWORKS

EDITED BY: Hua Lu, Jean Toby Greenberg and Loreto Holuigue
PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88919-827-6
DOI 10.3389/978-2-88919-827-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

SALICYLIC ACID SIGNALING NETWORKS

Topic Editors:
Hua Lu, University of Maryland Baltimore County, USA
Jean Toby Greenberg, The University of Chicago, USA
Loreto Holuigue, Pontificia Universidad Católica de Chile, Chile

The cover photo shows an example of some tree leaves with several infection sites. Often such sites show restricted lesion size due to the production of salicylic acid, a potent defense signal induced by infection.
Image by Nicolás M. Cecchini.

The small phenolic compound salicylic acid (SA) is critical for plant defense against a broad spectrum of pathogens. SA is also involved in multi-layered defense responses, from pathogen-associated molecular pattern triggered basal defense, resistance gene-mediated defense, to systemic acquired resistance. Recent decades have witnessed tremendous progress towards our understanding of SA-mediated signaling networks. Many genes have been identified to have direct or indirect effect on SA biosynthesis or to regulate SA accumulation. Several SA receptors have been identified and characterization of these receptors has shed light on the mechanisms of SA-mediated defense signaling, which encompass chromosomal remodeling, DNA repair, epigenetics, to transcriptional reprogramming. Molecules from plant-associated microbes have been identified, which manipulate SA levels and/or SA signaling. SA does not act alone. It engages in crosstalk with other signaling pathways, such as those mediated by other phytohormones, in an agonistic or antagonistic manner, depending on hormones and pathosystems. Besides affecting plant innate immunity, SA has also been implicated in other cellular processes, such as flowering time determination, lipid metabolism, circadian clock control, and abiotic stress responses, possibly contributing to the regulation of plant development. The multifaceted function of SA makes it critically important to further identify genes involved in SA signaling networks, understand their modes of action, and delineate interactions among the components of SA signaling networks. In addition, genetic

manipulation of genes involved in SA signaling networks has also provided a promising approach to enhance disease resistance in economically important plants.

This ebook collects articles in the research topic "Salicylic Acid Signaling Networks." For this collection we solicited reviews, perspectives, and original research articles that highlight recent exciting progress on the understanding of molecular mechanisms underlying SA-mediated defense, SA-crosstalk with other pathways and how microbes impact these events.

Citation: Lu, H., Greenberg, J. T., Holuigue, L., eds. (2016). Salicylic Acid Signaling Networks. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-827-6

Table of Contents

Chapter 6. Microbial manipulation of SA production and signaling.

Review Article:

Mini Review Article:

frontiers in Plant Science

EDITORIAL
published: 24 February 2016
doi: 10.3389/fpls.2016.00238

Editorial: Salicylic Acid Signaling Networks

Hua Lu[1]*, *Jean T. Greenberg*[2] *and Loreto Holuigue*[3]

[1] Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD, USA, [2] Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL, USA, [3] Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

Keywords: crosstalk, systemic acquired resistance, flowering, circadian clock, SA receptor, reactive oxygen species, effector, NPR1

The Editorial on the Research Topic

Salicylic Acid Signaling Networks

The small phenolic compound salicylic acid (SA) is critical for plant defense against a broad spectrum of pathogens and responses to different abiotic stress conditions. Particularly in response to pathogens, SA is involved in multiple processes, including basal and resistance gene-mediated defense as well as systemic acquired resistance (SAR). This Research Topic includes a collection of 18 articles for reviews, perspectives, and original research, to highlight recent exciting progress toward our understanding of molecular mechanisms underlying SA-mediated defense and SA-crosstalk to other pathways.

Seyfferth and Tsuda summarize the regulation of SA levels, perception, and transcriptional reprogramming (Seyfferth and Tsuda). Besides SA biosynthetic enzymes, the SA levels can be affected by multiple mechanisms mediated by some non-enzyme proteins (Lu, 2009; Dempsey et al., 2011). One of such mechanisms depends on calcium signaling. The calmodulin-binding transcription factor CBP60g and its close homolog SARD1 control expression of the SA biosynthetic gene *ICS1*, highlighting a role of calcium signaling in initiating SA synthesis (Seyfferth and Tsuda).

For SA-mediated transcriptional reprogramming, NPR1 has been demonstrated as a master co-activator that interacts with bZIP transcription factors in the TGA family (Seyfferth and Tsuda; Yan and Dong, 2014). SA controls NPR1 function by regulating its protein level in the nucleus, mainly through posttranslational modifications (Mou et al., 2003; Tada et al., 2008). Furniss and Spoel review the roles of ubiquitin-mediated protein degradation and sumoylation in modulating NPR1 function (Furniss and Spoel; Saleh et al., 2015). Recently two NPR1 homologs, NPR3 and NPR4, were shown to be SA receptors that have different SA-binding affinities and target NPR1 for ubiquitin-mediated protein degradation under high and low SA conditions, respectively (Fu et al., 2012). The primary working condition for NPR1 requires intermediate SA levels. Thus, creating SA gradient in the defense zone is critical for SA signaling. Interestingly, whether or not NPR1 itself is an SA receptor has been controversial (Fu et al., 2012; Wu et al., 2012). A perspective article compares SA-binding properties of NPR1, NPR3, and NPR4 under different laboratory conditions (Kuai et al.). Such information should help to clarify the controversy and highlight the possibility of NPR1 as another SA receptor. However, questions still remain about how multiple SA receptors coordinate with each other to transduce SA perception into signaling and ultimately transcriptional reprogramming.

A localized foliar infection of plants can lead to SAR, a long lasting resistance against a broad spectrum of pathogens at the systemic level. Gao and coworkers summarize the importance of SA in establishing SAR in plants (Gao et al.). Some mutants impaired in SA accumulation

OPEN ACCESS

Edited by:
Joshua L. Heazlewood, The University of Melbourne, Australia

Reviewed by:
Vincenzo Lionetti, "Sapienza" Università di Roma, Italy

***Correspondence:**
Hua Lu
hualu@umbc.edu

Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *19 November 2015*
Accepted: *12 February 2016*
Published: *24 February 2016*

Citation:
Lu H, Greenberg JT and Holuigue L (2016) Editorial: Salicylic Acid Signaling Networks. Front. Plant Sci. 7:238. doi: 10.3389/fpls.2016.00238

and/or signaling are compromised in SAR (Gao et al.). At least one SA derivative, methyl SA has been implicated in SAR (Park et al., 2007). Some SAR-inducing molecules require SA for the establishment or manifestation of SAR. For example, the SAR molecule azelaic acid acts by priming elevated SA production upon secondary infection (Jung et al., 2009). In addition, treating plants with the SAR-related molecule diterpenoid dehydrobietinal leads to SA accumulation in the absence of pathogen infection (Chaturvedi et al., 2012).

Given the critical roles of SA in plant defense and our lack of a complete understanding of SA signaling, it is important to uncover additional genes involved in SA-mediated defense. Two mutant screens are reported in this Research Topic for this purpose (Ding et al.; Manohar et al.). To look for SA binding proteins, Monahar and coworkers used a photo-reactive SA analog 4-AzidoSA (4AzSA) in a protein microarray (Manohar et al.). To look for genes affecting SA levels, Ding and coworkers used a biosensor-based method (Ding et al.). Different from some previous screens, these two screens were conducted at a large scale with high throughput and are anticipated to discover new and uncharacterized SA-related genes besides the ones that are already known.

While clearly representing a hub in plant defense signaling networks, SA is also known to exhibit crosstalk with other signaling pathways, such as those mediated by some phytohormones and reactive oxygen species (ROS). The antagonistic and synergistic relationship between SA and the phytohormone jasmonic acid (JA) is the focus of many discussions. Caarls and colleagues review the molecular mechanisms underlying transcriptional control of JA-induced genes by SA (Caarls et al.). The crosstalk between SA and JA is also dependent on the redox status of cells controlled by the TRX/GRX oxidoreductase enzymes as discussed by Herrera-Vasquez et al. Some SA transcriptional regulators, i.e., NPR1 and TGAs, are redox sensors and can be directly or indirectly affected by some TRX/GRX oxidoreductase enzymes, highlighting the interplay between SA, JA, and redox signaling (Caarls et al.; Herrera-Vasquez et al.). The research article by Westlake and co-workers reports a redox-sensing function of two SA binding proteins, TOP1 and TOP2, further underscoring the importance of ROS in SA signaling (Westlake et al.).

The crosstalk between SA and lipids is discussed in a collection of four papers in this Research Topic. Sanchez-Rangel and coworkers review the role of sphingolipids affecting SA accumulation (Sanchez-Rangel et al.). On the other hand, the research paper by Shi and coworkers show that SA could reciprocally influence the sphingolipid profile, using *in silico* Flux Balance Analysis and experimental validation (Shi et al.). The roles of two phospholipids, phosphatidic acid (PA) and phosphatidylinositol 4-phosphate, in affecting SA-mediated defense are reviewed by Zhang and Xiao. Janda and co-workers further show that one possible mechanism of PA function in SA defense is through affecting NPR1 localization (Janda et al.).

Emerging evidence shows that there is crosstalk between SA and the circadian clock, the internal time measuring machinery of plants to ensure growth, development, and proper responses to stresses. The circadian clock controls diurnal biosynthesis of SA and SA also feedback regulates clock activity (Goodspeed et al., 2012; Zheng et al., 2015; Zhou et al., 2015). The research article by Wang and co-workers reports a possible direct regulation of the defense gene *PHT4;1* by the core clock gene *CCA1* (Wang et al.), providing a potential molecular link for clock-defense crosstalk.

Crosstalk of SA to many signaling pathways suggests that SA could affect multiple cellular processes besides its central role in controlling immunity. Two articles in this Research Topic discuss the role of SA in affecting plant development with a focus on leaf senescence and flowering time control (Banday and Nandi; Carella et al.). Carella and coworkers also report that SA and some gene components in the SA pathway contribute to age-related resistance, a form of developmentally regulated pathogen resistance of plants (Carella et al.).

Because of the key role of SA in host defense activation, it is not surprising that the SA hub is hijacked by many pathogens in order to promote pathogen virulence and induce host susceptibility (Caarls et al.; Tanaka et al.). Bacterial and fungal pathogens are known to deliver effector proteins to the host cell and affect SA metabolism, SA signaling, and SA crosstalk with the JA pathway. It is not known yet though if pathogen effectors could bind directly to SA biosynthetic enzyme(s) and/or signaling proteins to modulate their activities and subsequently lead to altered SA levels and/or signaling. Besides effector proteins, pathogens can also produce chemicals to mimic host compounds in order to interfere with host signaling pathways. For instance, coronatine (COR) produced by *Pseudomonas syringae* is structurally similar to JA-Ile (the active form of JA). COR can activate host JA pathway and subsequently suppress SA accumulation and signaling (Zheng et al., 2012). Interestingly, while pathogens can use effectors and/or chemicals to target the SA hub for their own benefit, the host can also recognize some pathogen effectors and/or chemicals and subsequently activate strong defense responses to fight against the invaders. For instance, plant recognition of a cognate avirulence effector by a resistance protein activates much stronger and faster SA and ROS accumulation and cell death, leading to enhanced disease resistance (Hamdoun et al., 2013). In addition, plants treated with quorum sensing molecules, such as N-acyl homoserine lactones, are primed for stronger and faster defense activation upon further defense challenge (Baumgardt et al., 2014; Schenk and Schikora). Such defense priming is dependent on SA, JA, and JA related metabolites.

The articles collected in this Research Topic represent our current understanding of multifaceted function of SA and the complexity of SA signaling networks. They will serve as a catalyst for further discussions and discoveries. Many exciting advances are expected to come in the near future, such as identification of new players in the SA signaling networks, elucidation of molecular mechanisms underlying the crosstalk of SA with other pathways, and discovery of pathogen effectors that directly target SA pathway genes and proteins. The central role of SA in plant defense and its crosstalk to other physiological processes make it critically important to further understand SA signaling networks.

Manipulation of genes on the SA signaling networks provides a promising way to enhance disease resistance in economically important plants.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

HL is supported by a grant from National Science Foundation (NSF 1456140), JTG is supported by NSF grants (NSF IOS 1238201 and IOS 1456904), and LH is supported by the National Commission for Science and Technology CONICYT (FONDECYT 1141202) and the Millennium Science Initiative (NC130030). We thank all contributors to this Research Topic and apologize to those whose work could not be discussed in great detail due to space limitations.

REFERENCES

Baumgardt, K., Charoenpanich, P., McIntosh, M., Schikora, A., Stein, E., Thalmann, S., et al. (2014). RNase E affects the expression of the acyl-homoserine lactone synthase gene sinI in Sinorhizobium meliloti. *J. Bacteriol.* 196, 1435–1447. doi: 10.1128/JB.01471-13

Chaturvedi, R., Venables, B., Petros, R. A., Nalam, V., Li, M., Wang, X., et al. (2012). An abietane diterpenoid is a potent activator of systemic acquired resistance. *Plant J.* 71, 161–172. doi: 10.1111/j.1365-313X.2012.04981.x

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic Acid biosynthesis and metabolism. *Arabidopsis Book* 9:e0156. doi: 10.1199/tab.0156

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Goodspeed, D., Chehab, E. W., Min-Venditti, A., Braam, J., and Covington, M. F. (2012). Arabidopsis synchronizes jasmonate-mediated defense with insect circadian behavior. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4674–4677. doi: 10.1073/pnas.1116368109

Hamdoun, S., Liu, Z., Gill, M., Yao, N., and Lu, H. (2013). Dynamics of defense responses and cell fate change during Arabidopsis-*Pseudomonas syringae* interactions. *PLoS ONE* 8:e83219. doi: 10.1371/journal.pone.0083219

Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91. doi: 10.1126/science.1170025

Lu, H. (2009). Dissection of salicylic acid-mediated defense signaling networks. *Plant Signal. Behav.* 4, 713–717. doi: 10.4161/psb.4.8.9173

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Park, S. W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Saleh, A., Withers, J., Mohan, R., Marqués, J., Gu, Y., Yan, S., et al. (2015). Posttranslational modifications of the master transcriptional regulator NPR1 enable dynamic but tight control of plant immune responses. *Cell Host Microbe* 18, 169–182. doi: 10.1016/j.chom.2015.07.005

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., and Dong, X. (2008). Plant immunity requires conformational changes of NPR1 via S-Nitrosylation and Thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Yan, S., and Dong, X. (2014). Perception of the plant immune signal salicylic acid. *Curr. Opin. Plant Biol.* 20C, 64–68. doi: 10.1016/j.pbi.2014.04.006

Zheng, X. Y., Spivey, N. W., Zeng, W., Liu, P. P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596. doi: 10.1016/j.chom.2012.04.014

Zheng, X. Y., Zhou, M., Yoo, H., Pruneda-Paz, J. L., Spivey, N. W., Kay, S. A., et al. (2015). Spatial and temporal regulation of biosynthesis of the plant immune signal salicylic acid. *Proc. Natl. Acad. Sci. U.S.A.* 112, 9166–9173. doi: 10.1073/pnas.1511182112

Zhou, M., Wang, W., Karapetyan, S., Mwimba, M., Marqués, J., Buchler, N. E., et al. (2015). Redox rhythm reinforces the circadian clock to gate immune response. *Nature* 523, 472–476. doi: 10.1038/nature14449

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 09 December 2014
doi: 10.3389/fpls.2014.00697

Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming

Carolin Seyfferth and Kenichi Tsuda*

Department of Plant Microbe Interactions, Max Planck Institute for Plant Breeding Research, Cologne, Germany

Edited by:
Hua Lu, University of Maryland at Baltimore County, USA

Reviewed by:
Murray Grant, University of Exeter, UK
Zhixiang Chen, Purdue University, USA

****Correspondence:***
Kenichi Tsuda, Department of Plant Microbe Interactions, Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, D-50829 Cologne, Germany
e-mail: tsuda@mpipz.mpg.de

The phytohormone salicylic acid (SA) is a small phenolic compound that regulates diverse physiological processes, in particular plant resistance against pathogens. Understanding SA-mediated signaling has been a major focus of plant research. Pathogen-induced SA is mainly synthesized via the isochorismate pathway in chloroplasts, with ICS1 (ISOCHORISMATE SYNTHASE 1) being a critical enzyme. Calcium signaling regulates activities of a subset of transcription factors thereby activating nuclear *ICS1* expression. The produced SA triggers extensive transcriptional reprogramming in which NPR1 (NON-EXPRESSOR of PATHOGENESIS-RELATED GENES 1) functions as the central coactivator of TGA transcription factors. Recently, two alternative but not exclusive models for SA perception mechanisms were proposed. The first model is that NPR1 homologs, NPR3 and NPR4, perceive SA thereby regulating NPR1 protein accumulation. The second model describes that NPR1 itself perceives SA, triggering an NPR1 conformational change thereby activating SA-mediated transcription. Besides the direct SA binding, NPR1 is also regulated by SA-mediated redox changes and phosphorylation. Emerging evidence show that pathogen virulence effectors target SA signaling, further strengthening the importance of SA-mediated immunity.

Keywords: calcium, ICS1, NPR1, plant immunity, salicylic acid, SA perception, transcriptional reprogramming

INTRODUCTION

The phytohormone salicylic acid (SA) is a small phenolic compound that functions as an important signaling molecule during plant immunity (Vlot et al., 2009; Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). Since constitutive SA accumulation is often associated with stunted plant growth, resulting in reduction of plant fitness (Ishihara et al., 2008; Pajerowska-Mukhtar et al., 2012; Chandran et al., 2014), SA biosynthesis and SA-mediated signaling are tightly controlled.

The plant immune system comprises multiple layers, such as pattern-triggered immunity (PTI) and effector-triggered immunity (ETI; Jones and Dangl, 2006; Tsuda and Katagiri, 2010). PTI is triggered by recognition of common microbial components (MAMPs, microbe-associated molecular patterns), such as bacterial flagellin or the fungal cell wall component chitin (Boller and Felix, 2009; Macho and Zipfel, 2014). MAMP recognition stimulates generation of reactive oxygen species, intracellular calcium influx, transient activation of mitogen-activated protein kinases (MAPKs), and the production of SA (Tsuda et al., 2008a,b; Tsuda and Katagiri, 2010). Virulent pathogens, for example, the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000), however, can suppress PTI in *Arabidopsis* and tomato by effectors, injected via bacterial secretion systems into the plant cell (Lohou et al., 2013; Xin and He, 2013). Recent studies identified various effectors that interfere with SA signaling (Uppalapati et al., 2007; Djamei et al., 2011; Caillaud et al., 2013; Jiang et al., 2013; Rabe et al., 2013; Gimenez-Ibanez et al., 2014; Liu et al., 2014), highlighting the importance of SA signaling for plant immunity. To regain resistance, plants have acquired intracellular receptors [resistance (R) proteins], which induce the second layer of defense after effector recognition, termed ETI (Eitas and Dangl, 2010; Bonardi and Dangl, 2012; Jacob et al., 2013). Activation of ETI also induces SA accumulation and MAPK activation, which are also important for resistance against pathogens during ETI (Tsuda et al., 2013). Additionally, SA has vital roles in establishing systemic acquired resistance (SAR), a form of long-term and broad-spectrum resistance throughout the entire plant after local pathogen infection (Wang et al., 2006; Fu and Dong, 2013).

In this review, we summarize SA signal transduction from regulation of biosynthesis, perception, to transcriptional reprogramming during plant immunity. We also discuss compensation mechanisms that would provide robust immunity once SA signaling is compromised, for example, by pathogen effector attack. SA signaling pathway is highly interconnected with other phytohormone signaling such as mediated by jasmonates (JA), ethylene, and abscisic acid (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Derksen et al., 2013). For example, JA and ethylene signaling negatively regulate SA biosynthesis at the transcriptional level (Chen et al., 2009; Zheng et al., 2012). However, discussions on these are beyond the scope of this review.

THE BIOSYNTHESIS OF SA IN PLANTS

BIOSYNTHETIC PATHWAYS

Two major SA biosynthetic pathways in plants were identified: the isochorismate (IC) and the phenylalanine ammonia-lyase (PAL) pathways. Both pathways commonly utilize chorismate, the end product of the shikimate pathway, to produce SA (Dempsey et al., 2011). IC synthase (ICS) and PAL are critical enzymes for these pathways, respectively. Homologs of *ICS* and *PAL* genes are present throughout the plant kingdom, including *Arabidopsis*, tobacco, tomato, populus, sunflower, and pepper (Wildermuth et al., 2001; Cochrane et al., 2004; Uppalapati et al., 2007; Catinot et al., 2008; Yuan et al., 2009; Sadeghi et al., 2013; Kim and Hwang, 2014), suggesting the importance of these SA biosynthesis pathways to survive during the course of evolution. In *Arabidopsis*, mutations in *ICS1* lead to an almost complete loss of pathogen-induced SA accumulation (Wildermuth et al., 2001). However, *Arabidopsis* quadruple *PAL* mutants, in which PAL activity is reduced to 10%, also show lower SA accumulation (50%) compared to the wild type upon pathogen infection (Huang et al., 2010). Thus, while contribution of the PAL pathway is evident, the IC pathway is the major route for SA biosynthesis during plant immunity.

In chloroplasts, ICS catalyzes the conversion of chorismate into IC (Wildermuth et al., 2001; Strawn et al., 2007; Garcion et al., 2008), which is further converted to SA (Dempsey et al., 2011). In some bacteria, conversion of IC to SA is catalyzed by IC pyruvate lyases (IPLs; Dempsey et al., 2011). However, plant genomes encode no homologous genes to bacterial *IPLs*. Expression of bacterial enzymes catalyzing this conversion together with ICS in chloroplasts leads to constitutive accumulation of SA (Verberne et al., 2000; Mauch et al., 2001). Thus, it is conceivable that plants have yet-determined gene(s) whose product(s) possess IPL activity in chloroplasts. However, metabolic enzymes such as the acyl acid amido synthetase GH3.12 [also known as PBS3/WIN3/GDG1 (AVRPPHB SUSCEPTIBLE 3/HOPW1-INTERACTING 3/GH3-LIKE DEFENSE GENE 1); Nobuta et al., 2007; Zhang et al., 2007; Okrent et al., 2009; Westfall et al., 2010, 2012] and the acyltransferase EPS1 (ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1; Zheng et al., 2009) are involved in SA accumulation, perhaps by providing SA precursors or regulatory molecules for SA biosynthesis. Thus, SA biosynthesis may be more complex in plants compared to bacteria. SA export from chloroplasts is mediated by the MATE-transporter EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5; Serrano et al., 2013). This export seems important for SA accumulation and distribution in the cell since SA accumulation is compromised in *eds5* mutants (Nawrath et al., 2002; Ishihara et al., 2008).

REGULATION OF SA BIOSYNTHESIS

Salicylic acid biosynthesis is tightly regulated since constitutive SA accumulation has negative impacts on plant fitness (Ishihara et al., 2008; Pajerowska-Mukhtar et al., 2012; Chandran et al., 2014). Accumulating evidence show that transcriptional control of *ICS1* by calcium signaling is key for the initiation of SA biosynthesis (**Figure 1**). The concentration of calcium ions (Ca^{2+}) in the cytosol transiently increases upon immune receptor activation through Ca^{2+} channels. Elevation of intracellular Ca^{2+}, called Ca^{2+} signature, is decoded by Ca^{2+} sensor proteins, such as calmodulin (CaM) and Ca^{2+}-dependent protein kinases (CDPKs; Dodd et al., 2010; Boudsocq and Sheen, 2013; Poovaiah et al., 2013; Schulz et al., 2013). Binding of CaM regulates target protein activities thereby relaying Ca^{2+} signatures to downstream responses. During *Arabidopsis* immunity, the CaM-binding transcription factor CBP60g (CALMODULIN BINDING PROTEIN 60g) and its homolog SARD1 (SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1) control *ICS1* transcription (Wang et al., 2009, 2011; Zhang et al., 2010; Wan et al., 2012). CaM-binding is required for CBP60g function, whereas SARD1 does not appear to be a CaM-binding protein (Wang et al., 2009). Despite this difference, CBP60g and SARD1 are partially redundant for *ICS1* expression and SA accumulation during immunity. However, dual regulation of *ICS1* transcription by CBP60g and SARD1 seems important for temporal dynamics of SA biosynthesis: CBP60g mainly contributes to SA biosynthesis at early stages after *P. syringae* infection while SARD1 does at late stages (Wang et al., 2011). Another close homolog of CBP60g, CBP60a, negatively regulates *ICS1* expression upon CaM-binding (Truman et al., 2013). Conceivably, upon pathogen attack, CBP60g and SARD1 bind to the *ICS1* promoter and activate its expression, at least partly by removing the negative regulator CBP60a from the *ICS1* promoter.

Unlike CaM, CDPKs have both intrinsic Ca^{2+} sensing and responding sites thereby allowing individual CDPK proteins to relay Ca^{2+} signatures to downstream components via phosphorylation events. Recently, the CDPKs, CPK4, 5, 6, and 11, were shown to re-localize to the nucleus, and to interact with and phosphorylate the WRKY transcription factors, WRKY8, 28, and 48, during ETI mediated by the plasma membrane-associated immune receptors RPS2 (RESISTANCE TO P.SYRINGAE 2) or RPM1 (RESISTANCE TO P.SYRINGAE PV MACULICULA 1; Gao et al., 2013). Mutants in *WRKY8* or *WRKY48* are compromised in pathogen-induced *ICS1* expression. Furthermore, WRKY28 directly interacts with the *ICS1* promoter (van Verk et al., 2011), which might be regulated through phosphorylation by CPK4, 5, 6, or 11. Collectively, these results suggest that during ETI, these CDPKs relay Ca^{2+} signatures to activate *ICS1* transcription via WRKY transcription factors.

Besides *ICS1* regulation, calcium signaling also affects the maintenance of SA accumulation through transcriptional regulation of *EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1*; Du et al., 2009), encoding a central regulator of the positive feedback loop of SA accumulation (Feys et al., 2001). A CaM-binding transcription factor, CAMTA3/SR1 (CALMODULIN BINDING TRANSCRIPTION ACTIVATOR 3/SIGNAL-RESPONSIVE GENE 1), binds to the *EDS1* promoter to repress its transcription, and mutants of *CAMTA3/SR1* show elevated SA levels and enhanced immunity against *P. syringae* and the fungal pathogen *Botrytis cinerea*. Combinatorial mutant analysis indicates that CAMTA3/SR1 and its homologs CAMTA1/2 also suppress expression of *CBP60g*, *SARD1*, and *ICS1* (Kim et al., 2013). Thus, the three CAMTA homologs coordinately suppress SA accumulation, but it remains unknown if the CAMTA transcription factors directly target the promoters of *CBP60g*, *SARD1*, and *ICS1*. It was recently shown

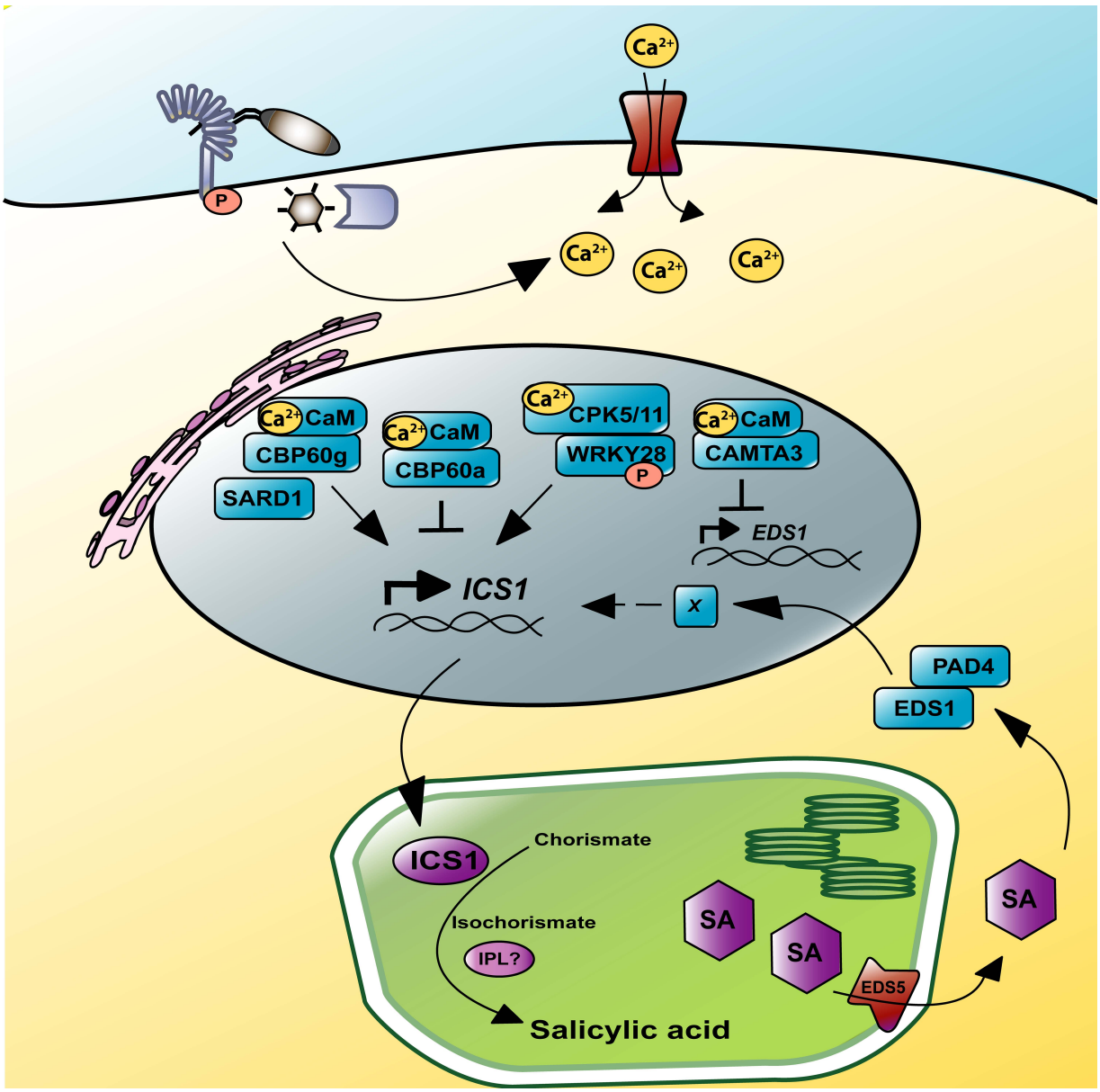


FIGURE 1 | Regulation of SA accumulation by calcium signaling. MAMP or effector recognition increases intracellular Ca^{2+} concentrations thereby regulating calcium sensor proteins, such as CaM and CDPKs. The CaM-binding transcription factors CBP60g and CBP60a are positive and negative regulators of *ICS1* transcription, respectively. A homolog of CBP60a/g, SARD1, is not a CaM-binding protein but functions redundantly with CBP60g for *ICS1* transcription. WRKY28, whose DNA-binding activity is regulated by the CDPKs CPK5 and CPK11, also contributes to *ICS1* expression. ICS1 mediates SA production in chloroplasts, by conversion of chorismate into the SA-precursor isochorismate. SA may be transported through the MATE-transporter EDS5 into the cytosol. The EDS1/PAD4 complex contributes to the positive feedback loop of SA accumulation. Repression of *EDS1* transcription by the Ca^{2+}/CaM-binding transcription factor CAMTA3 represents a fine-tuning mechanism for SA accumulation.

that a CAMTA3/SR1-interacting protein links CAMTA3/SR1 to ubiquitin-mediated protein degradation thereby enhancing *EDS1* expression and immunity against *P. syringae* (Zhang et al., 2014).

In summary, these results clearly indicate the importance of Ca^{2+} signaling in regulation of SA accumulation during immunity through transcriptional regulation of genes involved in SA biosynthesis and maintenance. However, how plants spatiotemporally coordinate positive and negative regulators of SA biosynthesis and accumulation remains to be investigated.

SA PERCEPTION

Identification of SA receptor(s) has been one of the major research interests for the last two decades. Considering its diverse functions in environmental stress response, plants may have multiple SA receptors. Indeed, biochemical approaches identified a num-

ber of SA-interacting proteins, and activities of these proteins were affected by SA-binding (Chen and Klessig, 1991; Chen et al., 1993; Durner and Klessig, 1995; Du and Klessig, 1997; Slaymaker et al., 2002; Kumar and Klessig, 2003; Forouhar et al., 2005; Park et al., 2009; Tripathi et al., 2010; Tian et al., 2012; Moreau et al., 2013). However, these SA-binding proteins do not fully explain SA response including SA-mediated transcriptional reprogramming. Recently, the three NPR (NON-EXPRESSOR of PATHOGENESIS-RELATED GENES) family members, NPR1, NPR3, and NPR4, were identified as *bona fide* SA receptors in *Arabidopsis* (Fu et al., 2012; Wu et al., 2012). In this section, we discuss how these NPR proteins function as SA receptors.

NPR1 is a master regulator of SA-mediated transcriptional reprogramming and immunity, functioning as a transcriptional coactivator (Pajerowska-Mukhtar et al., 2013). NPR1 comprises a BTB/POZ (broad-complex, tramtrack, and bric-à-brac/poxvirus and zinc-finger) domain, an ankyrin repeat domain, and a nuclear localization sequence. Mutations in *NPR1* lead to an almost complete loss of SA-mediated transcriptional reprogramming and great susceptibility to (hemi)-biotrophic pathogens (Shah et al., 1997; Volko et al., 1998; Dong, 2004). Therefore, it was not surprising but sensational that Wu et al. (2012) found NPR1 to be a *bona fide* SA receptor (**Figure 2A**). Using an equilibrium method, they showed that *Arabidopsis* NPR1 directly binds SA (Kd = 140 nM), but not inactive structural analogs, through $Cys^{521/529}$ via the transition metal copper. Consistently, $Cys^{521/529}$ were previously identified as key amino acid residues for *Arabidopsis* NPR1 function (Rochon et al., 2006). Biochemical approaches indicate that SA-binding triggers a conformational change in NPR1. Further protein deletion analyses suggest that the C-terminal transactivation domain of NPR1 is intramolecularly inhibited by the N-terminal BTB/POZ domain and that SA-binding releases the transactivation domain from BTB/POZ suppression. Thus, the study established a model with NPR1 as an SA receptor that also functions as a master signal transducer of SA signaling. However, $Cys^{521/529}$ are not conserved among

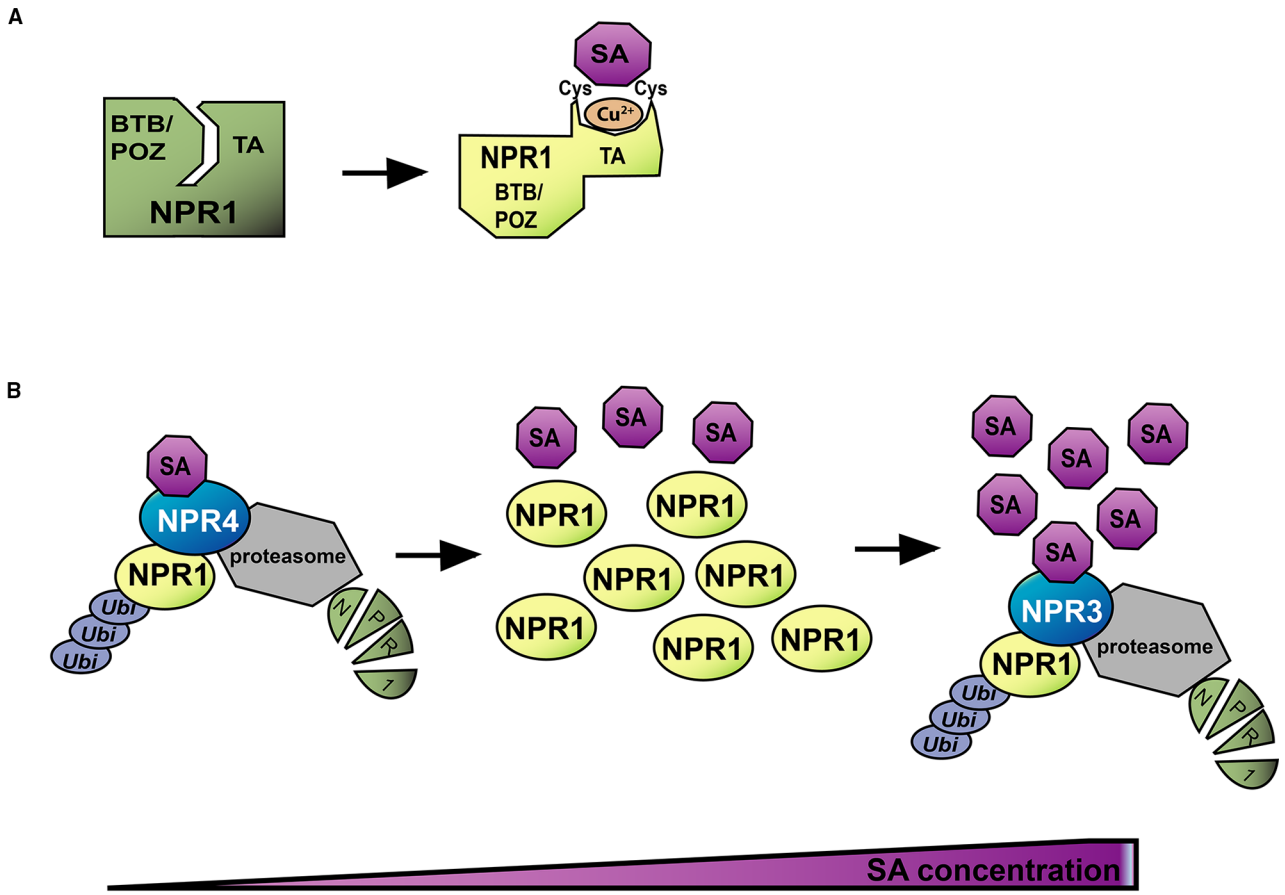


FIGURE 2 | Models for SA perception. (A) Direct SA binding to NPR1 modulates its activity. In unstressed conditions, the C-terminal transactivation domain of NPR1 is repressed by the N-terminal BTB/POZ domain, keeping NPR1 in an inactive state (green). NPR1 perceives SA through $Cys^{521/529}$ via the transition metal copper, which triggers a conformation change of NPR1, resulting in de-repression of the transactivation domain and activation of NPR1 (yellow). **(B)** NPR1 accumulation is regulated by SA through the SA receptors NPR3 and NPR4. Pathogen infection triggers SA accumulation. In the case of low SA, the SA-receptor NPR4 triggers NPR1 degradation through the 26S proteasome. When SA levels are intermediate, NPR1 protein accumulates. High SA-concentrations trigger the SA receptor NPR3-mediated NPR1 degradation. Thus, only intermediate levels of SA achieve NPR1 accumulation thereby activating SA-mediated transcriptional reprogramming.

plant species, raising an issue of the evolutionary significance of the SA perception mechanism via NPR1. In addition, another study showed that NPR1 does not bind SA in a conventional non-equilibrium ^{3}H-SA binding assay (Yan and Dong, 2014). Instead, Fu et al. (2012) identified two homologs of NPR1, NPR3 and NPR4, as SA receptors (**Figure 2B**; Fu et al., 2012). NPR1 is subject to degradation via the 26S proteasome pathway in the absence of SA (Spoel et al., 2009). Once SA increases upon pathogen infection, NPR1 is stabilized. However, full induction of SA-responsive genes also requires NPR1 turnover. Thus, regulation of NPR1 protein level is critical for SA response. Fu et al. (2012) found that NPR3 and NPR4 interact with NPR1 and are required for NPR1 degradation (Fu et al., 2012). NPR4 has a high SA affinity (Kd = 46 nM) whereas NPR3 shows a low affinity (Kd = 981 nM), suggesting differential regulations of NPR1 by NPR3 and NPR4. Interestingly, SA disrupts NPR1–NPR4 interaction, but facilitates NPR1-NPR3 interaction. These observations support a model in which NPR3 and NPR4 create an NPR1 protein concentration gradient in order to regulate NPR1–mediated transcription: in the absence of SA, NPR4-mediated NPR1 degradation prevents NPR1 accumulation whereas high SA levels also prevent NPR1 accumulation due to NPR3. Thus, NPR1-mediated signaling is active only at intermediate SA levels. This model is consistent with the observation that NPR1 protein highly accumulates at sites surrounding the infection site in a leaf. These regions are supposed to contain intermediate SA levels, while the infection site may have too high SA levels. Although this model is attractive, further validation is required.

Collectively, two alternative but not exclusive SA perception mechanisms in plant cells were identified, but further research is still required to address fundamental questions. For example, the subcellular location(s) of SA perception have not been addressed yet. The nuclear NPR1 pool is necessary for SA-mediated transcription (Mou et al., 2003). Consistently, NPR3 and NPR4 are nuclear proteins, and therefore SA is likely perceived by them in the nucleus to regulate nuclear NPR1 amount. On the other hand, the cytosolic NPR1 pool may regulate cross-talk between SA- and JA-mediated transcriptional reprogramming (Spoel et al., 2003), suggesting that SA is also perceived in the cytosol. Since SA perception by nuclear NPR3 and NPR4 does not explain this observation, cytosolic NPR1 activity may be regulated by the direct SA binding.

SA-MEDIATED TRANSCRIPTIONAL REPROGRAMMING

NPR1 controls expression of more than 95% of the responsive genes to the SA-analog benzothiadiazole (BTH; Wang et al., 2006). Functional regulation of NPR1 is not only mediated by the direct SA binding, but also by SA-triggered redox changes (Mou et al., 2003). In the absence of SA, NPR1 is present as an oligomer formed through intermolecular disulfide bonds. SA triggers changes in the cellular redox potential, thereby reducing cysteine residues in NPR1 through the thioredoxins TRXh3 and TRXh5, resulting in monomerization of NPR1 (Tada et al., 2008). Mutations in the cysteine residues (Cys^{82} or Cys^{216}) lead to constitutive monomerization and nuclear accumulation of NPR1, resulting in activation of *PR1* expression (Mou et al., 2003). Nuclear accumulation of NPR1 triggered by SA can be explained by stabilization of nuclear NPR1 or translocation of the NPR1 monomer from the cytosol to the nucleus. Thus, SA-triggered NPR1 monomerization and nuclear accumulation are important steps for NPR1-mediated transcription. However, forced nuclear localization of NPR1 is not sufficient for transcriptional reprogramming, as the presence of SA is additionally required for full *PR1* induction (Kinkema et al., 2000; Spoel et al., 2003). This can be explained by the observation that SA-binding triggers the NPR1 conformational change thereby allowing NPR1 to regulate gene expression (Wu et al., 2012). Additional regulation of NPR1 involves phosphorylation (Spoel et al., 2009). SA triggers phosphorylation of NPR1 at the N-terminus ($Ser^{11/15}$) in the nucleus via yet-determined kinase(s). NPR1 phosphorylation contributes to its recruitment to a ubiquitin ligase, resulting in proteasome-mediated NPR1 degradation. This degradation is required for the proper transcriptional control by NPR1, perhaps by allowing fresh NPR1 to reinitiate the next cycle of transcription.

NPR1 regulates transcription of SA-responsive genes through interactions with specific transcription factors (**Figure 3**). Identified major transcription factors belong to a subclass of the basic leucine zipper transcription factor family, TGA (Gatz, 2013). The *Arabidopsis* genome encodes 10 TGA transcription factors, which are structurally divided into five subgroups and all bind the consensus DNA sequence TGACG. Yeast-two-hybrid analyses with NPR1 and TGA transcription factors show interaction specificity for clade II TGAs (TGA2/TGA5/TGA6) and TGA3 (clade III; Zhou et al., 2000; Hepworth et al., 2005). Genetic analysis reveal that TGA2, TGA5, and TGA6 repress *PR1* transcription in the absence of SA, but on the other hand are required for

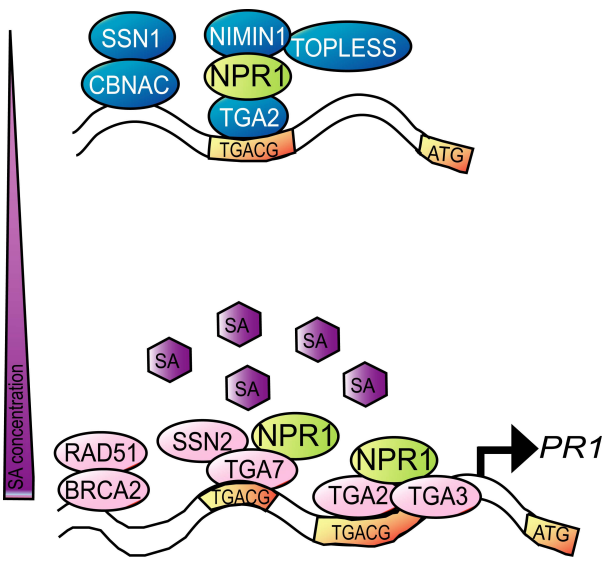


FIGURE 3 | SA-mediated transcriptional regulation of PR1 through NPR1. In the absence of SA, repression of *PR1* expression can be achieved by repressor complexes (blue), such as the TGA2-NPR1-NIMIN complex through the co-repressor TOPLESS or the CBNAC-SNI1 complex. SA triggers a conformational change of NPR1 and dissociation of NIMIN1, resulting in forming activator complexes (pink) including TGA transcription factors and SSN2. The DNA repair proteins BRCA2 and RAD51 are also involved in SA-mediated transcription.

PR1 induction in the presence of SA (Zhang et al., 2003). In the absence of SA, TGA2 binds to the *PR1* promoter thereby repressing its transcription (Rochon et al., 2006; Boyle et al., 2009). An NPR1-interacting protein, NIMIN1 (NPR1/NIM1-INTERACTING PROTEIN 1), can form a ternary complex with TGA2 through NPR1 at least in yeast (Weigel et al., 2005). Transcriptional repression by TGA2 may be achieved through NIMIN1 interacting with a transcriptional co-repressor, TOPLESS (Braun et al., 2011). Conceivably, SA allows NPR1 to form a different complex with TGA2 and other TGA factors, such as TGA3 thereby activating *PR1* transcription (Johnson et al., 2003). The NIMIN1–NPR1–TGA2 complex is dissociated in the presence of SA in yeast (Hermann et al., 2013). Thus, NIMIN1 dissociation from the NPR1–TGA transcriptional complex by SA may contribute to activation of the NPR1–TGA transcriptional complex. This transcriptional activation may be relayed through specific mediator subunits, such as the Mediator subunit MED15, since *med15* mutants are insensitive to SA (Canet et al., 2012).

A suppressor screen of *npr1* identified SNI1 (SUPPRESSOR OF *NPR1* INDUCIBLE 1) as another repressor of SA-responsive genes (e.g., *PR1*) in unstressed conditions (Li et al., 1999; Mosher et al., 2006). SNI1-mediated transcriptional repression may be achieved through the CaM-binding NAC (NAM, ATAF1,2, CUC2) transcription repressor CBNAC, since SNI1 directly interacts with CBNAC and enhances CBNAC-binding activity to the *PR1* promoter (Kim et al., 2012). Upon SA treatment, SNI1 is dissociated from the *PR1* promoter and replaced by the DNA repair protein SSN2 (Song et al., 2011). Although SSN2 contains a DNA-binding domain, its binding to the *PR1* promoter requires NPR1 and the transcription factor TGA7. These results suggest that SA triggers NPR1 activation through nuclear accumulation and conformational change, resulting in the formation of a TGA7–NPR1–SSN2 complex that activates *PR1* transcription. Additional DNA repair proteins, such as BRCA2A (BREAST CANCER 2A) and RAD51D, are also functionally associated with SA-mediated transcription (Durrant et al., 2007; Wang et al., 2010; Song et al., 2011). Interestingly, SA and *Pseudomonas* infection cause DNA damage, such as DNA double strand breaks, suggesting that DNA damage response is an intrinsic component of SA-mediated transcription during plant immunity (Yan et al., 2013; Song and Bent, 2014).

Besides functional regulation of transcription factors by NPR1 through complex formation, NPR1 also controls expression of transcription factors, such as WRKY transcription factors, which are required for SA-mediated transcriptional reprogramming (Wang et al., 2006; Pajerowska-Mukhtar et al., 2012). The *Arabidopsis* genome encodes 74 WRKY factors which bind the specific DNA sequence (C/TTGACT/C), termed the W-box (Rushton et al., 2010). WRKY factors form a complex interconnected regulatory network, containing recurring regulatory patterns, such as both positive and negative feedback and feedforward loops. This WRKY network ensures rapid and efficient signal amplification and allows tight control to limit the plant immune response. Furthermore, the presence of multiple W-boxes in the *NPR1* promoter suggests regulation of *NPR1* expression by WRKY factors, which is indeed supported by NPR1 promoter analysis (Yu et al., 2001). Thus, WRKY transcriptional regulatory networks downstream of NPR1 amplify and fine-tune SA-mediated transcriptional reprogramming.

COMPENSATION OF SA SIGNALING

The importance of SA signaling during immunity is reflected by the fact that pathogen effectors target it for virulence, either by preventing SA accumulation (Djamei et al., 2011; Rabe et al., 2013; Liu et al., 2014) or by dampen SA signaling and transcriptional regulation, using the antagonistic interaction between SA and JA signaling (Uppalapati et al., 2007; Caillaud et al., 2013; Jiang et al., 2013; Gimenez-Ibanez et al., 2014). It is reasonable to assume that plants have evolved compensatory mechanism(s) to circumvent weakened SA signaling upon effector attack, thereby ensuring robust immune response (Tsuda and Katagiri, 2010). For example, although it is believed that SA and JA signaling antagonize each other, a recent study suggests the compensation of SA accumulation by JA (Kim et al., 2014). The MAMP flg22 induces SA accumulation in an *ICS1*-dependent manner (Tsuda et al., 2008b). Additionally, a component of the SA amplification loop, PAD4, is required for full induction of SA (Zhou et al., 1998; Tsuda et al., 2008b). In agreement with the antagonistic relationship between JA and SA, single mutation in the JA biosynthesis gene *DDE2* leads to higher SA accumulation upon flg22 treatment. However, combined mutation in *DDE2* and *PAD4* diminishes SA accumulation comparable to that in *sid2*, suggesting that although JA suppresses SA accumulation through PAD4, it also supports SA accumulation once *PAD4* is compromised. Thus, JA signaling represents a compensation mechanism for SA accumulation during PTI.

In addition to JA, MAPK signaling also compensates SA signaling to secure transcriptional regulation of SA-responsive genes in ETI (Tsuda et al., 2013). Activation of the *Arabidopsis* MAPKs MPK3 and MPK6 is transient during PTI, but sustained during RPS2- and RPM1-mediated ETI (Tsuda et al., 2013), or upon *B. cinerea* infection (Han et al., 2010). While transient activation of MPK3 and MPK6 is not sufficient to overcome SA-dependency of a subset of SA-responsive genes such as *PR1*, prolonged activation of MPK3 and MPK6 facilitates their transcriptional regulation independent of SA. Furthermore, this compensation mechanism does not require NPR1 since *NPR1* mutation does not affect *PR1* induction mediated by prolonged MAPK activation. It can be assumed that prolonged MAPK activation bypasses the requirement of NPR1 to regulate transcription factor(s) involved in SA response. Although transcription factors shared by SA and the MAPK cascade are not known, large-scale protein target identifications of MPK3 and MPK6 would help to identify candidates (Popescu et al., 2009; Hoehenwarter et al., 2013). Among them, TGA transcription factors are reasonable candidates (Wang and Fobert, 2013). However, how this quantitative MAPK activation leads to qualitatively different transcriptional outputs still remains to be determined. One possible answer lays in temporal regulation of transcription factor(s). Hereby, the MAPKs first activate expression of transcription factor(s), and later on phosphorylate the accumulated transcription factor(s), representing a feedforward loop for activation of the transcription factor(s). In this case, only prolonged MAPK activation ensures activation of the transcription factor(s). Indeed, the MAPKs regulate expression of

a diverse transcription factor set (Mao et al., 2011; Li et al., 2012; Meng et al., 2013; Tsuda et al., 2013; Frei dit Frey et al., 2014), but whether the MAPKs also phosphorylate them is a future issue.

CONCLUSIONS AND PERSPECTIVES

Over the past decade a number of researches have shed light into our understanding of SA-mediated signaling, through the discoveries of calcium signaling as the major switch for SA biosynthesis, NPR family members as SA receptors, and the mechanism for NPR1-mediated transcriptional reprogramming. However, many questions are still unanswered, starting with identification of plant *IPL* gene(s) to further validate the IC pathway as the major route for SA biosynthesis in plants. The controversy for SA perception should also be solved in the future. In addition, information for temporal and spatial dynamics of SA biosynthesis and SA-mediated transcriptional reprogramming is missing. For this, systems approaches using time-series genomics data sets and tissue-specific analysis will help our conception (Mine et al., 2014). Most studies are based on experiments using the model plant *Arabidopsis*. Analysis of different plant species is necessary to understand evolutionary conservation and diversification of SA signal transduction. Finally, identification of the molecular components in MAPK-mediated SA/NPR1-independent gene regulation of SA-responsive genes in ETI will shed light on the molecular mechanism of SA compensation.

ACKNOWLEDGMENTS

We thank Akira Mine, Shajahan Anver, and Sajjad Khani for critical reading of the manuscript. This work was supported by the Max Planck Society (Carolin Seyfferth, Kenichi Tsuda) and a grant from the German Research Council SFB670 (Kenichi Tsuda).

REFERENCES

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Bonardi, V., and Dangl, J. L. (2012). How complex are intracellular immune receptor signaling complexes? *Front. Plant Sci.* 3:237. doi: 10.3389/fpls.2012.00237

Boudsocq, M., and Sheen, J. (2013). CDPKs in immune and stress signaling. *Trends Plant Sci.* 18, 30–40. doi: 10.1016/j.tplants.2012.08.008

Boyle, P., Le Su, E., Rochon, A., Shearer, H. L., Murmu, J., Chu, J. Y., et al. (2009). The BTB/POZ domain of the *Arabidopsis* disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell* 21, 3700–3713. doi: 10.1105/tpc.109.069971

Braun, P., Carvunis, A. R., Charloteaux, B., Dreze, M., Ecker, J. R., Hill, D. E., et al. (2011). Evidence for network evolution in an *Arabidopsis* interactome map. *Science* 333, 601–607. doi: 10.1126/science.1203877

Caillaud, M. C., Asai, S., Rallapalli, G., Piquerez, S., Fabro, G., and Jones, J. D. (2013). A downy mildew effector attenuates salicylic acid-triggered immunity in *Arabidopsis* by interacting with the host mediator complex. *PLoS Biol.* 11:e1001732. doi: 10.1371/journal.pbio.1001732

Canet, J. V., Dobon, A., and Tornero, P. (2012). Non-recognition-of-BTH4, an *Arabidopsis* mediator subunit homolog, is necessary for development and response to salicylic acid. *Plant Cell* 24, 4220–4235. doi: 10.1105/tpc.112.103028

Catinot, J., Buchala, A., Abou-Mansour, E., and Metraux, J. P. (2008). Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*. *FEBS Lett.* 582, 473–478. doi: 10.1016/j.febslet.2007.12.039

Chandran, D., Rickert, J., Huang, Y., Steinwand, M. A., Marr, S. K., and Wildermuth, M. C. (2014). Atypical E2F transcriptional repressor DEL1 acts at the intersection of plant growth and immunity by controlling the hormone salicylic acid. *Cell Host Microbe* 15, 506–513. doi: 10.1016/j.chom.2014.03.007

Chen, H., Xue, L., Chintamanani, S., Germain, H., Lin, H., Cui, H., et al. (2009). ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in *Arabidopsis*. *Plant Cell* 21, 2527–2540. doi: 10.1105/tpc.108.065193

Chen, Z., and Klessig, D. F. (1991). Identification of a soluble salicylic acid-binding protein that may function in signal transduction in the plant disease-resistance response. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8179–8183. doi: 10.1073/pnas.88.18.8179

Chen, Z., Silva, H., and Klessig, D. F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262, 1883–1886. doi: 10.1126/science.8266079

Cochrane, F. C., Davin, L. B., and Lewis, N. G. (2004). The *Arabidopsis* phenylalanine ammonia lyase gene family: kinetic characterization of the four PAL isoforms. *Phytochemistry* 65, 1557–1564. doi: 10.1016/j.phytochem.2004.05.006

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic Acid biosynthesis and metabolism. *Arabidopsis Book* 9, e0156. doi: 10.1199/tab.0156

Derksen, H., Rampitsch, C., and Daayf, F. (2013). Signaling cross-talk in plant disease resistance. *Plant Sci.* 207, 79–87. doi: 10.1016/j.plantsci.2013.03.004

Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahnt, J., et al. (2011). Metabolic priming by a secreted fungal effector. *Nature* 478, 395–398. doi: 10.1038/nature10454

Dodd, A. N., Kudla, J., and Sanders, D. (2010). The language of calcium signaling. *Annu. Rev. Plant Biol.* 61, 593–620. doi: 10.1146/annurev-arplant-070109-104628

Dong, X. (2004). NPR1, all things considered. *Curr. Opin. Plant Biol.* 7, 547–552. doi: 10.1016/j.pbi.2004.07.005

Du, H., and Klessig, D. F. (1997). Identification of a soluble, high-affinity salicylic acid-binding protein in tobacco. *Plant Physiol.* 113, 1319–1327.

Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S., et al. (2009). Ca^{2+}/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* 457, 1154–1158. doi: 10.1038/nature07612

Durner, J., and Klessig, D. F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 92, 11312–11316. doi: 10.1073/pnas.92.24.11312

Durrant, W. E., Wang, S., and Dong, X. (2007). *Arabidopsis* SNI1 and RAD51D regulate both gene transcription and DNA recombination during the defense response. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4223–4227. doi: 10.1073/pnas.0609357104

Eitas, T. K., and Dangl, J. L. (2010). NB-LRR proteins: pairs, pieces, perception, partners, and pathways. *Curr. Opin. Plant Biol.* 13, 472–477. doi: 10.1016/j.pbi.2010.04.007

Feys, B. J., Moisan, L. J., Newman, M. A., and Parker, J. E. (2001). Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. *EMBO J.* 20, 5400–5411. doi: 10.1093/emboj/20.19.5400

Forouhar, F., Yang, Y., Kumar, D., Chen, Y., Fridman, E., Park, S. W., et al. (2005). Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1773–1778. doi: 10.1073/pnas.0409227102

Frei dit Frey, N., Garcia, A. V., Bigeard, J., Zaag, R., Bueso, E., Garmier, M., et al. (2014). Functional analysis of *Arabidopsis* immune-related MAPKs uncovers a role for MPK3 as negative regulator of inducible defences. *Genome Biol.* 15, R87. doi: 10.1186/gb-2014-15-6-r87

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gao, X., Chen, X., Lin, W., Chen, S., Lu, D., Niu, Y., et al. (2013). Bifurcation of *Arabidopsis* NLR immune signaling via Ca^{2+}-dependent protein kinases. *PLoS Pathog.* 9:e1003127. doi: 10.1371/journal.ppat.1003127

Garcion, C., Lohmann, A., Lamodiere, E., Catinot, J., Buchala, A., Doermann, P., et al. (2008). Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of *Arabidopsis*. *Plant Physiol.* 147, 1279–1287. doi: 10.1104/pp.108.119420

Gatz, C. (2013). From pioneers to team players: TGA transcription factors provide a molecular link between different stress pathways. *Mol. Plant Microbe Interact.* 26, 151–159. doi: 10.1094/MPMI-04-12-0078-IA

Gimenez-Ibanez, S., Boter, M., Fernandez-Barbero, G., Chini, A., Rathjen, J. P., and Solano, R. (2014). The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in *Arabidopsis*. *PLoS Biol.* 12:e1001792. doi: 10.1371/journal.pbio.1001792

Han, L., Li, G. J., Yang, K. Y., Mao, G., Wang, R., Liu, Y., et al. (2010). Mitogen-activated protein kinase 3 and 6 regulate *Botrytis cinerea*-induced ethylene production in *Arabidopsis*. *Plant J.* 64, 114–127. doi: 10.1111/j.1365-313X.2010.04318.x

Hepworth, S. R., Zhang, Y., Mckim, S., Li, X., and Haughn, G. W. (2005). BLADE-ON-PETIOLE-dependent signaling controls leaf and floral patterning in *Arabidopsis*. *Plant Cell* 17, 1434–1448. doi: 10.1105/tpc.104.030536

Hermann, M., Maier, F., Masroor, A., Hirth, S., Pfitzner, A. J., and Pfitzner, U. M. (2013). The *Arabidopsis* NIMIN proteins affect NPR1 differentially. *Front. Plant Sci.* 4:88. doi: 10.3389/fpls.2013.00088

Hoehenwarter, W., Thomas, M., Nukarinen, E., Egelhofer, V., Rohrig, H., Weckwerth, W., et al. (2013). Identification of novel in vivo MAP kinase substrates in *Arabidopsis thaliana* through use of tandem metal oxide affinity chromatography. *Mol. Cell. Proteomics* 12, 369–380. doi: 10.1074/mcp.M112.020560

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y. H., et al. (2010). Functional analysis of the *Arabidopsis* PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiol.* 153, 1526–1538. doi: 10.1104/pp.110.157370

Ishihara, T., Sekine, K. T., Hase, S., Kanayama, Y., Seo, S., Ohashi, Y., et al. (2008). Overexpression of the *Arabidopsis thaliana* EDS5 gene enhances resistance to viruses. *Plant Biol.* 10, 451–461. doi: 10.1111/j.1438-8677.2008.00050.x

Jacob, F., Vernaldi, S., and Maekawa, T. (2013). Evolution and conservation of plant NLR functions. *Front. Immunol.* 4:297. doi: 10.3389/fimmu.2013.00297

Jiang, S., Yao, J., Ma, K. W., Zhou, H., Song, J., He, S. Y., et al. (2013). Bacterial effector activates jasmonate signaling by directly targeting JAZ transcriptional repressors. *PLoS Pathog.* 9:e1003715. doi: 10.1371/journal.ppat.1003715

Johnson, C., Boden, E., and Arias, J. (2003). Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in *Arabidopsis*. *Plant Cell* 15, 1846–1858. doi: 10.1105/tpc.012211

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kim, D. S., and Hwang, B. K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *J. Exp. Bot.* 65, 2295–2306. doi: 10.1093/jxb/eru109

Kim, H. S., Park, H. C., Kim, K. E., Jung, M. S., Han, H. J., Kim, S. H., et al. (2012). A NAC transcription factor and SNI1 cooperatively suppress basal pathogen resistance in *Arabidopsis thaliana*. *Nucleic Acids Res.* 40, 9182–9192. doi: 10.1093/nar/gks683

Kim, Y., Park, S., Gilmour, S. J., and Thomashow, M. F. (2013). Roles of CAMTA transcription factors and salicylic acid in configuring the low-temperature transcriptome and freezing tolerance of *Arabidopsis*. *Plant J.* 75, 364–376. doi: 10.1111/tpj.12205

Kim, Y., Tsuda, K., Igarashi, D., Hillmer, R. A., Sakakibara, H., Myers, C. L., et al. (2014). Mechanisms underlying robustness and tunability in a plant immune signaling network. *Cell Host Microbe* 15, 84–94. doi: 10.1016/j.chom.2013.12.002

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.1105/tpc.12.12.2339

Kumar, D., and Klessig, D. F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc. Natl. Acad. Sci. U.S.A.* 100, 16101–16106. doi: 10.1073/pnas.0307162100

Li, G., Meng, X., Wang, R., Mao, G., Han, L., Liu, Y., et al. (2012). Dual-level regulation of ACC synthase activity by MPK3/MPK6 cascade and its downstream WRKY transcription factor during ethylene induction in *Arabidopsis*. *PLoS Genet.* 8:e1002767. doi: 10.1371/journal.pgen.1002767

Li, X., Zhang, Y., Clarke, J. D., Li, Y., and Dong, X. (1999). Identification and cloning of a negative regulator of systemic acquired resistance, SNI1, through a screen for suppressors of npr1-1. *Cell* 98, 329–339. doi: 10.1016/S0092-8674(00)81962-5

Liu, T., Song, T., Zhang, X., Yuan, H., Su, L., Li, W., et al. (2014). Unconventionally secreted effectors of two filamentous pathogens target plant salicylate biosynthesis. *Nat. Commun.* 5, 4686. doi: 10.1038/ncomms5686

Lohou, D., Lonjon, F., Genin, S., and Vailleau, F. (2013). Type III chaperones & Co in bacterial plant pathogens: a set of specialized bodyguards mediating effector delivery. *Front. Plant Sci.* 4:435. doi: 10.3389/fpls.2013.00435

Macho, A. P., and Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Mol. Cell* 54, 263–272. doi: 10.1016/j.molcel.2014.03.028

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., and Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in *Arabidopsis*. *Plant Cell* 23, 1639–1653. doi: 10.1105/tpc.111.084996

Mauch, F., Mauch-Mani, B., Gaille, C., Kull, B., Haas, D., and Reimmann, C. (2001). Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. *Plant J.* 25, 67–77. doi: 10.1046/j.1365-313x.2001.00940.x

Meng, X., Xu, J., He, Y., Yang, K. Y., Mordorski, B., Liu, Y., et al. (2013). Phosphorylation of an ERF transcription factor by *Arabidopsis* MPK3/MPK6 regulates plant defense gene induction and fungal resistance. *Plant Cell* 25, 1126–1142. doi: 10.1105/tpc.112.109074

Mine, A., Sato, M., and Tsuda, K. (2014). Toward a systems understanding of plant-microbe interactions. *Front. Plant Sci.* 5:423. doi: 10.3389/fpls.2014.00423

Moreau, M., Westlake, T., Zampogna, G., Popescu, G., Tian, M., Noutsos, C., et al. (2013). The *Arabidopsis* oligopeptidases TOP1 and TOP2 are salicylic acid targets that modulate SA-mediated signaling and the immune response. *Plant J.* 76, 603–614. doi: 10.1111/tpj.12320

Mosher, R. A., Durrant, W. E., Wang, D., Song, J., and Dong, X. (2006). A comprehensive structure-function analysis of *Arabidopsis* SNI1 defines essential regions and transcriptional repressor activity. *Plant Cell* 18, 1750–1765. doi: 10.1105/tpc.105.039677

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Nawrath, C., Heck, S., Parinthawong, N., and Metraux, J. P. (2002). EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell* 14, 275–286.

Nobuta, K., Okrent, R. A., Stoutemyer, M., Rodibaugh, N., Kempema, L., Wildermuth, M. C., et al. (2007). The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent defense responses in *Arabidopsis*. *Plant Physiol.* 144, 1144–1156. doi: 10.1104/pp.107.097691

Okrent, R. A., Brooks, M. D., and Wildermuth, M. C. (2009). *Arabidopsis* GH3.*12* (PBS3) conjugates amino acids to 4-substituted benzoates and is inhibited by salicylate. *J. Biol. Chem.* 284, 9742–9754. doi: 10.1074/jbc.M806662200

Pajerowska-Mukhtar, K. M., Emerine, D. K., and Mukhtar, M. S. (2013). Tell me more: roles of NPRs in plant immunity. *Trends Plant Sci.* 18, 402–411. doi: 10.1016/j.tplants.2013.04.004

Pajerowska-Mukhtar, K. M., Wang, W., Tada, Y., Oka, N., Tucker, C. L., Fonseca, J. P., et al. (2012). The HSF-like transcription factor TBF1 is a major molecular switch for plant growth-to-defense transition. *Curr. Biol.* 22, 103–112. doi: 10.1016/j.cub.2011.12.015

Park, S. W., Liu, P. P., Forouhar, F., Vlot, A. C., Tong, L., Tietjen, K., et al. (2009). Use of a synthetic salicylic acid analog to investigate the roles of methyl salicylate and its esterases in plant disease resistance. *J. Biol. Chem.* 284, 7307–7317. doi: 10.1074/jbc.M807968200

Pieterse, C. M., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Poovaiah, B. W., Du, L., Wang, H., and Yang, T. (2013). Recent advances in calcium/calmodulin-mediated signaling with an emphasis on plant-microbe interactions. *Plant Physiol.* 163, 531–542. doi: 10.1104/pp.113.220780

Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., et al. (2009). MAPK target networks in *Arabidopsis thaliana* revealed using functional protein microarrays. *Genes Dev.* 23, 80–92. doi: 10.1101/gad.1740009

Rabe, F., Ajami-Rashidi, Z., Doehlemann, G., Kahmann, R., and Djamei, A. (2013). Degradation of the plant defence hormone salicylic acid by the biotrophic

fungus *Ustilago maydis*. *Mol. Microbiol.* 89, 179–188. doi: 10.1111/mmi.12269

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Rochon, A., Boyle, P., Wignes, T., Fobert, P. R., and Despres, C. (2006). The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *Plant Cell* 18, 3670–3685. doi: 10.1105/tpc.106.046953

Rushton, P. J., Somssich, I. E., Ringler, P., and Shen, Q. J. (2010). WRKY transcription factors. *Trends Plant Sci.* 15, 247–258. doi: 10.1016/j.tplants.2010.02.006

Sadeghi, M., Dehghan, S., Fischer, R., Wenzel, U., Vilcinskas, A., Kavousi, H. R., et al. (2013). Isolation and characterization of isochorismate synthase and cinnamate 4-hydroxylase during salinity stress, wounding, and salicylic acid treatment in *Carthamus tinctorius*. *Plant Signal. Behav.* 8:e27335. doi: 10.4161/psb.27335

Schulz, P., Herde, M., and Romeis, T. (2013). Calcium-dependent protein kinases: hubs in plant stress signaling and development. *Plant Physiol.* 163, 523–530. doi: 10.1104/pp.113.222539

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., et al. (2013). Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiol.* 162, 1815–1821. doi: 10.1104/pp.113.218156

Shah, J., Tsui, F., and Klessig, D. F. (1997). Characterization of a salicylic acid-insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. *Mol. Plant Microbe Interact.* 10, 69–78. doi: 10.1094/MPMI.1997.10.1.69

Slaymaker, D. H., Navarre, D. A., Clark, D., del Pozo, O., Martin, G. B., and Klessig, D. F. (2002). The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11640–11645. doi: 10.1073/pnas.182427699

Song, J., and Bent, A. F. (2014). Microbial pathogens trigger host DNA double-strand breaks whose abundance is reduced by plant defense responses. *PLoS Pathog.* 10:e1004030. doi: 10.1371/journal.ppat.1004030

Song, J., Durrant, W. E., Wang, S., Yan, S., Tan, E. H., and Dong, X. (2011). DNA repair proteins are directly involved in regulation of gene expression during plant immune response. *Cell Host Microbe* 9, 115–124. doi: 10.1016/j.chom.2011.01.011

Spoel, S. H., Koornneef, A., Claessens, S. M., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770. doi: 10.1105/tpc.009159

Spoel, S. H., Mou, Z., Tada, Y., Spivey, N. W., Genschik, P., and Dong, X. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* 137, 860–872. doi: 10.1016/j.cell.2009.03.038

Strawn, M. A., Marr, S. K., Inoue, K., Inada, N., Zubieta, C., and Wildermuth, M. C. (2007). *Arabidopsis* isochorismate synthase functional in pathogen-induced salicylate biosynthesis exhibits properties consistent with a role in diverse stress responses. *J. Biol. Chem.* 282, 5919–5933. doi: 10.1074/jbc.M605193200

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes [corrected] of NPR1 via *S*-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Tian, M., Von Dahl, C. C., Liu, P. P., Friso, G., Van Wijk, K. J., and Klessig, D. F. (2012). The combined use of photoaffinity labeling and surface plasmon resonance-based technology identifies multiple salicylic acid-binding proteins. *Plant J.* 72, 1027–1038. doi: 10.1111/tpj.12016

Tripathi, D., Jiang, Y. L., and Kumar, D. (2010). SABP2, a methyl salicylate esterase is required for the systemic acquired resistance induced by acibenzolar-*S*-methyl in plants. *FEBS Lett.* 584, 3458–3463. doi: 10.1016/j.febslet.2010.06.046

Truman, W., Sreekanta, S., Lu, Y., Bethke, G., Tsuda, K., Katagiri, F., et al. (2013). The CALMODULIN-BINDING PROTEIN60 family includes both negative and positive regulators of plant immunity. *Plant Physiol.* 163, 1741–1751. doi: 10.1104/pp.113.227108

Tsuda, K., Glazebrook, J., and Katagiri, F. (2008a). The interplay between MAMP and SA signaling. *Plant Signal. Behav.* 3, 359–361. doi: 10.4161/psb.3.6.5702

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., and Katagiri, F. (2008b). Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J.* 53, 763–775. doi: 10.1111/j.1365-313X.2007.03369.x

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* 13, 459–465. doi: 10.1016/j.pbi.2010.04.006

Tsuda, K., Mine, A., Bethke, G., Igarashi, D., Botanga, C. J., Tsuda, Y., et al. (2013). Dual regulation of gene expression mediated by extended MAPK activation and salicylic acid contributes to robust innate immunity in *Arabidopsis thaliana*. *PLoS Genet.* 9:e1004015. doi: 10.1371/journal.pgen.1004015

Uppalapati, S. R., Ishiga, Y., Wangdi, T., Kunkel, B. N., Anand, A., Mysore, K. S., et al. (2007). The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant Microbe Interact.* 20, 955–965. doi: 10.1094/MPMI-20-8-0955

van Verk, M. C., Bol, J. F., and Linthorst, H. J. (2011). WRKY transcription factors involved in activation of SA biosynthesis genes. *BMC Plant Biol.* 11:89. doi: 10.1186/1471-2229-11-89

Verberne, M. C., Verpoorte, R., Bol, J. F., Mercado-Blanco, J., and Linthorst, H. J. (2000). Overproduction of salicylic acid in plants by bacterial transgenes enhances pathogen resistance. *Nat. Biotechnol.* 18, 779–783. doi: 10.1038/77347

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Volko, S. M., Boller, T., and Ausubel, F. M. (1998). Isolation of new *Arabidopsis* mutants with enhanced disease susceptibility to *Pseudomonas syringae* by direct screening. *Genetics* 149, 537–548.

Wan, D., Li, R., Zou, B., Zhang, X., Cong, J., Wang, R., et al. (2012). Calmodulin-binding protein CBP60g is a positive regulator of both disease resistance and drought tolerance in *Arabidopsis*. *Plant Cell Rep.* 31, 1269–1281. doi: 10.1007/s00299-012-1247-7

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2:e123. doi: 10.1371/journal.ppat.0020123

Wang, L., and Fobert, P. R. (2013). *Arabidopsis* clade I TGA factors regulate apoplastic defences against the bacterial pathogen *Pseudomonas syringae* through endoplasmic reticulum-based processes. *PLoS ONE* 8:e77378. doi: 10.1371/journal.pone.0077378

Wang, L., Tsuda, K., Sato, M., Cohen, J. D., Katagiri, F., and Glazebrook, J. (2009). *Arabidopsis* CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. *PLoS Pathog.* 5:e1000301. doi: 10.1371/journal.ppat.1000301

Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen Le, V., Katagiri, F., et al. (2011). CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling. *Plant J.* 67, 1029–1041. doi: 10.1111/j.1365-313X.2011.04655.x

Wang, S., Durrant, W. E., Song, J., Spivey, N. W., and Dong, X. (2010). *Arabidopsis* BRCA2 and RAD51 proteins are specifically involved in defense gene transcription during plant immune responses. *Proc. Natl. Acad. Sci. U.S.A.* 107, 22716–22721. doi: 10.1073/pnas.1005978107

Weigel, R. R., Pfitzner, U. M., and Gatz, C. (2005). Interaction of NIMIN1 with NPR1 modulates PR gene expression in *Arabidopsis*. *Plant Cell* 17, 1279–1291. doi: 10.1105/tpc.104.027441

Westfall, C. S., Herrmann, J., Chen, Q., Wang, S., and Jez, J. M. (2010). Modulating plant hormones by enzyme action: the GH3 family of acyl acid amido synthetases. *Plant Signal. Behav.* 5, 1607–1612. doi: 10.4161/psb.5.12.13941

Westfall, C. S., Zubieta, C., Herrmann, J., Kapp, U., Nanao, M. H., and Jez, J. M. (2012). Structural basis for prereceptor modulation of plant hormones by GH3 proteins. *Science* 336, 1708–1711. doi: 10.1126/science.1221863

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Xin, X. F., and He, S. Y. (2013). *Pseudomonas syringae* pv. *tomato* DC3000: a model pathogen for probing disease susceptibility and hormone signaling

in plants. *Annu. Rev. Phytopathol.* 51, 473–498. doi: 10.1146/annurev-phyto-082712-102321

Yan, S., and Dong, X. (2014). Perception of the plant immune signal salicylic acid. *Curr. Opin. Plant Biol.* 20C, 64–68. doi: 10.1016/j.pbi.2014.04.006

Yan, S., Wang, W., Marques, J., Mohan, R., Saleh, A., Durrant, W. E., et al. (2013). Salicylic acid activates DNA damage responses to potentiate plant immunity. *Mol. Cell* 52, 602–610. doi: 10.1016/j.molcel.2013.09.019

Yu, D., Chen, C., and Chen, Z. (2001). Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* 13, 1527–1540. doi: 10.1105/tpc.13.7.1527

Yuan, Y., Chung, J. D., Fu, X., Johnson, V. E., Ranjan, P., Booth, S. L., et al. (2009). Alternative splicing and gene duplication differentially shaped the regulation of isochorismate synthase in Populus and *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22020–22025. doi: 10.1073/pnas.0906869106

Zhang, L., Du, L., Shen, C., Yang, Y., and Poovaiah, B. W. (2014). Regulation of plant immunity through ubiquitin-mediated modulation of Ca^{2+}-calmodulin-AtSR1/CAMTA3 signaling. *Plant J.* 78, 269–281. doi: 10.1111/tpj.12473

Zhang, Y., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653. doi: 10.1105/tpc.014894

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y. T., He, J., et al. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18220–18225. doi: 10.1073/pnas.1005225107

Zhang, Z., Li, Q., Li, Z., Staswick, P. E., Wang, M., Zhu, Y., et al. (2007). Dual regulation role of GH3.5 in salicylic acid and auxin signaling during *Arabidopsis-Pseudomonas syringae* interaction. *Plant Physiol.* 145, 450–464. doi: 10.1104/pp.107.106021

Zheng, X. Y., Spivey, N. W., Zeng, W., Liu, P. P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596. doi: 10.1016/j.chom.2012.04.014

Zheng, Z., Qualley, A., Fan, B., Dudareva, N., and Chen, Z. (2009). An important role of a BAHD acyl transferase-like protein in plant innate immunity. *Plant J.* 57, 1040–1053. doi: 10.1111/j.1365-313X.2008.03747.x

Zhou, J. M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., et al. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol. Plant Microbe Interact.* 13, 191–202. doi: 10.1094/MPMI.2000.13.2.191

Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F., and Glazebrook, J. (1998). PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell* 10, 1021–1030. doi: 10.1105/tpc.10.6.1021

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 October 2014; accepted: 24 November 2014; published online: 09 December 2014.

Citation: Seyfferth C and Tsuda K (2014) Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. Front. Plant Sci. 5:697. doi: 10.3389/fpls.2014.00697

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in Plant Science

REVIEW
published: 13 March 2015
doi: 10.3389/fpls.2015.00154

Cullin-RING ubiquitin ligases in salicylic acid-mediated plant immune signaling

James J. Furniss and Steven H. Spoel *

Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Edinburgh, UK

Plant immune responses against biotrophic pathogens are regulated by the signaling hormone salicylic acid (SA). SA establishes immunity by regulating a variety of cellular processes, including programmed cell death (PCD) to isolate and kill invading pathogens, and development of systemic acquired resistance (SAR) which provides long-lasting, broad-spectrum resistance throughout the plant. Central to these processes is post-translational modification of SA-regulated signaling proteins by ubiquitination, i.e., the covalent addition of small ubiquitin proteins. Emerging evidence indicates SA-induced protein ubiquitination is largely orchestrated by Cullin-RING ligases (CRLs), which recruit specific substrates for ubiquitination using interchangeable adaptors. Ligation of ubiquitin chains interlinked at lysine 48 leads to substrate degradation by the 26S proteasome. Here we discuss how CRL-mediated degradation of both nucleotide-binding/leucine-rich repeat domain containing immune receptors and SA-induced transcription regulators are critical for functional PCD and SAR responses, respectively. By placing these recent findings in context of knowledge gained in other eukaryotic model species, we highlight potential alternative roles for processive ubiquitination in regulating the activity of SA-mediated immune responses.

Keywords: Cullin-RING ligase (CRL), ubiquitin ligase, salicylic acid (SA), NPR1, plant immunity, proteasome, transcription activator, gene expression

OPEN ACCESS

Edited by:
Loreto Holuigue, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Kemal Kazan, Commonwealth Scientific and Industrial Research Organization, Australia
Roger W. Innes, Indiana University Bloomington, USA

***Correspondence:**
Steven H. Spoel, Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JR, UK
steven.spoel@ed.ac.uk

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *09 December 2014*
Paper pending published: *27 January 2015*
Accepted: *26 February 2015*
Published: *13 March 2015*

Citation:
Furniss JJ and Spoel SH (2015) Cullin-RING ubiquitin ligases in salicylic acid-mediated plant immune signaling. Front. Plant Sci. 6:154. doi: 10.3389/fpls.2015.00154

Introduction

Successful plant immune responses depend on the rapid recognition of the invading pathogen and subsequent local and systemic transmission of signals that induce resistance throughout all plant tissues. Pattern recognition receptors that recognize conserved pathogen-associated molecular patterns represent the first line of defense, leading to pattern-triggered immunity (Macho and Zipfel, 2014). To subvert immune responses, adapted pathogens have evolved an arsenal of effector proteins that suppress pattern-triggered immunity. The presence of these effector proteins can be sensed by intracellular nucleotide-binding/leucine-rich repeat domain containing (NLR) immune receptors, resulting in effector-triggered immunity (Jones and Dangl, 2006; van Ooijen et al., 2007). Effector-triggered immunity is characterized by rapid onset of programmed cell death (PCD) at the site of infection, which is thought to isolate and prevent proliferation of the invading pathogen. Following pathogen recognition, development of pattern- and effector-triggered immunity requires the immune signaling hormone salicylic acid (SA). Failure to accumulate SA upon pathogen attack results in severe disease susceptibility and inability to launch NLR receptor-mediated PCD (Delaney et al., 1994; Rairdan and Delaney, 2002). Additionally, SA

accumulates in tissues adjacent and distant to the site of infection where it induces systemic acquired resistance (SAR), a long-lasting immune response effective against a broad -spectrum of pathogens (Spoel and Dong, 2012; Fu and Dong, 2013). A major function of SA is to initiate reprogramming of the transcriptome to prioritize immune responses over other cellular functions. Accordingly, SA fine-tunes the activity of a network of SA-responsive transcriptional regulators, the concerted action of which establishes disease resistance (Moore et al., 2011).

Recent work has highlighted an important role for the ubiquitin-mediated proteasome system in regulating many aspects of SA-dependent immunity. In eukaryotic cells, post-translational modification by a single or polymeric chain of ubiquitin modulates protein function and stability (Komander and Rape, 2012). Ubiquitin is a highly conserved, small protein (8.5 kDa) that is covalently attached to a target substrate in a multistep enzymatic pathway. First, a ubiquitin-activating E1 enzyme forms a high-energy thioester linkage to a ubiquitin moiety, which is then passed onto an active-site cysteine residue of a ubiquitin-conjugating E2 enzyme. The E2 enzyme works in physical partnership with an E3 ligase to attach ubiquitin onto a specific lysine (Lys) ε-amino group within the target substrate (Smalle and Vierstra, 2004; Komander and Rape, 2012). Compared to many other eukaryotes, plant genomes encode for disproportionally large numbers of E3 ligases; for example, the *Arabidopsis* genome contains over 1400 different predicted E3 ligase components (Vierstra, 2009), suggesting that protein ubiquitination plays critical roles in plant biology. E3 ligases selectively recruit substrates for ubiquitination and thus provide an important level of specificity to the ubiquitination machinery. E3 ligases can be categorized into different classes based on the presence of a RING, U-box, or HECT domain, leading to distinct ways of binding a partner E2 conjugating enzyme. In addition to single polypeptide E3 ligases, the modular multi-subunit family of Cullin-RING Ligases (CRLs) plays prominent roles in protein ubiquitination. The Cullin subunit of CRLs acts as a scaffold to bring together the RING domain-containing protein and a variable adaptor that recruits the target protein (Santner and Estelle, 2009; Vierstra, 2009; Sadanandom et al., 2012). Emerging evidence suggests that plant immune signaling is predominantly mediated by CRL1 (also known as SCF for SKP1/Cullin1/F-box) and CRL3 [also denoted as BC3B for BTB (Bric-à-brac, Tramtrack, and Broad complex)/Cullin3/BTB], which recruit substrate adaptors that contain F-box motifs or BTB domains, respectively.

Although substrate ubiquitination by E3 ligases can have various functions depending on chain topology and length (Komander and Rape, 2012; Walsh and Sadanandom, 2014), ubiquitin chain linkage via Lys48 signals for degradation of the substrate by the 26S proteasome, a large (2.5 MDa) ATP-dependent chambered protease containing over 30 distinct subunits (Pickart and Cohen, 2004).

Several excellent comprehensive reviews are available on the role of ubiquitination in plant immune signaling in general (Trujillo and Shirasu, 2010; Marino et al., 2012; Duplan and Rivas, 2014). Instead, here we specifically focus on recent advances in understanding the function of ubiquitination in SA-induced immune signaling. How processive ubiquitination and degradation of transcription activators may underpin SA-responsive gene expression in local and systemic immunity will be discussed, as well as how CRLs play an integral part of cellular decisions of life and death upon pathogen recognition.

Ubiquitin-Mediated Suppression of SA-Responsive Gene Transcription

Genetic screens for SA-insensitive *Arabidopsis* mutants have repeatedly identified *npr1* (*non-expresser of PR genes*) mutant alleles (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). *NPR1* encodes a transcription coactivator that in resting cells forms a high molecular weight oligomer in the cytoplasm through intermolecular disulfide bonds between conserved cysteine residues, preventing it from entering the nucleus. Pathogen-induced SA accumulation triggers transient cellular redox changes, resulting in reduction of these disulfide bonds, and release of NPR1 monomers (Mou et al., 2003; Tada et al., 2008). NPR1 monomer translocates to the nucleus where it controls the expression of over 2,200 genes in *Arabidopsis* (Kinkema et al., 2000; Wang et al., 2006), in part by physically interacting with and transactivating TGA transcription factors that associate with SA-responsive gene promoters (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Boyle et al., 2009). NPR1 protein contains an N-terminal BTB domain and a C-terminal ankyrin repeat domain (Cao et al., 1997; Ryals et al., 1997; Aravind and Koonin, 1999). Interestingly, the presence of these domains in a single protein is a typical feature of a substrate adaptor for CRL3, in which the BTB domain mediates interaction with Cullin 3, while the ankyrin repeat recruits substrates for ubiquitination (Petroski and Deshaies, 2005). However, yeast two-hybrid studies were unable to find direct physical interaction between Cullin 3 and NPR1 (Dieterle et al., 2005). Co-immunoprecipitation experiments nevertheless showed that NPR1 associates with a CRL3 *in planta* (Spoel et al., 2009). These results suggested that NPR1 may not be in the substrate adaptor position of this E3 ligase. Indeed, in *Arabidopsis* cells, monomeric NPR1 is itself subject to ubiquitination by a CRL3 and is subsequently degraded in the nucleus. Blocking NPR1 degradation pharmacologically with proteasome inhibitors or genetically by mutation of Cullin 3 resulted in accumulation of NPR1 monomer, moderate induction of NPR1 target genes, and elevated resistance to pathogen infection (Spoel et al., 2009). This indicated that constitutive degradation of NPR1 monomer by CRL3 prevents autoimmunity in absence of a pathogen threat. This suppressive effect of CRL3 and the proteasome probably impacts a large proportion of the immune transcriptome, as many genes are co-regulated by SA and proteasome inhibitor (Spoel et al., 2010).

Ubiquitin-mediated protein degradation plays a similar role in SA-dependent immune responses in rice. Analogous to the function of *Arabidopsis* NPR1, *Oryza sativa* WRKY45 is an SA-induced transcription activator of several hundred

immune-related genes and confers resistance to bacterial and fungal pathogens (Shimono et al., 2007, 2012; Nakayama et al., 2013). Inhibition of the proteasome resulted in accumulation of polyubiquitinated OsWRKY45 in the nucleus and constitutive activation of its target genes in the absence of SA treatment (Matsushita et al., 2013). Although it remains unknown if OsWRKY45 is targeted for degradation by a CRL3, these findings indicate that constitutive turnover of this immune activator prevents autoimmune responses. SA also activates an NPR1-like protein, which functions in parallel with OsWRKY45 to regulate immune transcription in rice. By contrast to OsWRKY45, this OsNPR1 protein (also known as OsNH1) is thought to be predominantly involved in downregulation of gene expression, particularly those involved in photosynthetic activity (Sugano et al., 2010). Interestingly, OsNPR1 is not subject to constitutive proteasome-mediated degradation, intuitively suggesting that transcriptional repression does not require corepressor turnover. Hence, the presence of analogous proteasome-regulated modules consisting of unrelated transcription (co)activators in *Arabidopsis* and rice (i.e., NPR1 versus OsWRKY45) may reflect inherent constraints on how timely activation of SA-responsive immune genes can be achieved.

Ubiquitin-Mediated Activation of SA-Responsive Gene Transcription

Besides suppression of SA-responsive immune genes, the proteasome is also involved in gene activation. Pharmacological inhibition of the proteasome, genetic mutation of Cullin 3, and mutation of an NPR1 phosphorylation motif all stabilized the NPR1 protein but greatly reduced the SA-induced expression of its target genes in *Arabidopsis* (Spoel et al., 2009). Similarly, SA-induced transcriptional activity of OsWRKY45 in rice was impaired in the presence of proteasome inhibitor (Matsushita et al., 2013). Turnover of OsWRKY45 was dependent on a small 26 amino acid C-terminal region, which importantly was also required for its transactivation activity. Such overlap between transactivation domains and degradation motifs that signal ubiquitin-mediated proteasomal degradation has previously been discovered in transcription activators in both yeast and mammals (Salghetti et al., 2000). Fusion of well-defined degron motifs from yeast cyclin proteins to a DNA-binding domain even auto-activated gene transcription (Salghetti et al., 2000), suggesting that the intrinsic ability to activate transcription also makes activators a target for the ubiquitin-mediated proteasome. Additional work showed that like NPR1 and OsWRKY45, other activators also required turnover to unleash their full transcriptional potential (Spoel et al., 2010; Geng et al., 2012). This transcription process, sometimes dubbed 'destruction–activation', has been studied in more detail for GCN4 (General Control Non-inducible 4), a potent activator of genes involved in amino acid homeostasis. Upon amino acid starvation, the CDC4 F-box subunit of SCF^{CDC4} ligase targets GCN4 for ubiquitin-mediated degradation, a process required for recruitment of RNA Polymerase II (RNAPII) to GCN4 target genes (Lipford et al., 2005). Crucially, GCN4 was marked for degradation by the phosphorylative action of SRB10, a cyclin-dependent-kinase associated with the C-terminal domain of RNAPII (Liao et al., 1995; Chi et al., 2001). This indicates that when GCN4 initiates a round of transcription by recruiting RNAPII, it simultaneously triggers its own destruction. These results have led to the hypothesis that transcriptionally 'spent' activators may need to be cleared by the proteasome to reset target promoters and allow binding of 'fresh' activators (**Figure 1**; Lipford et al., 2005; Kodadek et al., 2006; Geng et al., 2012). A similar mode of regulation may control transcriptional activity of NPR1 and OsWRKY45 in plant immunity, as site-specific phosphorylation of a degron motif in NPR1was necessary for its ubiquitination and degradation, as well as for timely and sustained target gene expression (Spoel et al., 2009, 2010). Intriguingly, transcription initiation by MYC2, a transcription activator responsive to the developmental and immune hormone jasmonic acid, is also regulated by phosphorylation-induced proteasomal degradation (Zhai et al., 2013). These findings imply that proteasome-mediated regulation of transcription activators may be a general mechanism to control gene expression programs in plant immunity.

Elegant studies on the estrogen receptor ERα in mammalian cells have shed more light on why activators are turned over in the process of activating gene transcription. Upon ligand

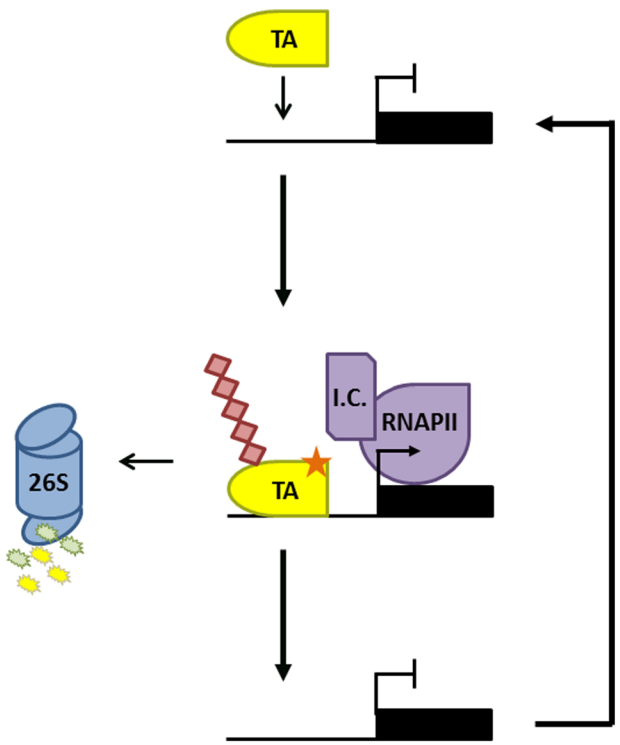


FIGURE 1 | Proteasome-mediated activator turnover activates transcription. Promoter binding of a transcription activator (TA) results in recruitment of the transcription initiation complex (IC) and RNA Polymerase II (RNAPII). The TA is subsequently phosphorylated (orange star) by a kinase within the IC, marking it for ubiquitination (red diamonds) and degradation by the 26S proteasome. This allows a new TA to bind the promoter and reinitiate a new round of gene transcription.

binding, nuclear localized ERα forms a stable dimer, and associates with cofactors on estrogen-responsive DNA elements to trigger gene transcription. Not only did inhibition of ERα proteolysis suppress its transcriptional activity, *vice versa* inhibition of RNAPII prevented degradation of ERα, indicating that activator turnover and transcriptional activity were interdependent (Reid et al., 2003). By following ERα transactivation over fine time scales by chromatin immunoprecipitation, it was proposed that ERα-mediated transcription may have distinct cyclical phases in which the ubiquitin-mediated proteasome plays key roles (Metivier et al., 2003). In this model, the first cycle is transcriptionally non-productive but results in ERα-induced remodeling of the promoter to commit it to transcription. In subsequent cycles ERα orchestrates the ordered recruitment of cofactors, ultimately resulting in gene transcription via recruitment of RNAPII. Importantly, experimental data showed that the proteasome was recruited to an ERα target promoter toward the end of each cycle and preceded the clearance of ERα and general transcription cofactors. Thus, proteasome activity is thought to be vital to allow ERα-dependent promoters to move from the transcriptionally non-productive to productive phase and to permit productive cycles to continue until transcription is no longer required (Metivier et al., 2003; Zhou and Slingerland, 2014). If these findings indeed represent a general model for transcription regulation, then the proteasome could have additional roles in SA-responsive gene transcription in plants, including promoter remodeling and ordered cofactor degradation.

But why would cyclical activation of transcription by unstable activators be advantageous over continuous activation by stable activators? Although the answer to this question remains at large, a recent mathematical and *in silico* analysis of proteasome involvement in transcription may have provided some clues (Lee et al., 2014). The gene targets of many mammalian transcription activators often include components of E3 ligases that promote proteolysis of that activator, generating a negative feedback loop to maintain appropriate levels of activator. Mathematical modeling of this feedback loop showed that cellular perturbations resulting in destabilization of the E3 ligase led to over-accumulation of activators and subsequent hyper-activation of gene expression. However, if the E3 ligase was modeled as a necessary transcription cofactor working in conjunction with the activator, a much more measured gene expression output was achieved upon cellular perturbation. These models suggest that the paradoxical involvement of E3 ligases in gene transcription activated by unstable activators may be necessary to provide a cellular safety mechanism. The authors of this work compared this to the principle of safety interlock devices in engineering, where a system will not function unless safety can be guaranteed (Lee et al., 2014). A similar system may be operational for NPR1- and OsWRKY45-dependent gene expression. Notably, interrogation of a list of NPR1-dependent genes provided by Wang et al. (2006) indicates that NPR1 activates the expression of genes encoding for its paralogues, NPR3, and NPR4. These BTB-containing proteins function as substrate adaptors that recruit NPR1 to CRL3 for ubiquitination and subsequent degradation (Fu et al., 2012). This suggests that similar to the mathematical system described above, a negative feedback loop may exist between NPR1 and $CRL3^{NPR3/NPR4}$. As CRL3 has a supportive role in NPR1-dependent gene transcription (Spoel et al., 2009), it may be part of a cellular safety mechanism to keep NPR1 activity in check when cellular perturbations are encountered. In support of this hypothesis, although genetic perturbations of $CRL3^{NPR3/NPR4}$ activity resulted in autoimmune phenotypes due to over-accumulation of NPR1 protein, this did not lead to over-activation of NPR1 target genes in the presence of SA (Spoel et al., 2009; Fu et al., 2012).

Processive Ubiquitination of Transcription Activators

In plants, research has mainly focused on polyubiquitination as a means of regulating protein degradation. However, recent advances in understanding processive ubiquitination in several eukaryotes have highlighted that ubiquitin may have additional important roles in the control of plant transcription factors. The notion that ubiquitin may be directly involved in transcription activation was first explored in yeast. Transcription induced by an artificial activator consisting of the yeast VP16 transactivation domain and the bacterial LexA DNA binding protein (LexA-VP16), was shown to require ubiquitination and degradation mediated by the F-box protein MET30. Strikingly, when ubiquitin was fused in-frame to LexA-VP16, the requirement for MET30 was completely bypassed (Salghetti et al., 2001), suggesting that ubiquitination has dual functions to both activate and destroy transcription activators. Subsequently, additional studies indicated roles for monoubiquitination in transcription activation (Bres et al., 2003; Greer et al., 2003; Burgdorf et al., 2004). Monoubiquitination does not usually signal for proteasome-mediated degradation, for which approximately four or more Lys48-linked ubiquitins are required (Thrower et al., 2000). Instead it was reported that promoter occupancy of the yeast prototypical transcription activator, GAL4, was stabilized by monoubiquitination (Ferdous et al., 2007; Archer et al., 2008b). Interestingly, unmodified GAL4 was destabilized by ATPase activity of the proteasome 19S regulatory particle, preventing transcription activation. Monoubiquitination limited the lifetime of physical interactions between the GAL4 activation domain and 19S subunits (**Figure 2A**; Archer et al., 2008a). This type of regulatory system likely extends to many other eukaryotes, as interactions between tumor suppressor protein p53, a transcription activator in mammalian cells, and its target promoters were also destabilized by 19S ATPases (Kim et al., 2009).

In contrast to these reports, examples of monoubiquitination leading to suppression of transcription activators have also emerged (Peloponese et al., 2004; Inui et al., 2011; Tang et al., 2011; Ndoja et al., 2014). Using the artificial LexA-VP16 activator described above, a recent report argued that in-frame fusion of ubiquitin to LexA-VP16 was susceptible to cleavage by deubiquitinases (DUBs). Preventing deubiquitination by introducing a non-cleavable ubiquitin-LexA-VP16 mutant resulted in suppression of transcriptional activity by the AAA^+ ATPase, CDC48, which stripped this activator from its target promoter (**Figure 2B**; Ndoja et al., 2014). These findings were extended from artificial

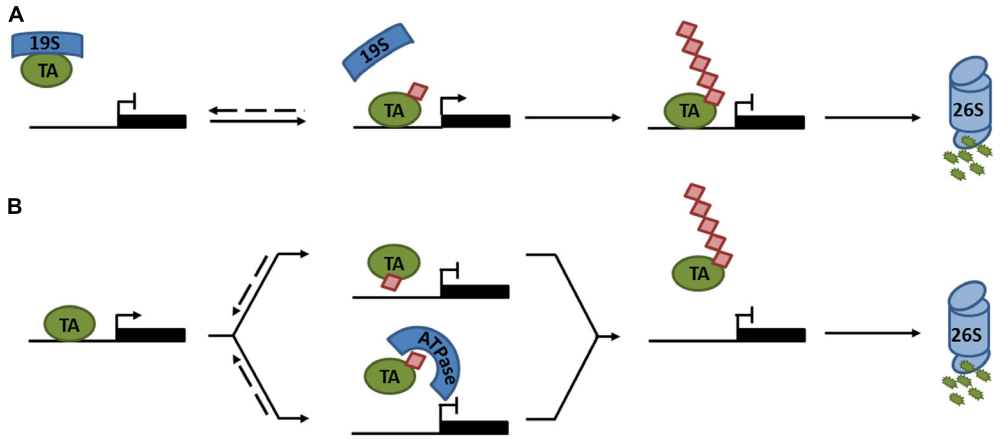


FIGURE 2 | Regulation of transcription activator activity by monoubiquitination. (A) The 19S proteasome subcomplex binds an unmodified transcription activator (TA), preventing it from associating with its target promoter. Monoubiquitination of the TA disrupts association with the 19S subcomplex, allowing the TA to bind its target promoter and activate gene expression. Subsequent polyubiquitination marks the TA for degradation by the 26S proteasome. **(B)** Monoubiquitination of a TA prevents transcription either by sterically hindering the binding to its target promoter (top) or by recruiting an ATPase that prevents it from associating with its target promoter (bottom). Subsequent polyubiquitination marks the TA for degradation by the 26S proteasome. Dashed lines indicate reversible steps.

to native transcription activators. CDC48 was implicated in yeast sulfur metabolism by removing the monoubiquitinated transcriptional activator, MET4, from its target promoters upon ubiquitination by SCF^{MET30} (Ndoja et al., 2014). Moreover, monoubiquitination of mammalian receptor-activated SMADs (R-SMAD), involved in TGF-β-mediated embryonic development and tissue homeostasis, attenuated its transcriptional activity by two possible mechanisms: (i) monoubiquitination prevented either R-SMAD transcription complex formation or DNA binding by steric hindrance; or (ii) the CDC48 homolog, p97, actively removed monoubiquitinated R-SMADs from the promoter (**Figure 2B**; Inui et al., 2011; Tang et al., 2011; Ndoja et al., 2014). Taken together, all these reports clearly illustrate that monoubiquitination can directly regulate the activity of transcription activators through a variety of different mechanisms (**Figure 2**). Additionally, monoubiquitination may indirectly regulate the activities of some activators by modulating their nucleocytoplasmic localization (van der Horst et al., 2006).

While monoubiquitination may play a regulatory role, processive ubiquitin chain elongation subsequently leads to activator turnover (Kodadek et al., 2006). This processive mono-to-polyubiquitination switch was explored in particular detail for the human Steroid Receptor Coactivator-3 (SRC-3). SRC-3 is an important developmental transcription coactivator, whose uncontrolled expression can lead to oncogenesis. SRC-3 was found to be subject to phosphorylation-dependent polyubiquitination by $SCF^{Fbw7\alpha}$, resulting in its transcription-coupled degradation. However, SRC-3 was also multi (mono)-ubiquitinated by $SCF^{Fbw7\alpha}$, which enhanced its transcriptional activity. Hence, it was proposed that biphasic, processive ubiquitination (i.e., transitioning from mono- to polyubiquitination) generates a timer for the functional lifetime of SRC-3 (Wu et al., 2007).

These intriguing findings relating to eukaryotic transcription indicate that ubiquitin-mediated control of transcription (co)activators in SA-dependent immunity is far more complex than generally appreciated. Current efforts in this field by several labs, including our own, may soon reveal additional roles for ubiquitin and ubiquitin ligases in the transcription activation of immune genes.

CRL3-Mediated Degradation of SA-Responsive Repressors?

In the past decade intimate relationships between plant hormone signaling and the ubiquitin-mediated proteasome have been uncovered. Recurring roles for CRL1 and CRL3 are found in jasmonic acid, ethylene, auxin, gibberellin, abscisic acid, strigolactone, and zeatin signaling (Kelley and Estelle, 2012). The role of CRL1 in jasmonic acid- and auxin-responsive gene expression is especially similar. In both cases the hormone facilitates physical interaction of the CRL1 F-box subunit with transcriptional repressors to form a hormone coreceptor complex. Hormone-dependent recruitment of repressors to CRL1 leads to their poly-ubiquitination and degradation, releasing the activity of transcriptional activators (Kelley and Estelle, 2012). A strikingly similar hormone perception mechanism regulates SA signaling, but instead utilizes CRL3. The CRL3 substrate adaptors NPR3 and NPR4 were shown to act as SA receptors. Whereas SA binding facilitated interaction between NPR3 and NPR1, it disrupted NPR4-NPR1 interaction. Moreover, genetic deletion of *NPR3* and *NPR4* severely impaired the ability to coimmunoprecipitate Cullin 3 and NPR1, indicating that NPR1 is the substrate of an SA-sensitive $CRL3^{NPR3/NPR4}$ (Fu et al., 2012).

It is likely that CRL3 complexes exist with roles that extend beyond targeting NPR1. In analogy to jasmonic acid and auxin signaling, CRL3 could target a number of transcription (co)repressors described for SA-responsive genes. For

example, TGA2 transcription factors act as repressors of *PR* genes (Zhang et al., 2003; Kesarwani et al., 2007). Moreover, NPR3 and NPR4 physically interact with TGA2 and other members of the TGA family (Liu et al., 2005; Zhang et al., 2006; Shi et al., 2013), implying that a $CRL3^{NPR3/4}$ might target TGA factors for degradation to activate SA-responsive genes. Other conceivable targets of CRL3 include SNI1 (Suppressor of NPR1, Inducible), a corepressor of mostly NPR1-depenent genes (Mosher et al., 2006). SNI1 was recently shown to associate with CBNAC, a calmodulin-binding NAC transcription factor. Genetic analysis suggested that CBNAC is a transcription repressor of SA-dependent immune responses. Interestingly, SNI1 facilitated the binding of CBNAC to a DNA-binding motif in the SA-responsive *PR-1* promoter (Kim et al., 2012). Finally, several NPR1-interacting NIMIN (NIM1/NPR1-Interacting) proteins act as corepressors, and their removal or inactivation is presumable necessary for activation of SA-responsive gene expression (Weigel et al., 2005). Thus, CRL3 targets could include SNI1, CBNAC, TGA factors, and NIMINs, but little is currently known about the stability of these (co)repressors. Analysis of transcription (co)factor interaction networks in rice between OsNPR paralogues, TGA factors, and NRR (Negative Regulator of Resistance) proteins that share limited homology to *Arabidopsis* NIMINs, paint a similar picture (Chern et al., 2014). All these factors formed a wide network of interactions in both yeast two-hydrid and split YFP assays, suggesting that involvement of CRL3 complexes in immunity may be functionally conserved in rice.

Alternative to direct targeting of (co)repressors by CRL3, a recent report suggests that these ubiquitin ligases can also promote the concurrent ubiquitination of multiple associated substrates. Upon light induction, the transcription factor PIF3 is recruited to $CRL3^{LRB}$ for ubiquitination. Strikingly, it was found that the PIF3 interaction partner, PhyB, was concomitantly recruited by $CRL3^{LRB}$ (Ni et al., 2014). CRL3 dimerisation through BTB domains might facilitate concurrent substrate degradation, essentially bringing together two active sites for substrate ubiquitination (Stogios et al., 2007). Hence, it plausible that $CRL3^{NPR3/NPR4}$ simultaneously targets complexes consisting of NPR1 and the transcriptional repressors that physically interact with NPR1.

Peculiarly, unlike NPR3 and NPR4, NPR1 has not yet been observed in the substrate adaptor position of a CRL3 (Dieterle et al., 2005; Fu et al., 2012). However, computational predictions of NPR1 protein structure suggest that it forms a typical BTB domain fold that should allow interaction with Cullin 3 (Tada et al., 2008). Moreover, immediately C-terminal to the BTB domain, NPR1, NPR3, and NPR4 all contain key elements of a conserved helical 3-box structure that, analogous to the F-box motif, was shown to stimulate Cullin 3 interaction by packing tightly against its N-terminus (Zhuang et al., 2009; Canning et al., 2013). Reports that NPR1 itself may directly sense SA or may also be a SA receptor (Maier et al., 2011; Wu et al., 2012) further suggests that NPR1 could be part of a CRL analogous to other plant hormone pathways, although definitive proof for NPR1 as an SA receptor was not supported by another study (Fu et al., 2012).

If NPR1 does indeed reside in a substrate adaptor position of CRL3, this would have important implications for the role of its own turnover in SA-responsive gene expression. First, this would create an additional layer of complexity whereby a $CRL3^{NPR3/NPR4}$ regulates the formation of a $CRL3^{NPR1}$. Secondly, CRL substrate adaptors often paradoxically exhibit instability themselves. In absence of a substrate, both F-box and BTB adaptors have been shown to be subject to auto-ubiquitination within their respective CRLs (Bosu and Kipreos, 2008). The necessity of NPR1 turnover in activation of SA-responsive genes may therefore reflect a requirement to allow switching of diverse NPR substrate adaptors within core CRL3 complexes (**Figure 3**).

CRLs in SA-Mediated Programmed Cell Death and Survival

Salicylic acid is an agonist of PCD responses induced by NLR immune receptors upon intracellular detection of pathogen effectors. In some cases cellular decisions to live or die upon pathogen infection are shaped by the activities of CRLs. Mutation of $CRL3^{NPR3/NPR4}$ components suggested that the stability of its substrate, NPR1, is an important determinant in PCD induced by the NLR receptors RPS2 and RPM1 (Fu et al., 2012). Indeed, analysis of pathogen-induced PCD in *npr1* mutants previously revealed that NPR1 suppressed PCD induced by these NLR receptors (Rate and Greenberg, 2001). Moreover, mutation of *NPR1* partially restored RPS2- and RPM1-induced PCD in *npr3* and *npr4* mutants (Fu et al., 2012). These results indicate that elevated levels of NPR1 promote cell survival and that its removal by $CRL3^{NPR3/NPR4}$ is required for successful PCD induced by at least some NLR receptor classes (**Figure 4**). In agreement with its role in promoting cell survival, genetic experiments have indicated that the presence of NPR1 is not essential for successful NLR receptor-induced PCD and immunity (Rairdan and Delaney, 2002).

In contrast to NPR1's pro-survival role, NLR receptors instigate PCD responses upon perception of pathogen effectors. In the absence of pathogen threats, NLR receptors must be kept tightly controlled to avoid autoimmune responses. Overexpression of the tomato NLR receptor, Prf, resulted in strong autoimmune phenotypes, including elevated SA levels and expression of SA-responsive genes in absence of pathogen attack (Oldroyd and Staskawicz, 1998). Moreover, overexpression of NLR receptors due to genomic duplication in the *Arabidopsis bal* variant also led to constitutive SA responses in absence of a pathogen as well as morphological defects such as severely stunted growth, highlighting the trade-off between growth and defense (Stokes et al., 2002; Yi and Richards, 2009). Similarly, mutation of the potential transcription corepressor, SRFR1, resulted in autoimmunity due to transcriptional upregulation of the co-regulated NLR receptors SNC1, RPS2, and RPS4 (Kwon et al., 2009; Kim et al., 2010; Li et al., 2010). Collectively these examples illustrate that the cellular protein levels of some NLR receptors are linked to their immune activities.

Recent work revealed that protein levels of several NLR receptors are tightly controlled by CRL activities. An early screen for mutations leading to SA-mediated autoimmune phenotypes

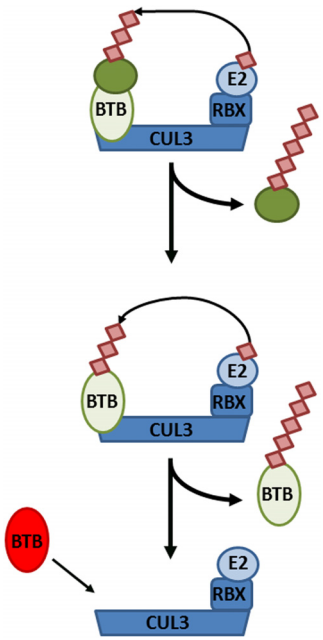


FIGURE 3 | Cullin-RING ligase 3 (CRL3) autoubiquitination and adaptor switching. (Top) A target substrate (dark green circle) is polyubiquitinated and targeted for degradation by CRL3, consisting of the CUL3 backbone, Bric-à-brac, Tramtrack, and Broad complex (BTB) domain-containing adaptor, RING-Box protein (RBX), and an E2 conjugating enzyme. **(Middle)** After all available substrates have been polyubiquitinated and degraded, the BTB adaptor itself becomes subject to autoubiquitination and degradation. **(Bottom)** Consequently, the CRL3 can now switch to a new BTB adaptor in order to polyubiquitinate different substrates.

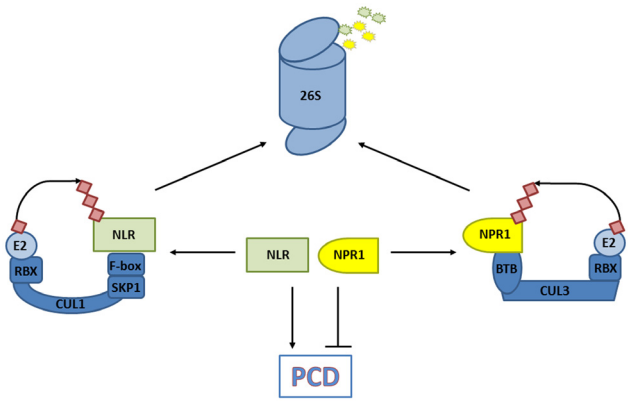


FIGURE 4 | CRLs decide on cell fate. The degradation of substrates that promote programmed cell death (PCD) in response to a pathogen [e.g., nucleotide-binding/leucine-rich repeat domain containing (NLR) immune receptors], and those that prevent PCD (e.g., non-expresser of PR genes, NPR1) are controlled by CRLs. The balance of substrate abundance between promoters and suppressors of PCD dictates cell fate, and is regulated by their ubiquitination and 26S proteasome-mediated degradation.

identified the *cpr1* (*constitutive expressor of PR genes*) mutant (Bowling et al., 1994). Importantly, protein levels of the NLR receptors SNC1 and RPS2 were inversely correlated with CPR1 activity, and loss-of-function mutations in *SNC1* largely suppressed the autoimmune phenotype of mutant *cpr1* plants. Cloning of *CPR1* revealed it encodes an F-box protein, suggesting it controls the abundance of specific NLR receptors by targeting them for proteasome-mediated degradation. Indeed, CPR1 directly interacted with SNC1 and RPS2, and in case of SNC1 this appeared to lead to its polyubiquitination and degradation by the proteasome (Cheng et al., 2011; Gou et al., 2012). NLR receptor signaling probably involves other CRL1 ubiquitin ligases as well but with distinct functions. Rather than eliciting autoimmunity, silencing of the F-box protein ACIF1 in tobacco and tomato compromised NLR receptor-mediated PCD and immunity (van den Burg et al., 2008). ACIF1 interacted with other CRL1 subunits, suggesting it can form a functional ubiquitin ligase but its direct targets remain unknown. Notably, several non-CRL ubiquitin ligases that regulate NLR accumulation or signaling have also been identified and are discussed in other excellent reviews (Marino et al., 2012; Duplan and Rivas, 2014). Hence, ubiquitin ligases – and CRLs in particular – play an integral role in cellular decisions of life and death by controlling the level of NLR receptors and PCD suppressors (**Figure 4**).

In addition to canonical ubiquitination pathway enzymes (E1, E2, E3), an E4 class of ubiquitin ligases has been described (Koegl et al., 1999). E4 ligases also polyubiquitinate substrates, but contrary to E3 ligases, they largely lack substrate specificity and rather function to elongate existing ubiquitin chains, thereby potentially promoting recognition of substrates by the proteasome. A recent forward genetic screen for mutants that enhanced autoimmunity of *snc1*, a mutation that renders this NLR receptor constitutively active, identified the E4 ligase MUSE3 (Mutant *snc1*-Enhancing; Huang et al., 2014). Mutant *muse3* plants exhibited elevated levels of SNC1 and RPS2, while overexpression of MUSE3 in itself did not lead to reduction of NLR receptor accumulation. However, coexpression of MUSE3 together with the F-box protein CPR1 resulted in a greater decrease in NLR receptor accumulation than observed with CPR1 expression alone, indicating that MUSE3 and SCF^{CPR1} function cooperatively to destabilize NLR receptors. In case of SNC1 but not RPS2, a direct physical association with MUSE3 was indeed found, suggesting that MUSE3 may recognize NLR receptors via distinct mechanisms. Thus, an intricate set of cooperative ubiquitin ligases underpin SA-dependent NLR receptor signaling to prevent autoimmunity and promote timely activation of immune responses.

The Road Ahead. . .

In this review we have discussed the emerging roles of ubiquitin ligases in aspects of SA-mediated immune signaling, including transcriptional reprogramming and cellular decisions of life and death. Similar to other hormone signaling pathways, members of the CRL class of ubiquitin ligases appear to fulfill particularly important tasks, although the targets of these CRLs still remain largely unknown. In SA-induced gene transcription the precise

role of CRL3-mediated turnover of NPR1 requires further investigation into processive ubiquitination events and it remains to be discovered if immune-induced CRL3 targets substrates other than NPR1 for proteasome-mediated degradation. Although the role of CRLs in controlling the accumulation of specific NLR receptors is becoming increasingly clear, it remains poorly understood why protein abundance is a key factor in determining NLR receptor activity. Conformational control of NLR receptors by highly conserved eukaryotic chaperone complexes is thought to keep receptors in a recognition-competent state and facilitate their activation upon pathogen perception (Shirasu, 2009; van Ooijen et al., 2010). It is plausible that uncontrolled accumulation of some NLR receptors could result in a shortage of available chaperones and consequent conformation-induced autoactivation of NLR receptors. Finally, many E3 ligases construct ubiquitin chain topologies distinct from proteasome-recognized Lys48-linkages. These alternative chain topologies serve a wide variety of different cellular signaling functions in eukaryotes, yet little is known about their existence and roles in plant biology (Walsh and Sadanandom, 2014). Hence, much remains to be discovered in the exciting field of plant ubiquitin signaling in general and in SA-mediated immune responses in particular.

Acknowledgments

We thank Dr. Gerben van Ooijen and Dr. Michael Skelly for critically reading the manuscript and apologize to colleagues whose work we were not able to include. JJF was supported by a studentship from the Biotechnology and Biological Sciences Research Council (BBSRC) and SHS by a BBSRC grant (BB/L006219/1) and Royal Society University Research Fellowship (UF090321).

References

Aravind, L., and Koonin, E. V. (1999). Fold prediction and evolutionary analysis of the POZ domain: structural and evolutionary relationship with the potassium channel tetramerization domain. *J. Mol. Biol.* 285, 1353–1361. doi: 10.1006/jmbi.1998.2394

Archer, C. T., Burdine, L., Liu, B., Ferdous, A., Johnston, S. A., and Kodadek, T. (2008a). Physical and functional interactions of monoubiquitylated transactivators with the proteasome. *J. Biol. Chem.* 283, 21789–21798. doi: 10.1074/jbc.M803075200

Archer, C. T., Delahodde, A., Gonzalez, F., Johnston, S. A., and Kodadek, T. (2008b). Activation domain-dependent monoubiquitylation of Gal4 protein is essential for promoter binding in vivo. *J. Biol. Chem.* 283, 12614–12623. doi: 10.1074/jbc.M801050200

Bosu, D. R., and Kipreos, E. T. (2008). Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell Div.* 3, 7. doi: 10.1186/1747-1028-3-7

Bowling, S. A., Guo, A., Cao, H., Gordon, A. S., Klessig, D. F., and Dong, X. (1994). A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *Plant Cell* 6, 1845–1857. doi: 10.1105/tpc.6.12.1845

Boyle, P., Le Su, E., Rochon, A., Shearer, H. L., Murmu, J., Chu, J. Y., et al. (2009). The BTB/POZ domain of the *Arabidopsis* disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell* 21, 3700–3713. doi: 10.1105/tpc.109.069971

Bres, V., Kiernan, R. E., Linares, L. K., Chable-Bessia, C., Plechakova, O., Treand, C., et al. (2003). A non-proteolytic role for ubiquitin in Tat-mediated transactivation of the HIV-1 promoter. *Nat. Cell Biol.* 5, 754–761. doi: 10.1038/ncb1023

Burgdorf, S., Leister, P., and Scheidtmann, K. H. (2004). TSG101 interacts with apoptosis-antagonizing transcription factor and enhances androgen receptor-mediated transcription by promoting its monoubiquitination. *J. Biol. Chem.* 279, 17524–17534. doi: 10.1074/jbc.M313703200

Canning, P., Cooper, C. D. O., Krojer, T., Murray, J. W., Pike, A. C., Chaikuad, A., et al. (2013). Structural basis for Cul3 protein assembly with the BTB-Kelch family of E3 ubiquitin ligases. *J. Biol. Chem.* 288, 7803–7814. doi: 10.1074/jbc.M112.437996

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 6, 1583–1592. doi: 10.1105/tpc.6.11.1583

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., and Dong, X. (1997). The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57–63. doi: 10.1016/S0092-8674(00)81858-9

Cheng, Y. T., Li, Y., Huang, S., Huang, Y., Dong, X., Zhang, Y., et al. (2011). Stability of plant immune-receptor resistance proteins is controlled by SKP1-CULLIN1-F-box (SCF)-mediated protein degradation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 14694–14699. doi: 10.1073/pnas.1105685108

Chern, M., Bai, W., Ruan, D., Oh, T., Chen, X., and Ronald, P. C. (2014). Interaction specificity and coexpression of rice NPR1 homologs 1 and 3 (NH1 and NH3), TGA transcription factors and Negative Regulator of Resistance (NRR) proteins. *BMC Genomics* 15:461. doi: 10.1186/1471-2164-15-461

Chi, Y., Huddleston, M. J., Zhang, X., Young, R. A., Annan, R. S., Carr, S. A., et al. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev.* 15, 1078–1092. doi: 10.1101/gad.867501

Delaney, T. P., Friedrich, L., and Ryals, J. A. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6602–6606. doi: 10.1073/pnas.92.14.6602

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. (1994). A central role of salicylic acid in plant disease resistance. *Science* 266, 1247–1250. doi: 10.1126/science.266.5188.1247

Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P. R. (2000). The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12, 279–290. doi: 10.1105/tpc.12.2.279

Dieterle, M., Thomann, A., Renou, J. P., Parmentier, Y., Cognat, V., Lemonnier, G., et al. (2005). Molecular and functional characterization of *Arabidopsis* Cullin 3A. *Plant J.* 41, 386–399. doi: 10.1111/j.1365-313X.2004.02302.x

Duplan, V., and Rivas, S. (2014). E3 ubiquitin-ligases and their target proteins during the regulation of plant innate immunity. *Front. Plant Sci.* 5:42. doi: 10.3389/fpls.2014.00042

Ferdous, A., Sikder, D., Gillette, T., Nalley, K., Kodadek, T., and Johnston, S. A. (2007). The role of the proteasomal ATPases and activator monoubiquitylation in regulating Gal4 binding to promoters. *Genes Dev.* 21, 112–123. doi: 10.1101/gad.1493207

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Geng, F., Wenzel, S., and Tansey, W. P. (2012). Ubiquitin and proteasomes in transcription. *Annu. Rev. Biochem.* 81, 177–201. doi: 10.1146/annurev-biochem-052110-120012

Glazebrook, J., Rogers, E. E., and Ausubel, F. M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 143, 973–982.

Gou, M., Shi, Z., Zhu, Y., Bao, Z., Wang, G., and Hua, J. (2012). The F-box protein CPR1/CPR30 negatively regulates R protein SNC1 accumulation. *Plant J.* 69, 411–420. doi: 10.1111/j.1365-313X.2011.04799.x

Greer, S. F., Zika, E., Conti, B., Zhu, X. S., and Ting, J. P. (2003). Enhancement of CIITA transcriptional function by ubiquitin. *Nat. Immunol.* 4, 1074–1082. doi: 10.1038/ni985

Huang, Y., Minaker, S., Roth, C., Huang, S., Hieter, P., Lipka, V., et al. (2014). An E4 ligase facilitates polyubiquitination of plant immune receptor resistance proteins in *Arabidopsis*. *Plant Cell* 26, 485–496. doi: 10.1105/tpc.113.119057

Inui, M., Manfrin, A., Mamidi, A., Martello, G., Morsut, L., Soligo, S., et al. (2011). USP15 is a deubiquitylating enzyme for receptor-activated SMADs. *Nat. Cell Biol.* 13, 1368–1375. doi: 10.1038/ncb2346

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kelley, D. R., and Estelle, M. (2012). Ubiquitin-mediated control of plant hormone signaling. *Plant Physiol.* 160, 47–55. doi: 10.1104/pp.112.200527

Kesarwani, M., Yoo, J., and Dong, X. (2007). Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in *Arabidopsis*. *Plant Physiol.* 144, 336–346. doi: 10.1104/pp.106.095299

Kim, H. S., Park, H. C., Kim, K. E., Jung, M. S., Han, H. J., Kim, S. H., et al. (2012). A NAC transcription factor and SNI1 cooperatively suppress basal pathogen resistance in *Arabidopsis thaliana*. *Nucleic Acids Res.* 40, 9182–9192. doi: 10.1093/nar/gks683

Kim, S. H., Gao, F., Bhattacharjee, S., Adiasor, J. A., Nam, J. C., and Gassmann, W. (2010). The *Arabidopsis* resistance-like gene SNC1 is activated by mutations in SRFR1 and contributes to resistance to the bacterial effector AvrRps4. *PLoS Pathog.* 6:e1001172. doi: 10.1371/journal.ppat.1001172

Kim, Y. C., Wu, S. Y., Lim, H. S., Chiang, C. M., and Kodadek, T. (2009). Non-proteolytic regulation of p53-mediated transcription through destabilization of the activator•promoter complex by the proteasomal ATPases. *J. Biol. Chem.* 284, 34522–34530. doi: 10.1074/jbc.M109.017277

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.1105/tpc.12.12.2339

Kodadek, T., Sikder, D., and Nalley, K. (2006). Keeping transcriptional activators under control. *Cell* 127, 261–264. doi: 10.1016/j.cell.2006.10.002

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* 96, 635–644. doi: 10.1016/S0092-8674(00)80574-7

Komander, D., and Rape, M. (2012). The ubiquitin code. *Annu. Rev. Biochem.* 81, 203–229. doi: 10.1146/annurev-biochem-060310-170328

Kwon, S. I., Kim, S. H., Bhattacharjee, S., Noh, J. J., and Gassmann, W. (2009). SRFR1, a suppressor of effector-triggered immunity, encodes a conserved tetratricopeptide repeat protein with similarity to transcriptional repressors. *Plant J.* 57, 109–119. doi: 10.1111/j.1365-313X.2008.03669.x

Lee, D., Kim, M., and Cho, K. H. (2014). A design principle underlying the paradoxical roles of E3 ubiquitin ligases. *Sci. Rep.* 4, 5573. doi: 10.1038/srep05573

Li, Y., Li, S., Bi, D., Cheng, Y. T., Li, X., and Zhang, Y. (2010). SRFR1 negatively regulates plant NB-LRR resistance protein accumulation to prevent autoimmunity. *PLoS Pathog.* 6:e1001111. doi: 10.1371/journal.ppat.1001111

Liao, S. M., Zhang, J., Jeffery, D. A., Koleske, A. J., Thompson, C. M., Chao, D. M., et al. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. *Nature* 374, 193–196. doi: 10.1038/374193a0

Lipford, J. R., Smith, G. T., Chi, Y., and Deshaies, R. J. (2005). A putative stimulatory role for activator turnover in gene expression. *Nature* 438, 113–116. doi: 10.1038/nature04098

Liu, G., Holub, E. B., Alonso, J. M., Ecker, J. R., and Fobert, P. R. (2005). An *Arabidopsis* NPR1-like gene, NPR4, is required for disease resistance. *Plant J.* 41, 304–318. doi: 10.1111/j.1365-313X.2004.02296.x

Macho, A. P., and Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Mol. Cell* 54, 263–272. doi: 10.1016/j.molcel.2014.03.028

Maier, F., Zwicker, S., Hückelhoven, A., Meissner, M., Funk, J., Pfitzner, A. J. P., et al. (2011). NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) and some NPR1-related proteins are sensitive to salicylic acid. *Mol. Plant Pathol.* 12, 73–91. doi: 10.1111/j.1364-3703.2010.00653.x

Marino, D., Peeters, N., and Rivas, S. (2012). Ubiquitination during plant immune signaling. *Plant Physiol.* 160, 15–27. doi: 10.1104/pp.112.199281

Matsushita, A., Inoue, H., Goto, S., Nakayama, A., Sugano, S., Hayashi, N., et al. (2013). Nuclear ubiquitin proteasome degradation affects WRKY45 function in the rice defense program. *Plant J.* 73, 302–313. doi: 10.1111/tpj.12035

Metivier, R., Penot, G., Hubner, M. R., Reid, G., Brand, H., Kos, M., et al. (2003). Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 115, 751–763. doi: 10.1016/S0092-8674(03)00934-6

Moore, J. W., Loake, G. J., and Spoel, S. H. (2011). Transcription dynamics in plant immunity. *Plant Cell* 23, 2809–2820. doi: 10.1105/tpc.111.087346

Mosher, R. A., Durrant, W. E., Wang, D., Song, J., and Dong, X. (2006). A comprehensive structure-function analysis of *Arabidopsis* SNI1 defines essential regions and transcriptional repressor activity. *Plant Cell* 18, 1750–1765. doi: 10.1105/tpc.105.039677

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Nakayama, A., Fukushima, S., Goto, S., Matsushita, A., Shimono, M., Sugano, S., et al. (2013). Genome-wide identification of WRKY45-regulated genes that mediate benzothiadiazole-induced defense responses in rice. *BMC Plant Biol.* 13:150. doi: 10.1186/1471-2229-13-150

Ndoja, A., Cohen, R. E., and Yao, T. (2014). Ubiquitin signals proteolysis-independent stripping of transcription factors. *Mol. Cell* 53, 893–903. doi: 10.1016/j.molcel.2014.02.002

Ni, W., Xu, S. L., Tepperman, J. M., Stanley, D. J., Maltby, D. A., Gross, J. D., et al. (2014). A mutually assured destruction mechanism attenuates light signaling in *Arabidopsis*. *Science* 344, 1160–1164. doi: 10.1126/science.1250778

Oldroyd, G. E., and Staskawicz, B. J. (1998). Genetically engineered broad-spectrum disease resistance in tomato. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10300–10305. doi: 10.1073/pnas.95.17.10300

Peloponese, J. M. Jr., Iha, H., Yedavalli, V. R. K., Miyazato, A., Li, Y., Haller, K., et al. (2004). Ubiquitination of human T-cell leukemia virus type 1 tax modulates its activity. *J. Virol.* 78, 11686–11695. doi: 10.1128/JVI.78.21.11686-11695.2004

Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of Cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* 6, 9–20. doi: 10.1038/nrm1547

Pickart, C. M., and Cohen, R. E. (2004). Proteasomes and their kin: proteases in the machine age. *Nat. Rev. Mol. Cell Biol.* 5, 177–187. doi: 10.1038/nrm1336

Rairdan, G. J., and Delaney, T. P. (2002). Role of salicylic acid and NIM1/NPR1 in race-specific resistance in *Arabidopsis*. *Genetics* 161, 803–811.

Rate, D. N., and Greenberg, J. T. (2001). The *Arabidopsis* aberrant growth and death2 mutant shows resistance to Pseudomonas syringae and reveals a role for NPR1 in suppressing hypersensitive cell death. *Plant J.* 27, 203–211. doi: 10.1046/j.0960-7412.2001.1075

Reid, G., Hubner, M. R., Metivier, R., Brand, H., Denger, S., Manu, D., et al. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ERα on responsive promoters is an integral feature of estrogen signaling. *Mol. Cell* 11, 695–707. doi: 10.1016/S1097-2765(03)00090-X

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H. Y., et al. (1997). The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor IkB. *Plant Cell* 9, 425–439.

Sadanandom, A., Bailey, M., Ewan, R., Lee, J., and Nelis, S. (2012). The ubiquitin-proteasome system: central modifier of plant signalling. *New Phytol.* 196, 13–28. doi: 10.1111/j.1469-8137.2012.04266.x

Salghetti, S. E., Caudy, A. A., Chenoweth, J. G., and Tansey, W. P. (2001). Regulation of transcriptional activation domain function by ubiquitin. *Science* 293, 1651–1653. doi: 10.1126/science.1062079

Salghetti, S. E., Muratani, M., Wijnen, H., Futcher, B., and Tansey, W. P. (2000). Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3118–3123. doi: 10.1073/pnas.97.7.3118

Santner, A., and Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. *Nature* 459, 1071–1078. doi: 10.1038/nature08122

Shah, J., Tsui, F., and Klessig, D. F. (1997). Characterization of a salicylic acid-insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. *Mol. Plant Microbe Interact.* 10, 69–78. doi: 10.1094/MPMI.1997.10.1.69

Shi, Z., Maximova, S., Liu, Y., Verica, J., and Guiltinan, M. J. (2013). The salicylic acid receptor NPR3 is a negative regulator of the transcriptional defense response during early flower development in *Arabidopsis*. *Mol. Plant* 6, 802–816. doi: 10.1093/mp/sss091

Shimono, M., Koga, H., Akagi, A., Hayashi, N., Goto, S., Sawada, M., et al. (2012). Rice WRKY45 plays important roles in fungal and bacterial disease resistance. *Mol. Plant Pathol.* 13, 83–94. doi: 10.1111/j.1364-3703.2011.00732.x

Shimono, M., Sugano, S., Nakayama, A., Jiang, C. J., Ono, K., Toki, S., et al. (2007). Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance. *Plant Cell* 19, 2064–2076. doi: 10.1105/tpc.106.046250

Shirasu, K. (2009). The HSP90-SGT1 chaperone complex for NLR immune sensors. *Annu. Rev. Plant Biol.* 60, 139–164. doi: 10.1146/annurev.arplant.59.032607.092906

Smalle, J., and Vierstra, R. D. (2004). The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.* 55, 555–590. doi: 10.1146/annurev.arplant.55.031903.141801

Spoel, S. H., and Dong, X. N. (2012). How do plants achieve immunity? defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Spoel, S. H., Mou, Z., Tada, Y., Spivey, N. W., Genschik, P., and Dong, X. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* 137, 860–872. doi: 10.1016/j.cell.2009.03.038

Spoel, S. H., Tada, Y., and Loake, G. J. (2010). Post-translational protein modification as a tool for transcription reprogramming. *New Phytol.* 186, 333–339. doi: 10.1111/j.1469-8137.2009.03125.x

Stogios, P. J., Chen, L., and Prive, G. G. (2007). Crystal structure of the BTB domain from the LRF/ZBTB7 transcriptional regulator. *Protein Sci.* 16, 336–342. doi: 10.1110/ps.062660907

Stokes, T. L., Kunkel, B. N., and Richards, E. J. (2002). Epigenetic variation in *Arabidopsis* disease resistance. *Genes Dev.* 16, 171–182. doi: 10.1101/gad.952102

Sugano, S., Jiang, C. J., Miyazawa, S. I., Masumoto, C., Yazawa, K., Hayashi, N., et al. (2010). Role of OsNPR1 in rice defense program as revealed by genome-wide expression analysis. *Plant Mol. Biol.* 74, 549–562. doi: 10.1007/s11103-010-9695-3

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Tang, L.-Y., Yamashita, M., Coussens, N. P., Tang, Y., Wang, X., Li, C., et al. (2011). Ablation of Smurf2 reveals an inhibition in TGF-β signalling through multiple mono-ubiquitination of Smad3. *EMBO J.* 30, 4777–4789. doi: 10.1038/emboj.2011.393

Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19, 94–102. doi: 10.1093/emboj/19.1.94

Trujillo, M., and Shirasu, K. (2010). Ubiquitination in plant immunity. *Curr. Opin. Plant Biol.* 13, 402–408. doi: 10.1016/j.pbi.2010.04.002

van den Burg, H. A., Tsitsigiannis, D. I., Rowland, O., Lo, J., Rallapalli, G., Maclean, D., et al. (2008). The F-box protein ACRE189/ACIF1 regulates cell death and defense responses activated during pathogen recognition in tobacco and tomato. *Plant Cell* 20, 697–719. doi: 10.1105/tpc.107.056978

van der Horst, A., de Vries-Smits, A. M. M., Brenkman, A. B., van Triest, M. H., van den Broek, N., Colland, F., et al. (2006). FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Nat. Cell Biol.* 8, 1064–1073. doi: 10.1038/ncb1469

van Ooijen, G., Lukasik, E., Van Den Burg, H. A., Vossen, J. H., Cornelissen, B. J. C., and Takken, F. L. W. (2010). The small heat shock protein 20 RSI2 interacts with and is required for stability and function of tomato resistance protein I-2. *Plant J.* 63, 563–572. doi: 10.1111/j.1365-313X.2010.04260.x

van Ooijen, G., van den Burg, H. A., Cornelissen, B. J. C., and Takken, F. L. W. (2007). Structure and function of resistance proteins in solanaceous plants. *Annu. Rev. Phytopathol.* 45, 43–72. doi: 10.1146/annurev.phyto.45.062806.094430

Vierstra, R. D. (2009). The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat. Rev. Mol. Cell Biol.* 10, 385–397. doi: 10.1038/nrm2688

Walsh, C. K., and Sadanandom, A. (2014). Ubiquitin chain topology in plant cell signaling: a new facet to an evergreen story. *Front. Plant Sci.* 5:122. doi: 10.3389/fpls.2014.00122

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2:e123. doi: 10.1371/journal.ppat.0020123

Weigel, R. R., Pfitzner, U. M., and Gatz, C. (2005). Interaction of NIMIN1 with NPR1 modulates PR gene expression in *Arabidopsis*. *Plant Cell* 17, 1279–1291. doi: 10.1105/tpc.104.027441

Wu, R. C., Feng, Q., Lonard, D. M., and O'Malley, B. W. (2007). SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. *Cell* 129, 1125–1140. doi: 10.1016/j.cell.2007.04.039

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Yi, H., and Richards, E. J. (2009). Gene duplication and hypermutation of the pathogen resistance gene SNC1 in the *Arabidopsis* bal variant. *Genetics* 183, 1227–1234. doi: 10.1534/genetics.109.105569

Zhai, Q., Yan, L., Tan, D., Chen, R., Sun, J., Gao, L., et al. (2013). Phosphorylation-coupled proteolysis of the transcription factor MYC2 is important for jasmonate-signaled plant immunity. *PLoS Genet.* 9:e1003422. doi: 10.1371/journal.pgen.1003422

Zhang, Y., Cheng, Y. T., Qu, N., Zhao, Q., Bi, D., and Li, X. (2006). Negative regulation of defense responses in *Arabidopsis* by two NPR1 paralogs. *Plant J.* 48, 647–656. doi: 10.1111/j.1365-313X.2006.02903.x

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528. doi: 10.1073/pnas.96.11.6523

Zhang, Y., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653. doi: 10.1105/tpc.014894

Zhou, J. M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., et al. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol. Plant Microbe Interact.* 13, 191–202. doi: 10.1094/MPMI.2000.13.2.191

Zhou, W., and Slingerland, J. M. (2014). Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy. *Nat. Rev. Cancer* 14, 26–38. doi: 10.1038/nrc3622

Zhuang, M., Calabrese, M. F., Liu, J., Waddell, M. B., Nourse, A., Hammel, M., et al. (2009). Structures of SPOP-substrate complexes: insights into molecular architectures of BTB-Cul3 ubiquitin ligases. *Mol. Cell* 36, 39–50. doi: 10.1016/j.molcel.2009.09.022

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

PERSPECTIVE
published: 10 April 2015
doi: 10.3389/fpls.2015.00235

Integrating data on the *Arabidopsis* NPR1/NPR3/NPR4 salicylic acid receptors; a differentiating argument

Xiahezi Kuai[†], *Brandon J. MacLeod*[†] *and Charles Després*[*]

Department of Biological Sciences, Brock University, St. Catharines, ON, Canada

Salicylic acid (SA) is a mandatory plant metabolite in the deployment of systemic acquired resistance (SAR), a broad-spectrum systemic immune response induced by local inoculation with avirulent pathogens. The NPR1 transcription co-activator is the central node positively regulating SAR. SA was the last of the major hormones to be without a known receptor. Recently, NPR1 was shown to be the direct link between SA and gene activation. This discovery seems to be controversial. NPR1 being an SA-receptor is reminiscent of the mammalian steroid receptors, which are transcription factors whose binding to DNA is dependent on the interaction with a ligand. Unlike steroid receptors, NPR1 does not bind directly to DNA, but is recruited to promoters by the TGA family of transcription factors to form an enhanceosome. In *Arabidopsis*, NPR1 is part of a multigene family in which two other members, NPR3 and NPR4, have also been shown to interact with SA. NPR3/NPR4 are negative regulators of immunity and act as substrate adaptors for the recruitment of NPR1 to an E3-ubiquitin ligase, leading to its subsequent degradation by the proteasome. In this perspective, we will stress-test in a friendly way the current NPR1/NPR3/NPR4 model.

Keywords: NPR1, *Arabidopsis*, salicylic acid, receptor, plant immunity

OPEN ACCESS

Edited by:
Loreto Holuigue, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Kemal Kazan, Commonwealth Scientific and Industrial Research Organization, Australia
Shahid Mukhtar, University of Alabama at Birmingham, USA

***Correspondence:**
Charles Després, Department of Biological Sciences, Brock University, St. Catharines, ON L2S 3A1, Canada
cdespres@brocku.ca

[†]*These authors have contributed equally for this work.*

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *15 January 2015*
Accepted: *24 March 2015*
Published: *10 April 2015*

Citation:
Kuai X, MacLeod BJ, and Després C (2015) Integrating data on the Arabidopsis NPR1/NPR3/NPR4 salicylic acid receptors; a differentiating argument. Front. Plant Sci. 6:235. doi: 10.3389/fpls.2015.00235

New Insights into SA Signaling

Salicylic acid (SA) is an endogenous plant hormone essential to the deployment of a long-lasting, broad-based immunity termed systemic acquired resistance (SAR). SA protects plants from a wide range of phytopathogens by mediating immune response at both local and systemic level (Vlot et al., 2009). SA has also been found to participate in abiotic stress responses. For instance, exogenous SA applications induce tolerance to copper toxicity (Mostofa and Fujita, 2013). In addition to its role in biotic and abiotic stress resistances, SA can influence plant flowering and thermogenesis (Vlot et al., 2009). Due to its biological significance, the synthesis and signal transduction of SA has been intensely studied. Still, not much is known about the molecular details of the SA signaling pathway and the SA receptor remained unidentified for decades.

In 2012, two independent groups contributed new insights into the SA-perception and signaling-cascade. Interestingly, these advances are all centered on the NPR1 protein. One study showed that NPR1 can directly bind SA and acts as an SA-receptor (Wu et al., 2012). The other group proposed that two NPR1 paralogs, NPR3, and NPR4, bind SA and control the proteasome-mediated degradation of NPR1 through their interaction with NPR1 (Fu et al., 2012). Both groups however, demonstrated the indispensable role of NPR1 in SA signaling. The focus of this perspective centers on NPR1 as the mediator of SA-perception, while comparing the SA-binding properties and molecular mechanisms of NPR1, NPR3, and NPR4. These data are compiled in **Table 1**.

TABLE 1 | Comparison of salicylic acid (SA)-binding properties between NPR1, NPR3, and NPR4.

	NPR1	NPR3	NPR4
Method used to study SA-binding	Equilibrium dialysis (Non-equilibrium methods not working suggesting fast on/off rates)	Conventional non-equilibrium ligand binding assay (Slow off rates)	Conventional non-equilibrium ligand binding assay (Slow off rates)
Affinity	Kd = 140 ± 10 nM (High affinity)	Kd = 981 ± 409 nM (Low affinity)	Kd = 46.2 ± 2.35 nM (High affinity)
Secondary binding method	Scintillation proximity assay (Wu et al., 2012). Surface Plasmon Resonance, photoaffinity labeling, and size-exclusion chromatography (Manohar et al., 2015)	No	No
Ligand interface	$Cys^{521/529}$	Not known	Not known
Stoichiometry	−**SA:** Oligomer +**SA:** Dimer	Not known	−**SA:** Tetramer +**SA:** Tetramer
Conformation change and molecular properties after SA-binding	−**SA:** N-terminal BTB domain interacts with C-terminal transactivation domain to inhibit the transcription activity of NPR1. +**SA:** Disruption of the interaction between BTB and C-terminus converting NPR1 into a transcription co-activator.	No conformation change known. −**SA:** Does not interact with NPR1. +**SA:** Interacts with NPR1.	No conformation change known. −**SA:** Interacts with NPR1. +**SA:** Does not interact with NPR1.
Metal requirement for SA-binding	Requires copper	No	No
Crystal structure	Not determined	Not determined	Not determined

Furthermore, we will address some shortcomings in our current understanding of the SA-signaling pathway in the context of plant immunity.

NPR1 at the Core of the SA-Signaling Network

NPR1 is a central regulator of plant immunity, which controls both local resistance and SAR. Plants lacking a functional NPR1 protein are unable to undergo SAR or express the SAR-marker gene *PR1*, and as a result succumb to biotrophic pathogenic challenges (Cao et al., 1994; Delaney et al., 1995). Later, it was shown that NPR1 is a transcription coactivator (Rochon et al., 2006). The molecular mechanisms of NPR1 function are best understood in the case of *PR1*. Transcription of *PR1* is repressed by TGA2 transcription factor under SA concentration existing in naïve cells (Zhang et al., 2003; Rochon et al., 2006). Upon build-up of SA, NPR1 activates *PR1* transcription by forming an enhansome with TGA2 on the promoter and negating the repressor activity of TGA2 (Boyle et al., 2009). The formation of the enhanceosome is well understood. However, the exact role played by SA leading to its formation remains unclear.

Structurally, NPR1 contains an N-terminal BTB/POZ domain, an ankyrin repeat domain, a C-terminal transactivation domain and a nuclear localization sequence. The ankyrin repeats of NPR1 are responsible for its interaction with TGA2 (Zhang et al., 1999). The BTB/POZ also contacts TGA2 masking its repressor domain (Boyle et al., 2009). Besides its role in converting TGA2 from a repressor to an activator, the BTB/POZ also acts as an autoinhibitory domain. In the absence of SA, it interacts with the NPR1 C-terminal transactivation domain, and inhibits the transcription co-activator function of NPR1 (Wu et al., 2012). Two cysteines (Cys521 and Cys529), located in the C-terminus of NPR1, are crucial for the SA-induced transactivation activity of NPR1 (Rochon et al., 2006). These same Cys are required for the direct binding of SA to *Arabidopsis* NPR1 (Wu et al., 2012). Mechanistically, the binding of SA leads to the disruption of the interaction between the BTB/POZ and the C-terminus, thus releasing the C-terminal transactivation domain from autoinhibition by the BTB/POZ domain and converting NPR1 into an activated transcription co-activator.

A novel and interesting feature of NPR1, aside from being a newly discovered and important phytohormone-receptor, is the requirement of the transition metal copper for SA-binding. Mutation of Cys521 and Cys529 of the C-terminal transactivation domain not only disrupts the SA-binding capacity of NPR1, but also eliminates the recruitment of copper by NPR1 (Wu et al., 2012). This is the first plant example of a copper-binding protein acting as a transcription regulator. The fact that NPR1 is a metalloprotein explains why it took so long to identify it as an SA receptor. Many researchers, by default, include EDTA as a chelator when preparing buffers. However, recruitment of SA by NPR1 is EDTA-sensitive and its presence in buffers precludes SA from binding to NPR1 (Wu et al., 2012). Despite the fact that NPR1 is the first copper-binding transcription-regulator discovered in plant, it is not the first time that copper is found to play a critical function in hormone signal-transduction pathway. The high-affinity binding-activity of the gaseous plant hormone, ethylene, to the ethylene receptor, ETR1, also requires copper as a cofactor (Rodriguez et al., 1999). As is the case of SA in NPR1, ethylene is coordinated to copper in the ETR1 hormone-binding pocket.

NPR3 and NPR4: The Newer Kids on the Block

NPR1 is a positive regulator of SAR. Recently, additional members of the NPR family, NPR3, and NPR4, were shown to negatively regulate SAR (Liu et al., 2005; Zhang et al., 2006; Fu et al., 2012). Analysis of conceptual gene products revealed that NPR3 and NPR4, respectively, share 34.5 and 36.0% amino acid-conservation with NPR1, specifically in the BTB/POZ and ankyrin repeat domains (Liu et al., 2005). Protein alignments indicate that all three NPR share four (4) conserved Cys in their BTB/POZ domain, and a stretch of five (5) variable basic-amino acids at the C-terminal, that may be involved in nuclear localization (Shi et al., 2013). The structural similarities among these three protein appears to extend to their functional roles including SA-perception and interaction with members of the TGA family of transcription factors (Després et al., 2000; Kinkema et al., 2000; Subramaniam et al., 2001; Fan and Dong, 2002; Mou et al., 2003; Rochon et al., 2006).

At the organ level, expression of NPR1/NPR3/NPR4 appears to occur in different locations. Promoter-driven GFP expression observed with fluorescence stereomicroscopy, demonstrated that NPR1 was detectable only in leaves, NPR4 only in mature siliques and roots, while NPR3 was expressed in relatively high quantities in the young flower (Shi et al., 2013). At the subcellular level, NPR3/NPR4-TGA2 interactions have been observed primarily in the nucleus, when studied in onion epidermal cells and *Arabidopsis* mesophyll protoplasts (Zhang et al., 2006). While nuclear localization of NPR1 has been shown definitively, differing reports have suggested that NPR1 can also be observed in the cytoplast as well (Després et al., 2000).

The pathology surrounding *npr1/npr3/npr4* mutants has displayed different phenotypes under the exact and differential conditions. Early experiments infecting *npr4-1* plants with the fungi *Erysiphe cichoracearum* (powdery mildew) and bacterium *Pseudomonas syringae* pv. *Tomato DC3000* (*Pst* DC3000) indicated that these plants were compromised in disease resistance (Liu et al., 2005). However, an independent study from Zhang et al. (2006), partially disagreed, rather observing that the *npr4-3* and *npr4-2* plants were not more susceptible to *Pst* DC3000 or *P. syringae* pv. *maculicola* ES4326 (*Psm* ES4326). When combined with the *npr3-1* mutant (*npr3-1npr4-3)* plants were found to be more resistant (Zhang et al., 2006). Corroborating the results of Zhang et al. (2006), single *npr3* or *npr4* mutants showed little difference in SAR response when compared to Col-0. Furthermore, the double mutant (*npr3npr4)* was highly resistant in basal and induced SAR states (Fu et al., 2012). At the basal level, NPR3 deficient backgrounds have compromised fitness when measured by primary root length, average growth rates, and seed production. Most recently an *npr3-3* mutant was generated and found to not differ from Col-0 plants in terms of quantity of bacterial growth when leaves were infiltrated with *Pst* DC3000, consistent with previous data. Conversely, transgenic plants overexpressing NPR3 were more susceptible to inoculation (Shi et al., 2013). Interestingly, the quantity of NPR3 transcripts was approximately threefold lower in flower petals when taken from the *npr3-3* background in comparison to the *npr3-2* mutant (Shi et al., 2013). Although, both backgrounds were created from homozygous T-DNA insertions in the third exon, the *npr3-2* plant may nonetheless be a "weak allele" in flowers, at least (Shi et al., 2013). The discrepancies observed between laboratories when testing the same pathogens reflect the complexity of the disease resistance phenotype compared to the analysis of the SAR-marker gene *PR1*. Differences may result from the use of different mutant alleles. However, the theme emerging from these data is the functional redundancy, at least in leaves, of NPR3 and NPR4 with respect to immunity. This somewhat contrasts with the proposed role of NPR4 and NPR3 functioning as independent SA-receptors under low and high SA concentrations, respectively.

NPR1/NPR3/NPR4, SA and the Regulation of SAR: Some Shortcomings

Contemporary analysis suggests that NPR4 is a CUL3 E3-ligase substrate-adapter in naïve cells, which can interact with NPR1, allowing for the continuous ubiquitylation and turnover of NPR1 by the proteasome. During SAR, the cellular accumulation of SA allows NPR4 to bind the hormone, disrupting the NPR4–NPR1 interaction and abolishing the adaptor-substrate complex. Conversely, NPR3 responds to the abundance of SA by presumably binding to the hormone allowing NPR3 to interact with NPR1, resuming ubiquitylation of NPR1, targeting it for degradation (Fu et al., 2012). Hence, NPR3 and NPR4 would function as both substrate adaptors and SA- receptors that mediate the degradation of NPR1 in the SAR induced and naïve cells, respectively.

Despite the attractiveness of this model, it has yet to be demonstrated how NPR3 or NPR4 actually interact and bind SA. Furthermore, no structural changes in these proteins were directly observed upon binding SA. Such conformational changes are the usual hallmark of receptor-ligand interactions. In what appears to be a controversial finding, the study by Fu et al. (2012) suggested that NPR1 was unable to bind SA. Interestingly, using the same non-equilibrium method (see **Table 1**), Wu et al. (2012) came to the same conclusion, as they also found that NPR1 could not bind SA under these conditions. However, NPR1 clearly binds SA under equilibrium conditions when appropriate methodologies are used and chelating agents are omitted from experimental buffers (Wu et al., 2012; **Table 1**). Furthermore, while this manuscript was under review, Manohar et al. (2015), demonstrated, using three alternative methods, that NPR1 binds SA, bringing to five the total number of methods tested to demonstrate that NPR1 is an SA-receptor (**Table 1**). While these data clearly confirm that NPR1 binds SA and should put an end to the controversy, they also clearly show the need to confirm that NPR3 and NPR4 can indeed bind SA, especially given the fact that they do not undergo conformational changes upon binding SA. Therefore, considering that NPR1 is also an SA-receptor that binds the hormone with a relatively high affinity in the presence of copper, it is also unclear *in vivo* whether the interaction between NPR1–NPR3/NPR4 is a result of SA bound to NPR1 or to NPR3/NPR4. Since yeast-two-hybrid assays were used to study the SA-dependent interactions between NPR1 and NPR3/NPR4,

it is possible that cellular copper was present at quantities sufficient to allow NPR1 to bind SA. Transient BiFC assays in naïve onion epidermal cells have also indicated an interaction between NPR1 and NPR3. However, it is unclear whether basal levels of SA were present at sufficient concentrations in the naïve onion epidermal cells to allow SA perception by NPR1 or NPR3, making it unclear whether or not the interaction requires NPR1 bound SA, NPR3 bound SA, or whether the interaction requires the presence of SA at all *in vivo* (Shi et al., 2013). However, because NPR1 has a higher affinity for SA than NPR3, as observed by the respective dissociation constants, it would follow that NPR1 would outcompete NPR3 for the interaction with SA (Fu et al., 2012; Wu et al., 2012). Given that NPR1 is the only NPR (among NPR1/NPR3/NPR4) shown to display a conformational change upon binding to its ligand, NPR1 may in fact be the decisional entity responsible for dictating whether interaction with NPR3/NPR4 occurs, regardless of the SA-status of the system.

Although NPR3 and NPR4 appear to degrade NPR1 in an SA-dependent and independent model, respectively, the biochemical and phenotypic data observed from the *npr3, npr4,* and *npr3npr4* mutant plants are not always in agreement with this hypothesis. For example, in the *in vivo* NPR1 degradation experiment (Figure 1A in Fu et al., 2012), in the *npr4* mutant, in which the NPR3-mediated NPR1-degradation is not affected, NPR1 accumulates to the highest levels after 8 h SA application. This indicates that NPR4 and not NPR3 is responsible for degrading NPR1 under SA conditions, which is not consistent with the model. Furthermore, although NPR1 accumulates to some extent in *npr3npr4* mutant before SA application, NPR1 accumulates to even greater extent in the *npr3npr4* double mutant in response to SA treatment, which indicates that the *npr3npr4* mutant is not completely insensitive to SA, suggesting that there is(are) other SA receptor(s) which mediate or trigger the accumulation of NPR1. Another indication, illustrating the presence of central receptor(s) of SA other than NPR3/NPR4, is the data showing that *Psm* ES4326 growth is significantly decreased in the *npr3npr4* double mutant plant even without SAR induction (Figure 4A in Fu et al., 2012). This does not suggest that SAR is defective as proposed by the authors, but rather that SAR is already established in the *npr3npr4* double mutant. Further inconsistencies with the model are revealed by the SAR sets of experiments. Although SA accumulation was not quantified in these experiments, treatment with the *Psm avrRpt2* strain would presumably induce SAR and thus promote SA accumulation. Therefore the model would predict that, if NPR4 is a CUL3 substrate adaptor only in the absence of SA, the *npr3npr4* mutant should not be more resistant than the single *npr3* mutant.

On the *PR1* front, the relative expression of the gene in naïve cells shows a slightly higher than wild-type induction in the *npr3* plants and about the same induction as wild-type in *npr4* plants. By contrast *PR1* induction was several folds greater in the *npr3npr4* plants when compared with wild-type or the single *npr3* or *npr4* mutants. The current NPR3/NPR4–NPR1 degradation model would predict rather that the *npr4* plants should display similar *PR1* induction as the *npr3npr4* plants and that the *npr3* plants should be no different from the wild-type. This is expected because of the lack of NPR3-targeted degradation of NPR1 in naïve cells. As proposed by Zhang et al. (2006), NPR3 and NPR4 appear to have redundant functions with respect to immunity, as opposed to the model proposed by Fu et al. (2012), where they have distinct non-overlapping functions.

Final Thoughts

The NPR3/NPR4-mediated NPR1 degradation is reminiscent of the emerging trend of ubiquitylation in plant hormone signaling (Santner and Estelle, 2009). Auxins act by stimulating the degradation of Aux/IAA transcriptional repressor through the ubiquitin-ligase complex SCF^{TIR1} (Gray et al., 2001). Jasmonates activate downstream gene transcription by promoting degradation of the JAZ family of repressors through SCF^{COL1} E3 ubiquitin-ligase (Chini et al., 2007). The gibberellin receptor GID1 mediates ubiquitylation and degradation of DELLA repressor, thus activating gibberellin-responsive gene transcription (Griffiths et al., 2006). It seems that in many signaling pathways, plants use ubiquitin and the proteasome pathway to regulate the abundance of negative regulators of the corresponding system. However, in contrast to the aforementioned pathways, in the case of SA signaling, the proteasome targets the positive regulator NPR1. Although the biological importance and molecular mechanism of SA-regulated NPR1-degradation needs further investigation, ubiquitylation also plays a role in mediating SA signaling (Fu et al., 2012).

Cys521 and Cys529 responsible for the binding of SA to the *Arabidopsis* NPR1 are not universally conserved in NPR1 orthologs, such as those found in crops. However, metal interaction with proteins is not limited to Cys, since any amino acid harboring electronegative elements in its side chain can potentially participate in metal interaction (Wu et al., 2012; Figure S2 therein). Objectively, this leaves us with three possible scenarios: (1) NPR1 from crops could bind SA through metal-coordination, as does the *Arabidopsis* NPR1, using amino-acids other than Cys; (2) NPR1 from crops could bind SA without coordination through a metal; (3) NPR1 from crops are not receptors for SA. Further research on crop NPR1 should prove invaluable in assessing whether, in the case of NPR1, *Arabidopsis* can serve as a model system or whether it is the exception to the rule.

Since NPR1 is a transcriptional coactivator (Rochon et al., 2006), the discovery that two NPR1 family members are Cul3 substrate-adaptors (Fu et al., 2012) came as a surprise. Given that NPR3 and NPR4, just like NPR1, interact with the TGA family of transcription factors (Liu et al., 2005; Zhang et al., 2006), a role for these proteins in transcription regulation would have been anticipated. Nevertheless, as proposed (Zhang et al., 2006), a regulatory function for NPR3 and NPR4 involving transcriptional control may still be revealed in the future.

Acknowledgments

CD is supported by the National Science and Engineering Research Council (NSERC) Discovery Grant and Accelerator Grant Programs. BM was supported by an NSERC Undergraduate Student Research Award.

References

Boyle, P., Le, S. E., Rochon, A., Shearer, H. L., Murmu, J., Chu, J. Y., et al. (2009). The BTB/POZ domain of the *Arabidopsis* disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell* 21, 3700–3713. doi: 10.1105/tpc.109.069971

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 6, 1583–1592. doi: 10.1105/tpc.6.11.1583

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J. M., Lorenzo, O., et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448, 666–671. doi: 10.1038/nature06006

Delaney, T. P., Friedrich, L., and Ryals, J. A. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6602–6606. doi: 10.1073/pnas.92.14.6602

Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P. R. (2000). The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12, 279–290. doi: 10.1105/tpc.12.2.279

Fan, W., and Dong, X. (2002). In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in *Arabidopsis*. *Plant Cell* 14, 1377–1389. doi: 10.1105/tpc.001628

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gray, W. M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. *Nature* 414, 271–276. doi: 10.1038/35104500

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z. L., Powers, S. J., et al. (2006). Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. *Plant Cell* 18, 3399–3414. doi: 10.1105/tpc.106.047415

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.1105/tpc.12.12.2339

Liu, G., Holub, E. B., Alonso, J. M., Ecker, J. R., and Fobert, P. R. (2005). An *Arabidopsis* NPR1-like gene, NPR4, is required for disease resistance. *Plant J.* 41, 304–318. doi: 10.1111/j.1365-313X.2004.02296.x

Manohar, M., Tian, M., Moreau, M., Park, S. W., Choi, H. W., Fei, Z., et al. (2015). Identification of multiple salicylic acid-binding proteins using two high throughput screens. *Front. Plant Sci.* 5:777. doi: 10.3389/fpls.2014.00777

Mostofa, M. G., and Fujita, M. (2013). Salicylic acid alleviates copper toxicity in rice (*Oryza sativa* L.) seedlings by up-regulating antioxidative and glyoxalase systems. *Ecotoxicology* 22, 959–973. doi: 10.1007/s10646-013-1073-x

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Rochon, A., Boyle, P., Wignes, T., Fobert, P. R., and Despres, C. (2006). The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *Plant Cell* 18, 3670–3685. doi: 10.1105/tpc.106.046953

Rodriguez, F. I., Esch, J. J., Hall, A. E., Binder, B. M., Schaller, G. E., and Bleecker, A. B. (1999). A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*. *Science* 283, 996–998. doi: 10.1126/science.283.5404.996

Santner, A., and Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. *Nature* 459, 1071–1078. doi: 10.1038/nature08122

Shi, Z., Maximova, S., Liu, Y., Verica, J., and Guiltinan, M. J. (2013). The salicylic acid receptor NPR3 is a negative regulator of the transcriptional defense response during early flower development in *Arabidopsis*. *Mol. Plant* 6, 802–816. doi: 10.1093/mp/sss091

Subramaniam, R., Desveaux, D., Spickler, C., Michnick, S. W., and Brisson, N. (2001). Direct visualization of protein interactions in plant cells. *Nat. Biotechnol.* 19, 769–772. doi: 10.1038/90831

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Zhang, Y., Cheng, Y. T., Qu, N., Zhao, Q., Bi, D., and Li, X. (2006). Negative regulation of defense responses in *Arabidopsis* by two NPR1 paralogs. *Plant J.* 48, 647–656. doi: 10.1111/j.1365-313X.2006.02903.x

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528. doi: 10.1073/pnas.96.11.6523

Zhang, Y., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653. doi: 10.1105/tpc.014894

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 07 January 2015
doi: 10.3389/fpls.2014.00763

A large-scale genetic screen for mutants with altered salicylic acid accumulation in Arabidopsis

Yezhang Ding, Danjela Shaholli and Zhonglin Mou*

Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, USA

Edited by:
Hua Lu, University of Maryland Baltimore County, USA

Reviewed by:
Jian Hua, Cornell University, USA
Yuelin Zhang, University of British Columbia, Canada

****Correspondence:***
Zhonglin Mou, Department of Microbiology and Cell Science, University of Florida, Museum Road, Building 981, Gainesville, FL 32611, USA
e-mail: zhlmou@ufl.edu

Salicylic acid (SA) is a key defense signal molecule against biotrophic and hemibiotrophic pathogens in plants, but how SA is synthesized in plant cells still remains elusive. Identification of new components involved in pathogen-induced SA accumulation would help address this question. To this end, we performed a large-scale genetic screen for mutants with altered SA accumulation during pathogen infection in Arabidopsis using a bacterial biosensor *Acinetobacter* sp. ADPWH_*lux*-based SA quantification method. A total of 35,000 M_2 plants in the *npr1-3* mutant background have been individually analyzed for the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326-induced SA accumulation. Among the mutants isolated, 19 had SA levels lower than *npr1* (*sln*) and two exhibited increased SA accumulation in *npr1* (*isn*). Complementation tests revealed that seven of the *sln* mutants are new alleles of *eds5*/*sid1*, two are *sid2*/*eds16* alleles, one is allelic to *pad4*, and the remaining seven *sln* and two *isn* mutants are new non-allelic SA accumulation mutants. Interestingly, a large group of mutants (in the *npr1-3* background), in which *Psm* ES4326-induced SA levels were similar to those in the wild-type Columbia plants, were identified, suggesting that the signaling network fine-tuning pathogen-induced SA accumulation is complex. We further characterized the *sln1* single mutant and found that *Psm* ES4326-induced defense responses were compromised in this mutant. These defense response defects could be rescued by exogenous SA, suggesting that *SLN1* functions upstream of SA. The *sln1* mutation was mapped to a region on the north arm of chromosome I, which contains no known genes regulating pathogen-induced SA accumulation, indicating that *SLN1* likely encodes a new regulator of SA biosynthesis. Thus, the new *sln* and *isn* mutants identified in this genetic screen are valuable for dissecting the molecular mechanisms underlying pathogen-induced SA accumulation in plants.

Keywords: salicylic acid, genetic screen, *NPR1*, *Arabidopsis thaliana*, disease resistance, *sln* mutant, *isn* mutant

INTRODUCTION

As sessile organisms, plants are under constant attack from diverse microbes including bacteria, fungi, oomycetes, and viruses. To ward off pathogens, plants activate their immune system to mount multiple defense responses, which are similar to animal innate immunity (Jones and Dangl, 2006). Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors results in PAMP-triggered immunity (PTI). To achieve successful colonization, adapted pathogens can deliver effector molecules directly into the plant cells to suppress PTI, resulting in effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). On the other hand, plants have evolved resistance (R) proteins to detect the presence of certain pathogen effector molecules, inducing effector-triggered immunity (ETI). Activation of PTI or ETI leads to generation of mobile signals, which induce a long-lasting broad-spectrum immune response known as systemic acquired resistance (SAR) (Durrant and Dong, 2004).

The phytohormone salicylic acid (SA) plays an essential role in these defense response pathways (Vlot et al., 2009). Exogenous application of SA or its analogs induces expression of defense genes including *PR* (*pathogenesis-related*) genes and disease resistance (White, 1979; Dong, 2004), whereas transgenic plants carrying the bacterial *NahG* gene, which encodes an SA hydroxylase, are hypersusceptible to pathogen infection and fail to develop SAR (Gaffney et al., 1993; Delaney et al., 1994; Lawton et al., 1995). Furthermore, Arabidopsis mutants with impaired SA biosynthesis during pathogen infection, such as *sid2* (*salicylic acid induction-deficient2*) (Nawrath and Métraux, 1999; Wildermuth et al., 2001), *eds5* (*enhanced disease susceptibility5*) (Nawrath and Métraux, 1999; Nawrath et al., 2002), and *pad4* (*phytoalexin deficient4*) (Zhou et al., 1998; Jirage et al., 1999), show compromised defense responses. In contrast, mutants with elevated levels of SA, such as *acd* (*accelerated cell death*) (Greenberg et al., 1994; Rate et al., 1999), *cpr* (*constitutive expressor of PR genes*) (Bowling et al., 1997; Clarke et al., 1998), and *ssi* (*suppressor of salicylate insensitivity of npr1-5*) (Shah et al., 1999, 2001), display constitutive expression of *PR* genes and SAR.

Previous research has revealed that plants mainly utilize two distinct enzymatic pathways to synthesize SA, the phenylalanine

ammonia-lyase (PAL) pathway and the isochorismate (IC) pathway (Vlot et al., 2009; Dempsey et al., 2011). Both pathways require the primary metabolite chorismate, which is derived from the shikimate pathway. Earlier studies using isotope feeding suggested that SA is synthesized from phenylalanine via either benzoate intermediates or coumaric acid catalyzed by a series of enzymes including PAL, benzoic acid 2-hydroxylase, and other unknown enzymes (León et al., 1995; Dempsey et al., 2011). SA can also be synthesized through isochorismate catalyzed by isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL). Two ICS enzymes, ICS1 and ICS2, exist in Arabidopsis, and ICS1 has been shown to play a major role in SA biosynthesis (Garcion et al., 2008). Intriguingly, no plant genes encoding IPL have been identified. In comparison to the PAL pathway, the IC pathway plays a more important role in synthesis of both basal and induced SA in Arabidopsis (Mauch-Mani and Slusarenko, 1996; Garcion et al., 2008). However, neither pathway has been fully defined so far.

Nawrath and Métraux (1999) conducted a forward genetic screen in Arabidopsis for mutants with altered levels of total SA after infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 carrying the avirulence gene *avrRpm1*. Two mutants, *sid1* and *sid2*, were identified, which did not accumulate SA during the infection (Nawrath and Métraux, 1999). The *sid1* and *sid2* mutants were shown to be allelic to *eds5* and *eds16*, respectively, which were identified in another genetic screen for enhanced disease susceptibility (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999). *EDS5*/*SID1* encodes a chloroplast MATE (multidrug and toxin extrusion) transporter (Nawrath et al., 2002), and *SID2*/*EDS16* encodes an SA biosynthetic enzyme ICS1 (Wildermuth et al., 2001). In this screen, an HPLC (high performance liquid chromatography)-based method was used to quantify SA levels in pathogen-infected leaf tissues from about 4500 individual M_2 plants. Obviously, the genetic screen did not reach saturation.

The HPLC-based method used by Nawrath and Métraux (1999) is extremely costly and time-consuming, which would not be practical for a large-scale genetic screen. Recently, an SA biosensor, named *Acinetobacter* sp. ADPWH_*lux*, was developed (Huang et al., 2005). This bacterial strain was derived from *Acinetobacter* sp. ADP1 and contains a chromosomal integration of an SA-inducible *lux-CDABE* operon, which encodes a luciferase (LuxA and LuxB) and the enzymes that produce its substrate (LuxC, LuxD, and LuxE). In the presence of SA, methylsalicylic acid, and acetylsalicylic acid, the operon is activated, resulting in emission of 490-nm light (Huang et al., 2005). Measurement of SA from tobacco mosaic virus-infected tobacco leaves with the biosensor and gas chromatography/mass spectrometry (GC/MS) yielded similar results, demonstrating that this strain is suitable for quantification of SA in plants (Huang et al., 2006). DeFraia et al. developed an improved methodology for *Acinetobacter* sp. ADPWH_*lux*-based SA quantification for both free SA and SA *O*-β-glucoside (SAG) in crude plant extracts (Defraia et al., 2008). Based on this, Marek et al. (2010) established a further simplified protocol for estimation of free SA levels in crude plant extracts in a high-throughput format (Marek et al., 2010). The efficacy and effectiveness of the newly developed SA biosensor-based method were confirmed by HPLC and verified in a small-scale mutant screen.

To better understand SA biology, we conducted a large-scale forward genetic screen aimed at isolating more Arabidopsis mutants with altered SA accumulation upon pathogen infection. We expected that mutants accumulating significantly altered levels of SA during pathogen infection will help study how SA is synthesized in plant cells and uncover important regulators of plant immunity. This screen allowed us to identify nine new mutants with significantly altered levels of pathogen-induced SA in the *npr1-3* genetic background. Among them, seven produced SA levels lower than *npr1* (*sln*) and two displayed increased SA accumulation in *npr1* (*isn*). Enhanced disease resistance tests demonstrated that the seven new *sln npr1-3* mutants are more susceptible to bacterial pathogen infection, while both *isn npr1-3* mutants are more resistant than *npr1-3*. We further characterized the *sln1* single mutant and found that the *sln1* mutation compromised the bacterial pathogen *P. syringae* pv. *maculicola* (*Psm*) ES4326-induced defense responses. Moreover, exogenous SA induced both *PR* gene expression and disease resistance in *sln1*, indicating that *SLN1* functions upstream of SA. Finally, the *sln1* mutation was mapped to a region on the north arm of chromosome I, which contains no known genes involved in regulating pathogen-induced SA accumulation, suggesting that *SLN1* encodes a new SA pathway component.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

The wild type used was the *Arabidopsis thaliana* (L.) Heynh. Columbia (Col-0) ecotype, and the mutant alleles used were *npr1-3* (Glazebrook et al., 1996), *npr1*-L (GT_5_89558), *eds5-1* (Nawrath et al., 2002), *sid2-1* (Nawrath and Métraux, 1999; Wildermuth et al., 2001), *pad4-1* (Glazebrook et al., 1996; Jirage et al., 1999), *eps1-1* (Zheng et al., 2009), and *pbs3-1* (Nobuta et al., 2007). The *eds5-1 npr1-3*, *sid2-1 npr1-3*, and *pad4-1 npr1-3* double mutants were created by crossing *npr1-3* with *eds5-1*, *sid2-1*, and *pad4-1*, respectively. Homozygous plants were identified by genotyping (Tables S1 and S2). Arabidopsis seeds were sown on autoclaved soil (Sunshine MVP, Sun Gro Horticulture, http://www.sungro.com) and cold-treated at 4°C for 3 days. Plants were grown at approximately 22°C under a 16-h light/8-h dark regime.

PATHOGEN INFECTION

The bacterial strains *Psm* ES4326 and *Pst* DC3000/*avrRpt2* were grown overnight in liquid King's B medium. Bacterial cells were collected by centrifugation and diluted in 10 mM $MgCl_2$. Inoculation of plants was performed by pressure infiltration with a 1 mL needleless syringe (Clarke et al., 1998). For SA measurement, *Psm* ES4326 and *Pst* DC3000/*avrRpt2* suspensions with an OD_{600} of 0.001 were used for inoculation. The susceptibility phenotype was tested using a low-titer inoculum ($OD_{600} = 0.0001$) of *Psm* ES4326. *In planta* growth of *Psm* ES4326 was assayed 3 days after inoculation as previously described (Clarke et al., 1998). For SA-induced resistance assay, SA-treated plants were inoculated with a *Psm* ES4326 suspension ($OD_{600} = 0.001$) and the bacterial growth was determined 3 days post-inoculation.

SA MEASUREMENT

Free SA measurement using the SA biosensor was conducted as described by Marek et al. (2010). SA measurement with HPLC was performed as described by Verberne et al. (2002).

RNA EXTRACTION AND QUANTITATIVE PCR

RNA extraction was carried out as described previously (Cao et al., 1997). For reverse transcription (RT), ∼10 μg of total RNA was treated with DNase I (Ambion) at 37°C for 30 min for digestion of contaminating DNA. After inactivation of the DNase, ∼2 μg of total RNA was used as a template for first-strand cDNA synthesis using the M-MLV Reverse Transcriptase first-strand synthesis system (Promega). The resulting cDNA products were diluted 20-fold with autoclaved distilled water, and 2.5 μL of the diluted solution was used for quantitative PCR (qPCR). qPCR was performed in an Mx3005P qPCR system (Stratagene). All qPCR reactions were performed with a 12.5 μL reaction volume using the SYBR Green protocol under the following conditions: denaturation program (95°C for 10 min), amplification and quantification program repeated for 40 cycles (95°C for 30 s, 55°C for 1 min, 72°C for 1 min), and melting curve program (95°C for 1 min, 55°C for 30 s, and 95°C for 30 s). The primers used for qPCR in this study are listed in Table S2.

STATISTICAL METHODS

Statistical analyses were performed with Prism 5 (GraphPad Software, Inc., La Jolla, CA). One-Way analysis of variance (ANOVA) was used to determine statistical significance among genotypes or treatments. In addition, two-way analysis of variance was used to examine the effects of genotypes, treatments, and the interaction of these two factors on disease resistance. Post-hoc comparison was performed using Fisher's least significant difference LSD test and represented by different letters. Alternatively, statistical analyses were performed using Student's *t*-test for comparison of two data sets (Assuming Unequal Variances).

ACCESSION NUMBER

The locus numbers for the genes discussed in this study are as follows: *NPR1* (At1g64280), *EDS5* (At4g39030), *ICS1* (At1g74710), *PAD4* (At3g52430), *EPS1* (At5g67160), *PBS3* (At5g13320), *PR1* (At2g14610), *PR2* (At3g57260), *PR5* (At1g75040), *UBQ5* (At3g62250).

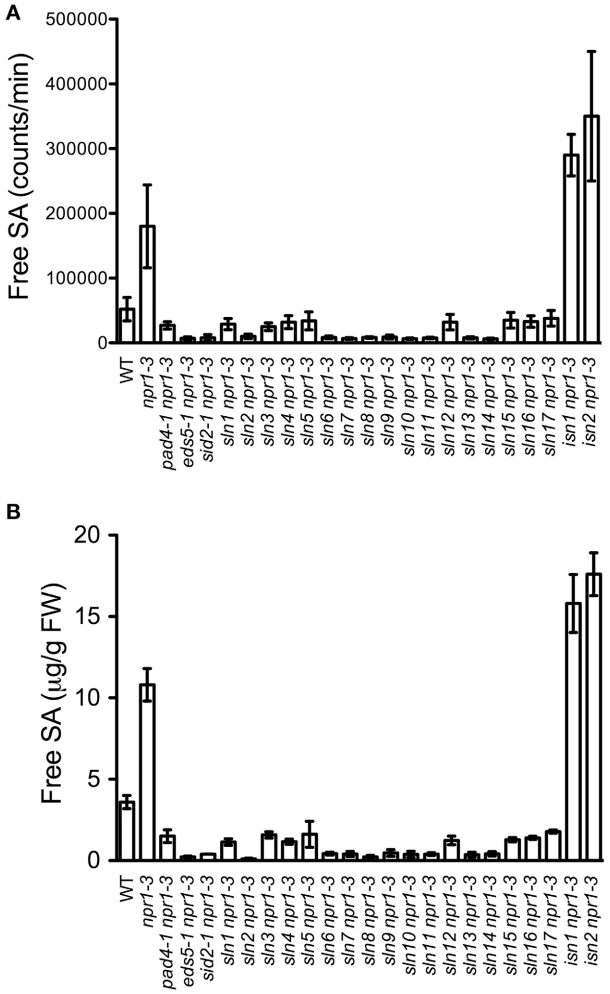


FIGURE 1 | Pathogen-induced free SA levels in the SA accumulation mutants. (A) Luminescence from crude extracts of *Psm* ES4326-infected wild-type, *npr1-3, pad4-1 npr1-3, eds5-1 npr1-3, sid2-1 npr1-3*, and 19 putative mutant leaf tissues measured with the SA biosensor. **(B)** Free SA levels in *Psm* ES4326-infected wild-type, *npr1-3, pad4-1 npr1-3, eds5-1 npr1-3, sid2-1 npr1-3*, and 19 putative mutant plants detected by the HPLC-based method. Values are the mean of eight **(A)** or three **(B)** samples with standard deviation (SD). The experiments were repeated three times with similar results.

RESULTS

ISOLATION OF SA ACCUMULATION MUTANTS

In order to identify new components involved in pathogen-induced SA accumulation, we took advantage of the SA biosensor-based method to screen for mutants with altered levels of pathogen-induced SA in Arabidopsis. Approximately 35,000 M_2 plants from an ethyl methanesulfonate-mutagenized population (20 pools, each from ∼500 M_1 plants) in the *npr1-3* mutant background were individually analyzed for free SA levels after infection with the bacterial pathogen *Psm* ES4326. The *npr1-3* mutant was used as the starting material for the genetic screen, because it accumulates significantly higher levels of SA than wild type upon bacterial pathogen infection (**Figures 1A,B**; Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997; Zhang et al., 2010). Plants that accumulated significantly higher or lower levels of pathogen-induced SA than *npr1-3* were considered to be putative SA accumulation mutants. Approximately 350 such mutants were identified in the primary screen. To confirm these putative mutants, eight plants of each mutant line were tested for *Psm* ES4326-induced SA accumulation using the SA biosensor in the M_3 generation (Marek et al., 2010). Nineteen mutants with drastically altered levels of pathogen-induced SA, including 17 *sln npr1-3* and two *isn npr1-3* mutants, were chosen for further analysis (**Figure 1A**). SA levels accumulated in the remaining mutants were significantly lower than those in *npr1-3*, but slightly higher than those in the wild-type plants (data not shown).

Contamination from other mutants in the lab was excluded by checking the mutant plants under ultraviolet (UV) illumination, since the *npr1-3* mutant carries a *fuhl-2* allele, which lacks sinapoyl malate in the leaf epidermis and appears red under UV light (Chapple et al., 1992; Glazebrook et al., 1996). In addition, the presence of the *npr1-3* mutation in the identified mutants was confirmed with a derived cleaved amplification polymorphism sequence (dCAPS) marker (Table S1).

To confirm that the 19 mutants accumulate altered levels of SA after pathogen infection, we measured free SA levels accumulated in these mutants after *Psm* ES4326 infection using HPLC. Similarly to the results obtained using the SA biosensor, upon *Psm* ES4326 infection, the 17 *sln npr1-3* mutants accumulated dramatically lower levels of free SA and the two *isn npr1-3* mutants produced higher levels of free SA than the *npr-3* mutant (**Figure 1B**). These results suggest that the *sln* mutations may reside in genes that are required for pathogen-induced SA biosynthesis, whereas the *isn* mutations may be located in suppressors of SA accumulation.

PATHOGEN RESISTANCE OF THE SA ACCUMULATION MUTANTS

SA accumulation is generally associated with resistance to biotrophic and hemibiotrophic bacterial pathogens (An and Mou, 2011). To investigate whether susceptibility or resistance to bacterial pathogens in the 19 SA accumulation mutants described above is also affected, we inoculated 4-week-old plants with a low-titer inoculum ($OD_{600} = 0.0001$) of the virulent bacterial pathogen *Psm* ES4326. Interestingly, all *sln npr1-3* mutants developed enhanced disease symptoms (data not shown) and supported more bacterial growth (2- to 7-fold) compared with the *npr1-3* mutant (**Figure 2**), suggesting that the *SLN* genes are required for resistance to the bacterial pathogen. In contrast, the two *isn npr1-3* mutants supported less *Psm* ES4326 growth than *npr1-3*, although the bacteria still grew to a slightly higher titer in the *isn npr1-3* mutants than in the wild-type plants (**Figure 2**), indicating that the increased levels of SA in the *isn npr1-3* mutants may activate NPR1-independent disease resistance.

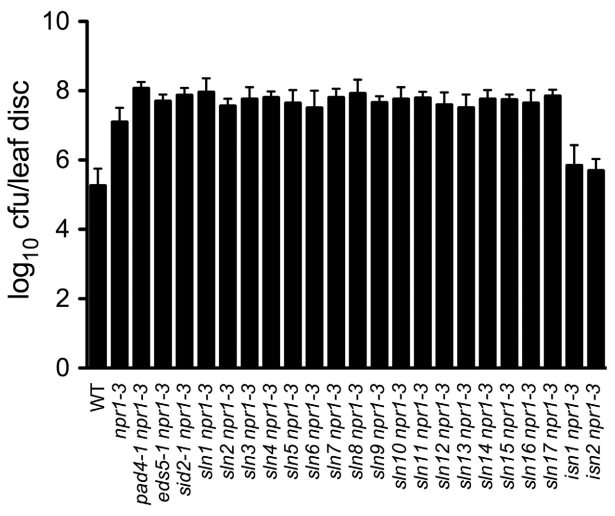


FIGURE 2 | Pathogen growth in the SA accumulation mutants. Leaves of 4-week-old plants were inoculated with a *Psm* ES4326 suspension ($OD_{600} = 0.0001$). The *in planta* bacterial titers were determined 3 days post-inoculation. Data represent the mean of eight independent samples with SD. cfu, colony-forming units. The experiment was repeated three times with similar results.

ALLELISM TEST

Analyses of the F_1 plants from crosses between the 19 SA accumulation mutants and *npr1-3* indicated that all *sln* and *isn* mutations are recessive. Several recessive mutations, including *eds5* (Nawrath and Métraux, 1999; Nawrath et al., 2002), *sid2* (Nawrath and Métraux, 1999; Wildermuth et al., 2001), *pad4* (Glazebrook et al., 1996; Zhou et al., 1998; Jirage et al., 1999), *eds1* (Parker et al., 1996; Falk et al., 1999), *eps1* (Zheng et al., 2009), and *pbs3*/*win3*/*gdg1* (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007), have been shown to compromise pathogen-induced SA accumulation. We reasoned that the *sln* mutants are unlikely alleles of *eps1*, *pbs3*, and *eds1*, since no difference in pathogen-induced free SA levels was detected between *eps1-1* or *pbs3-1* and the wild type using the SA biosensor (**Figure S1**), and two *EDS1* genes are present in the Arabidopsis ecotype Col-0 (Feys et al., 2005). We therefore tested for allelism between the *sln* mutants and *eds5*, *sid2*, or *pad4*. Pathogen-induced SA levels in F_1 plants were measured using the SA biosensor and compared with those in their parents. These allelism tests revealed that seven *sln* mutants are alleles of *eds5*, two are *sid2* alleles, and one is allelic to *pad4* (**Table 1**).

We also performed complementation tests for allelism among the remaining seven *sln* mutants. They were crossed to each other and the resulting F_1 plants were tested for the ability to accumulate SA after *Psm* ES4326 infection using the SA biosensor. We found that the *sln* mutations complemented each other, suggesting that they are located in different genes required for pathogen-induced SA accumulation (**Table 1**). Moreover, complementation test indicated that the two *isn* mutations reside in two different genes, which are likely involved in suppressing pathogen-induced SA accumulation (**Table 1**).

CHARACTERIZATION OF THE *sln1 npr1-3* MUTANT

To have a better understanding of the *sln* mutations, we further characterized one of the newly identified SA accumulation

Table 1 | Mutants identified in this genetic screen.

Gene/locus	Alleles/new mutants
SID1/EDS5	*sln2, sln6, sln8, sln9, sln11, sln13, sln14*
SID2/EDS16	*sln7, sln10*
PAD4	*sln12*
SLN1	*sln1*
SLN3	*sln3*
SLN4	*sln4*
SLN5	*sln5*
SLN15	*sln15*
SLN16	*sln16*
SLN17	*sln17*
ISN1	*isn1*
ISN2	*isn2*

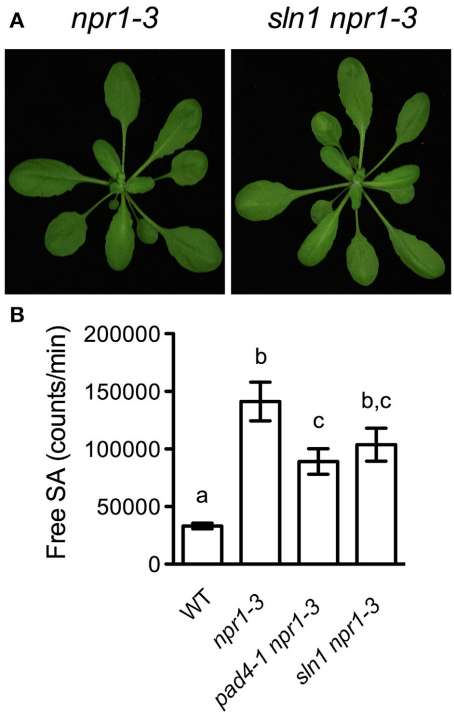


FIGURE 3 | Further characterization of *sln1 npr1-3*. (A) Photos of 4-week-old soil-grown *npr1-3* and *sln1 npr1-3* plants. **(B)** Luminescence from crude extracts of *Pst* DC3000/*avrRpt2*-infected wild-type, *npr1-3*, *pad4-1 npr1-3*, *sln1 npr1-3* leaf tissues measured with the SA biosensor. Values are the mean of eight independent samples with SD. Different letters above the bars indicate significant differences ($P < 0.05$, One-Way ANOVA). The experiment was repeated three times with similar results.

mutants, *sln1 npr1-3*. The *sln1 npr1-3* mutant was morphologically similar to *npr1-3* (**Figure 3A**). F_1 plants from a backcross of *sln1 npr1-3* and *npr1-3* accumulated similar levels of free SA as *npr1-3*, suggesting that *sln1* is recessive. SA analysis of F_2 progeny showed that *sln1* segregated as a single Mendelian locus (high SA:low SA, 33:8; $\chi^2 = 0.6585$, $0.25 < P < 1$).

It was reported that the *pad4* mutation does not affect free SA accumulation in response to the avirulent bacterial pathogen *Pst* DC3000/*avrRpt2* (Zhou et al., 1998). To test whether the *sln1* mutation influences the avirulent pathogen-induced SA accumulation, we challenged *sln1 npr1-3* plants with *Pst* DC3000/*avrRpt2*. As shown in **Figure 3B**, *Pst* DC3000/*avrRpt2* induced significant SA accumulation in both *sln1 npr1-3* and *pad4-1 npr1-3* plants. Although free SA levels accumulated in the *sln1 npr1-3* plants were still slightly lower than those in the *npr1-3* plants, the difference was not as dramatic as that detected in the *Psm* ES4326-infected plants (**Figure 1**). These results indicate that the avirulent pathogen *Pst* DC3000/*avrRpt2*-triggered SA accumulation is largely independent of *SLN1*.

SA ACCUMULATION IN THE *sln1* SINGLE MUTANT

Since the *sln1* mutation is able to reduce SA accumulation in *npr1-3*, it may affect SA accumulation in the presence of *NPR1*. To test this, we isolated *sln1* single mutant in the F_2 progeny of a cross between *sln1 npr1-3* and the wild-type Col-0 using the *npr1-3* dCAPS marker (Table S1) and based on SA levels accumulated in the plants upon *Psm* ES4326 infection. As shown in **Figures 4A,B**, both free SA and total SA levels accumulated in the *sln1* single mutant plants after *Psm* ES4326 infection were significantly lower than those in the wild type. We also found that *Psm* ES4326-induced expression of *ICS1*, which is responsible for pathogen-induced SA accumulation (Wildermuth et al., 2001), was significantly reduced in the *sln1* single mutant compared with that in the wild type (**Figure 4C**), indicating that *SLN1* may regulate SA accumulation through *ICS1*.

PATHOGEN RESISTANCE OF THE *sln1* SINGLE MUTANT

We then investigated pathogen growth in the *sln1* single mutant. After infected with a low-titer inoculum ($OD_{600} = 0.0001$) of *Psm* ES4326, the *sln1* single mutant plants developed enhanced disease symptoms (**Figure 5A**), and supported ∼15-fold more bacterial growth than the wild type (**Figure 5B**). We also tested pathogen-induced *PR* gene expression in the *sln1* single mutant. As shown in **Figures 5C–E**, *Psm* ES4326-induced *PR1* expression was significantly reduced in the *sln1* single mutant, but the induction of *PR2* and *PR5* in *sln1* was comparable to that in the wild type. Taken together, these results indicate that *SLN1* is required for defense responses against the bacterial pathogen *Psm* ES4326.

Since the *sln1* mutation inhibits pathogen-induced SA accumulation, exogenous SA may restore defense responses in *sln1* plants. Indeed, SA treatment induced similar levels of *PR1* gene expression and resistance to *Psm* ES4326 in the *sln1* single mutant and the wild-type plants (**Figures 6A,B**). Based on these results, we concluded that the signaling pathway downstream of SA in *sln1* is intact. Thus, *SLN1* most likely functions in a signal amplification loop upstream of SA.

PRELIMINARY MAPPING OF THE *sln1* MUTATION

To map the *sln1* mutation, *sln1 npr1-3* (in the Col-0 genetic background) was crossed with *npr1*-L (an *npr1* T-DNA insertion mutant in the polymorphic ecotype Landsberg *erecta*) to generate a segregating population. Preliminary mapping using 74 F_2 plants, which accumulated extremely low levels of SA after *Psm* ES4326 infection, revealed that *sln1* is located between gene At1g01448 and the molecular marker PAI1.2 (**Figure 7**). To our knowledge, this region does not contain any known genes regulating pathogen-induced SA accumulation. Therefore, *SLN1* likely encodes a new regulator of SA biosynthesis. Further fine-mapping and/or whole genome sequencing will help identify the *sln1* mutation.

DISCUSSION

In this study, we performed a forward genetic screen for Arabidopsis mutants with altered SA accumulation during pathogen infection using the newly developed SA biosensor method (Marek et al., 2010). Compared with the HPLC and GC/MS approaches, the SA biosensor method is much faster and less expensive (Malamy et al., 1992; Verberne et al., 2002; Marek et al., 2010). Using this method, we screened a large population (35,000) of M_2 plants in less than 1 year. Approximately 350 putative SA accumulation mutants in the *npr1-3* genetic background were identified. Among them, 17 are *sln npr1-3* mutants,

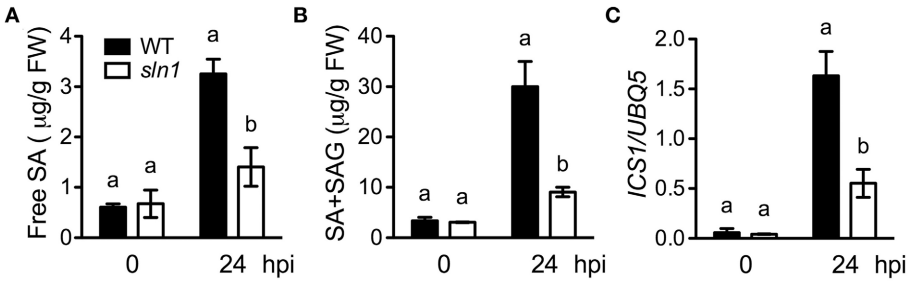


FIGURE 4 | Pathogen-induced SA levels and *ICS1* expression in the *sln1* single mutant. Leaves of 4-week-old soil-grown wild-type and *sln1* plants were infiltrated with a suspension of *Psm* ES4326 ($OD_{600} = 0.001$). The inoculated leaves were harvested 24 h post-inoculation (hpi) for SA measurement using HPLC or *ICS1* expression analysis using qPCR. **(A)** Free SA levels in *Psm* ES4326-infected wild-type and *sln1* plants. **(B)** Total SA levels in *Psm* ES4326-infected wild-type and *sln1* plants. **(C)** *ICS1* expression levels in *Psm* ES4326-infected wild-type and *sln1* plants. Values are the mean of three independent samples with SD. Different letters above the bars indicate significant differences ($P < 0.05$, Student's *t*-test). The comparison was made separately for each time point. Expression of *ICS1* in **(C)** was normalized against constitutively expressed *UBQ5*. The experiments were repeated three times with similar results.

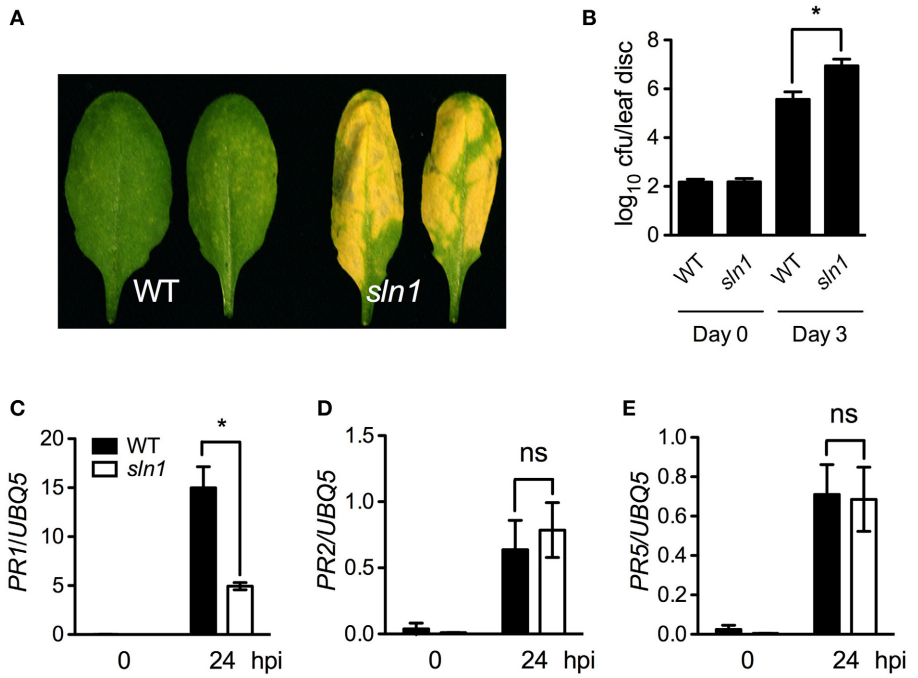


FIGURE 5 | Defense responses in the *sln1* single mutant. (A) Disease symptoms of *Psm* ES4326-infected wild-type and *sln1* leaves. Four-week-old soil-grown plants were inoculated with a suspension of *Psm* ES4326 ($OD_{600} = 0.0001$). Photos were taken 3 days post-inoculation. **(B)** Growth of *Psm* ES4326 in wild-type and *sln1* plants. Four-week-old soil-grown plants were inoculated with a suspension of *Psm* ES4326 ($OD_{600} = 0.0001$). The *in planta* bacterial titers were determined immediately and 3 days post-inoculation. Data represent the mean of eight independent samples with SD. **(C–E)** *Psm* ES4326-induced *PR1* **(C)**, *PR2* **(D)**, and *PR5* **(E)** gene expression in wild-type and *sln1* plants. Four-week-old soil-grown plants were inoculated with a suspension of *Psm* ES4326 ($OD_600 = 0.001$). Total RNA was extracted from leaf tissues collected at 24 hpi and subjected to qPCR analysis. Data represent the mean of three independent samples with SD. An asterisk (*) above the bars indicates significant differences ($P < 0.05$, Student's *t*-test). ns, not significant. All experiments were repeated three times with similar results.

producing significantly lower levels of SA than *npr1-3* after *Psm* ES4326 infection, and two are *isn npr1-3* mutants, accumulating higher levels of SA than *npr1-3* (**Figures 1A,B**). Interestingly, upon *Psm* ES4326 infection, SA levels accumulated in the remaining putative mutants (in the *npr1-3* background) were significantly lower than those in *npr1-3*, but slightly higher than those in the wild-type plants, suggesting the existence of a larger number of regulatory components involved in pathogen-induced SA accumulation. Indeed, genetic studies have uncovered a complicated signaling network that regulates SA accumulation. This consists of upstream SA signaling components (such as EDS1, PAD4, and NDR1), downstream SA signaling components (such as NPR1),

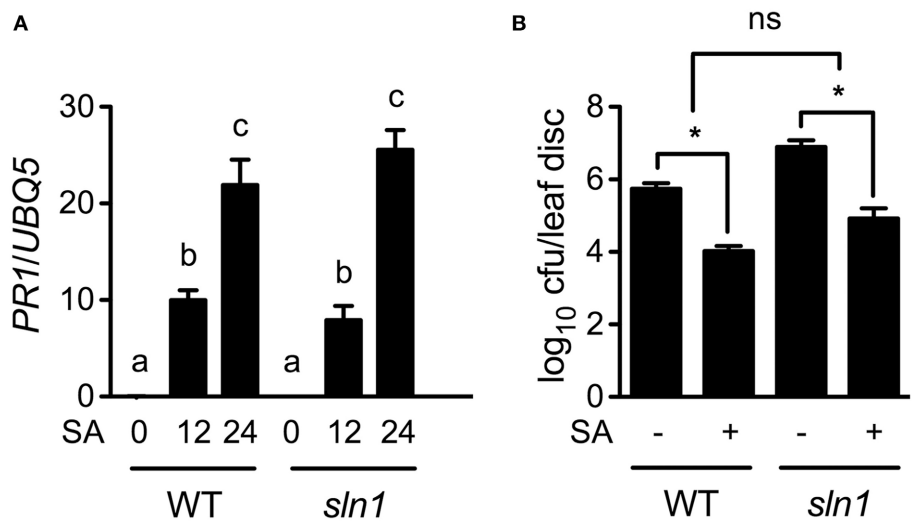


FIGURE 6 | Exogenous SA-induced *PR* gene expression and resistance in *sln1*. **(A)** Exogenous SA-induced *PR1* expression in wild-type and *sln1* plants. Four-week-old soil-grown wild-type and *sln1* plants were soaked with an SA water solution (1 mM). Total RNA was extracted from leaf tissues collected at the indicated time points and analyzed for *PR1* expression using qPCR. Values are the mean of three independent samples with SD. Different letters above the bars indicate significant differences ($P < 0.05$, One-Way ANOVA). The comparison was made separately for each genotype. **(B)** Exogenous SA-induced resistance to *Psm* ES4326 in wild-type and *sln1* plants. Plants were treated as in **(A)**. Twelve hours later, the plants were inoculated with a suspension of *Psm* ES4326 ($OD_{600} = 0.001$). The *in planta* bacterial titers were determined 3 days post-inoculation. Values are the mean of eight independent samples with SD. An asterisk (*) above the bars indicates significant differences ($P < 0.05$, Two-Way ANOVA). ns, not significant. These experiments were repeated three times with similar results.

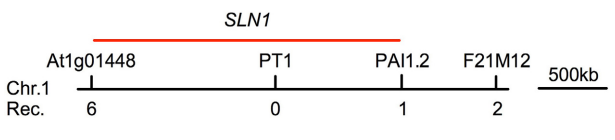


FIGURE 7 | Preliminary mapping of the *sln1* mutation. A total of 74 F_2 progeny homozygous for *sln1* were used to determine the approximate position of the *sln1* mutation using bulked segregant analysis. The *sln1* mutation was linked to the molecular marker PT1 on chromosome 1. Out of the 74 F_2 plants, six were heterozygous at gene At1g01448, and one was heterozygous at the molecular marker PAI1.2. The heterozygotes found by these two markers were mutually exclusive. No heterozygotes were found at PT1. The *SLN1* gene is likely located in the vicinity of PT1, as indicated by the red bar. Rec., recombinant.

transcription factors (such as CBP60g and SARD1), metabolic enzymes (such as EPS1 and PBS3), and various positive and negative feedback loops (Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997; Zhou et al., 1998; Jirage et al., 1999; Shapiro and Zhang, 2001; Wildermuth et al., 2001; Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007; Zheng et al., 2009; Zhang et al., 2010; Wang et al., 2011).

The SA accumulation phenotype of the *sln* mutants is similar to that of *eds5*, *sid2*, and *pad4* mutants (Zhou et al., 1998; Jirage et al., 1999; Nawrath and Métraux, 1999; Wildermuth et al., 2001; Nawrath et al., 2002). *EDS5* and *SID2* encode a chloroplast MATE transporter and an SA biosynthetic enzyme ICS1, respectively, which are two important components in the SA biosynthesis pathway. PAD4 is a lipase-like protein involved in an SA positive signal-amplification loop required for activation of defense responses (Jirage et al., 1999). Complementation tests indicated that seven out of the 17 *sln* mutants are new alleles of *eds5*, two are alleles of *sid2*, and one is allelic to *pad4*, and the other seven *sln* and two *isn* mutants are new non-allelic mutants (**Table 1**). Although this is a large-scale genetic screen, the low frequency of alleles for the new *sln* and *isn* mutants indicates that our genetic screen has not been saturated.

Several other recessive mutations have also been reported to impair pathogen-induced SA accumulation. In the *eps1-1* mutant, pathogen-induced accumulation of SAG was greatly reduced, but free SA levels were comparable to those in the wild type. *EPS1* encodes a novel member of the BAHD acyltransferase superfamily, which is predicted to be directly involved in the synthesis of a precursor or regulatory molecule for SA biosynthesis (Zheng et al., 2009). Similarly, the *pbs3-1* mutant displayed decreased pathogen-induced accumulation of SAG, but varied in free SA accumulation between studies (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007). PBS3 belongs to the acyl adenylate/thioesterforming enzyme superfamily. The exact functions of both EPS1 and PBS3 in SA biosynthesis, however, have not been clearly defined. Consistent with these studies, we found that free SA levels in the *eps1-1* and *pbs3-1* mutants were comparable to those in the wild type when assayed with the SA biosensor (**Figure S1**). Thus, the *sln* mutations are unlikely located in either *EPS1* or *PBS3*, since these mutations greatly influenced *Psm* ES4326-induced free SA accumulation (**Figures 1A,B**). Additionally, although the *eds1* mutation significantly affects pathogen-induced accumulation of both free SA and SAG (Falk et al., 1999), the *sln* mutants are unlikely alleles of *eds1*, because there are two *EDS1* genes lying in tandem on chromosome 3

of the Arabidopsis ecotype Col-0 (Feys et al., 2005). Therefore, the *SLN* genes may encode new signaling components downstream of recognition of pathogen infection, or new enzymes directly involved in the synthesis of a precursor and/or regulatory molecule for SA biosynthesis.

In addition to components upstream of SA biosynthesis, the downstream component, NPR1 (nonexpressor of *PR* genes1), which has been shown to be an important regulator of defense responses (Cao et al., 1997; Dong, 2004), also regulates SA levels. Mutations in the *NPR1* gene enhance SA accumulation during pathogen infection, suggesting that NPR1 is a feedback inhibitor of SA biosynthesis (**Figures 1A,B**; Clarke et al., 2000; Wildermuth et al., 2001; Zhang et al., 2010). Here we found that *eds5 npr1-3*, *sid2 npr1-3*, *pad4 npr1-3*, and *sln npr1-3* double mutants accumulated significantly lower levels of SA than *npr1-3* (**Figures 1A,B**), suggesting that these mutations (*eds5*, *sid2*, *pad4*, and *sln*) suppress *npr1*-mediated SA hyperaccumulation. On the other hand, these double mutants were more susceptible to *Psm* ES4326 than *npr1-3* (**Figure 2**), indicating that *EDS5*, *SID2*, *PAD4*, and the *SLN* genes may contribute to NPR1-independent defense responses (Glazebrook, 2001). NPR1-independent defense signaling pathways have been shown to be activated in various Arabidopsis mutants, including *sni1* (Li et al., 1999), *snc1* (Li et al., 2001), *ssi* (Shah et al., 1999, 2001), and *cpr* (Bowling et al., 1997; Clarke et al., 1998). The two *isn* mutations appear to also activate NPR1-independent disease resistance (**Figure 2**).

We further isolated and characterized the *sln1* single mutant. The *sln1* plants exhibited significantly reduced levels of *Psm* ES4326-induced SA and supported more *Psm* ES4326 growth than the wild-type plants (**Figures 4A,B**, **5B**), suggesting that *SLN1* plays an important role in activation of defense responses against this pathogen. Interestingly, the *sln1* mutation appears to differentially influence pathogen-induced *PR1*, *PR2*, and *PR5* expression. *Psm* ES4326-induced *PR1* expression was greatly reduced in *sln1* plants, but induction of *PR2* and *PR5* was nearly unaffected (**Figures 5C–E**). In this regard, *sln1* is also similar to *eds5*, *sid2*, and *pad4*, which cause reduced induction of *PR1*, but have no effect on the expression of *PR2* and *PR5* (Rogers and Ausubel, 1997; Zhou et al., 1998; Nawrath and Métraux, 1999). On the other hand, pathogen-induced expression of *PR1*, *PR2*, and *PR5* is strongly reduced in *NahG* transgenic plants (Nawrath and Métraux, 1999), which argues against the idea that an SA-independent pathway exists to control *PR2* and *PR5* expression. It is possible that the low levels of SA accumulated in the SA biosynthesis mutants are sufficient for induction of *PR2* and *PR5*, but not for *PR1*.

In summary, we identified a group of new SA accumulation mutants, including seven *sln* mutants and two *isn* mutants, in a genetic screen using the newly developed SA biosensor-based method. Further characterization of these *sln* and *isn* mutants and cloning of the *SLN* and *ISN* genes will shed new light on the molecular mechanisms underlying pathogen-induced SA accumulation and SA-mediated defense signaling in plants.

AUTHOR CONTRIBUTIONS

Yezhang Ding and Zhonglin Mou designed research. Yezhang Ding and Danjela Shaholli performed mutant screen. Yezhang Ding characterized mutants and analyzed data. Yezhang Ding and Zhonglin Mou wrote the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Hui Wang (NERC/Centre for Ecology and Hydrology-Oxford, Oxford, UK) for the SA biosensor strain *Acinetobacter* sp. ADPWH_*lux* and Dr. Sixue Chen (University of Florida) for access to the HPLC equipment. We are grateful to Dr. Roger Innes (Indiana University) for the *pbs3-1* mutant and Dr. Zhixiang Chen (Purdue University) for the *eps1-1* mutant. We thank the Arabidopsis Biological Resource Center at Ohio State University for providing the T-DNA insertion line GT_5_89558 (*npr1*-L). This work was supported by a grant from the National Science Foundation (IOS-0842716) awarded to Zhonglin Mou.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00763/abstract

Figure S1 | SA accumulation in *eps1* and *pbs3*. Luminescence from crude extracts of *Psm* ES4326-infected wild-type, *npr1-3, eps1-1*, and *pbs3-1* leaf tissues measured with the SA biosensor. Values are the mean of six samples with standard deviation (SD). The experiment was repeated with similar results.

REFERENCES

An, C., and Mou, Z. (2011). Salicylic acid and its function in plant immunity. *J. Integr. Plant Biol.* 53, 412–428. doi: 10.1111/j.1744-7909.2011.01043.x

Bowling, S. A., Clarke, J. D., Liu, Y., Klessig, D. F., and Dong, X. (1997). The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9, 1573–1584. doi: 10.1105/tpc.9.9.1573

Cao, H., Glazebrook, J., Clark, J. D., Volko, S., and Dong, X. (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57–63. doi: 10.1016/S0092-8674(00)81858-9

Chapple, C. C., Vogt, T., Ellis, B. E., and Somerville, C. R. (1992). An *Arabidopsis* mutant defective in the general phenylpropanoid pathway. *Plant Cell* 4, 1413–1424. doi: 10.1105/tpc.4.11.1413

Clarke, J. D., Liu, Y., Klessig, D. F., and Dong, X. (1998). Uncoupling *PR* gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis *cpr6-1* mutant. *Plant Cell* 10, 557–569.

Clarke, J. D., Volko, S. M., Ledford, H., Ausubel, F. M., and Dong, X. (2000). Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in *Arabidopsis*. *Plant Cell* 12, 2175–2190. doi: 10.1105/tpc.12.11.2175

Defraia, C. T., Schmelz, E. A., and Mou, Z. (2008). A rapid biosensor-based method for quantification of free and glucose-conjugated salicylic acid. *Plant Methods* 4, 28. doi: 10.1186/1746-4811-4-28

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. (1994). Central role of salicylic acid in plant disease resistance. *Science* 266, 1247–1250. doi: 10.1126/science.266.5188.1247

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arabidopsis Book* 9:e0156. doi: 10.1199/tab.0156

Dong, X. (2004). NPR1, all things considered. *Curr. Opin. Plant Biol.* 7, 547–752. doi: 10.1016/j.pbi.2004.07.005

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

Falk, A., Feys, B. J., Frost, L. N., Jones, J. D., Daniels, M. J., and Parker, J. E. (1999). EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3292–3297. doi: 10.1073/pnas.96.6.3292

Feys, B. J., Wiermer, M., Bhat, R. A., Moisan, L. J., Medina-Escobar, N., Neu, C., et al. (2005). *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stablizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell* 17, 2601–2613. doi: 10.1105/tpc.105.033910

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., et al. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261, 754–756. doi: 10.1126/science.261.5122.754

Garcion, C., Lohmann, A., Lamodière, E., Catinot, J., Buchala, A., Doermann, P., et al. (2008). Characterization and biological function of the *ISOCHORISMATE SYNTHASE2* gene of *Arabidopsis*. *Plant Physiol.* 147, 1279–1287. doi: 10.1104/pp.108.119420

Glazebrook, J. (2001). Genes controlling expression of defense responses in *Arabidopsis*–2001 status. *Curr. Opin. Plant Biol.* 4, 301–308. doi: 10.1016/S1369-5266(00)00177-1

Glazebrook, J., Rogers, E. E., and Ausubel, F. M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 143, 973–982.

Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. (1994). Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. *Cell* 7, 551–563. doi: 10.1016/0092-8674(94)90217-8

Huang, W. E., Huang, L., Preston, G. M., Martin, N., Carr, J. P., Li, Y. H., et al. (2006). Quantitative *in situ* assay of salicylic acid in tobacco leaves using a genetically modified biosensor strain of *Acinetobacter* sp. ADP1. *Plant J.* 46, 1073–1083. doi: 10.1111/j.1365-313X.2006.02758.x

Huang, W. E., Wang, H., Zheng, H., Huang, L., Singer, A. C., Thompson, I., et al. (2005). Chromosomally located gene fusions constructed in *Acinetobacter* sp. ADP1 for the detection of salicylate. *Environ. Microbiol.* 7, 1339–1348. doi: 10.1111/j.1462-5822.2005.00821.x

Jagadeeswaran, G., Raina, S., Acharya, B. R., Maqbool, S. B., Mosher, S. L., Appel, H. M., et al. (2007). Arabidopsis *GH3-LIKE DEFENSE GENE 1* is required for accumulation of salicylic acid, activation of defense responses and resistance to *Pseudomonas syringae*. *Plant J.* 51, 234–246. doi: 10.1111/j.1365-313X.2007.03130.x

Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., et al. (1999). *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13583–13588. doi: 10.1073/pnas.96.23.13583

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Lawton, K. A., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol. Plant Microbe Interact.* 8, 863–870. doi: 10.1094/MPMI-8-0863

Lee, M. W., Lu, H., Jung, H. W., and Greenberg, J. T. (2007). A key role for the Arabidopsis WIN3 protein in disease resistance triggered by *Pseudomonas syringae* that secrete AvrRpt2. *Mol. Plant Microbe Interact.* 20, 1192–1200. doi: 10.1094/MPMI-20-10-1192

León, J., Shulaev, V., Yalpani, N., Lawton, M. A., and Raskin, I. (1995). Benzoic acid 2-hydroxylase, a soluble oxygenase from tobacco, catalyzes salicylic acid biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10413–10417 doi: 10.1073/pnas.92.22.10413

Li, X., Clarke, J. D., Zhang, Y., and Dong, X. (2001). Activation of an EDS1-mediated R-gene pathway in the *snc1* mutant leads to constitutive, NPR1-independent pathogen resistance. *Mol. Plant Microbe Interact.* 14, 1131–1139. doi: 10.1094/MPMI.2001.14.10.1131

Li, X., Zhang, Y., Clarke, J. D., Li, Y., and Dong, X. (1999). Identification and cloning of a negative regulator of systemic acquired resistance, *SNI1*, through a screen for suppressors of *npr1-1*. *Cell* 98, 329–339. doi: 10.1016/S0092-8674(00)81962-5

Malamy, J., Hennig, J., and Klessig, D. F. (1992). Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. *Plant Cell* 4, 359–366. doi: 10.1105/tpc.4.3.359

Marek, G., Carver, R., Ding, Y., Sathyanarayan, D., Zhang, X., and Mou, Z. (2010). A high-throughput method for isolation of salicylic acid metabolic mutants. *Plant Methods* 6, 21. doi: 10.1186/1746-4811-6-21

Mauch-Mani, B., and Slusarenko, A. J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell* 8, 203–212. doi: 10.1105/tpc.8.2.203

Nawrath, C., Heck, S., Parinthawong, N., and Métraux, J. P. (2002). EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell* 14, 275–286. doi: 10.1105/tpc.010376

Nawrath, C., and Métraux, J. P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11, 1393–1404.

Nobuta, K., Okrent, R. A., Stoutemyer, M., Rodibaugh, N., Kempema, L., Wildermuth, M. C., et al. (2007). The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent defense responses in *Arabidopsis*. *Plant Physiol.* 144, 1144–1156. doi: 10.1104/pp.107.097691

Parker, J. E., Frost, L. N., Falk, A., Gunn, N. D., and Daniels, M. J. (1996). Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. *Plant Cell* 8, 2033–2046. doi: 10.1105/tpc.8.11.2033

Rate, D. N., Cuenca, J. V., Bowman, G. R., Guttman, D. S., and Greenberg, J. T. (1999). The gain-of-function *Arabidopsis acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defense, and cell growth. *Plant Cell* 11, 1695–1708. doi: 10.1105/tpc.11.9.1695

Rogers, E. E., and Ausubel, F. M. (1997). *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. *Plant Cell* 9, 305–316. doi: 10.1105/tpc.9.3.305

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H. Y., et al. (1997). The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I_kB. *Plant Cell* 9, 425–439.

Shah, J., Kachroo, P., and Klessig, D. F. (1999). The Arabidopsis *ssi1* mutation restores pathogenesis-related gene expression in *npr1* plants and renders Defensin gene expression SA dependent. *Plant Cell* 11, 191–206. doi: 10.1105/tpc.11.2.191

Shah, J., Kachroo, P., Nandi, A., and Klessig, D. F. (2001). A recessive mutation in the *Arabidopsis SSI2* gene confers SA- and NPR1-independent expression of *PR* genes and resistance against bacterial and oomycete pathogens. *Plant J.* 25, 563–574. doi: 10.1046/j.1365-313x.2001.00992.x

Shah, J., Tsui, F., and Klessig, D. F. (1997). Characterization of a *salicylic acid-insensitive* mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Mol. Plant Microbe Interact.* 10, 69–78. doi: 10.1094/MPMI.1997.10.1.69

Shapiro, A. D., and Zhang, C. (2001). The role of *NDR1* in avirulence gene-directed signaling and control of programmed cell death in Arabidopsis. *Plant Physiol.* 127, 1089–1101. doi: 10.1104/pp.010096

Verberne, M. C., Brouwer, N., Delbianco, F., Linthorst, H. J., Bol, J. F., and Verpoorte, R. (2002). Method for the extraction of the volatile compound salicylic acid from tobacco leaf material. *Phytochem. Anal.* 13, 45–50. doi: 10.1002/pca.615

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen le, V., Katagiri, F., et al. (2011). CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling. *Plant J.* 67, 1029–1041. doi: 10.1111/j.1365-313X.2011.04655.x

White, R. F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* 99, 410–412. doi: 10.1016/0042-6822(79)90019-9

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Zhang, X., Chen, S., and Mou, Z. (2010). Nuclear localization of NPR1 is required for regulation of salicylate tolerance, *isochorismate synthase 1* expression and salicylate accumulation in Arabidopsis. *J. Plant Physiol.* 167, 144–148. doi: 10.1016/j.jplph.2009.08.002

Zheng, Z., Qualley, A., Fan, B., Dudareva, N., and Chen, Z. (2009). An important role of a BAHD acyl transferase-like protein in plant innate immunity. *Plant J.* 57, 1040–1053. doi: 10.1111/j.1365-313X.2008.03747.x

Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F., and Glazebrook, J. (1998). PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell* 10, 1021–1030. doi: 10.1105/tpc.10.6.1021

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 October 2014; paper pending published: 04 November 2014; accepted: 10 December 2014; published online: 07 January 2015.

*Citation: Ding Y, Shaholli D and Mou Z (2015) A large-scale genetic screen for mutants with altered salicylic acid accumulation in Arabidopsis. Front. Plant Sci. **5**:763. doi: 10.3389/fpls.2014.00763*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 12 January 2015
doi: 10.3389/fpls.2014.00777

Identification of multiple salicylic acid-binding proteins using two high throughput screens

Murli Manohar[1‡], Miaoying Tian[1†‡], Magali Moreau[1†‡], Sang-Wook Park[1†], Hyong Woo Choi[1], Zhangjun Fei[1,2], Giulia Friso[3], Muhammed Asif[1†], Patricia Manosalva[1], Caroline C. von Dahl[1†], Kai Shi[1†], Shisong Ma[4], Savithramma P. Dinesh-Kumar[4], Inish O'Doherty[1†], Frank C. Schroeder[1], Klass J. van Wijk[3] and Daniel F. Klessig[1]*

[1] Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY, USA
[2] Plant, Soil, and Nutrition Laboratory, United States Department of Agriculture, Ithaca, NY, USA
[3] Department of Plant Biology, Cornell University, Ithaca, NY, USA
[4] Department of Plant Biology and Genome Center, University of California, Davis, Davis, CA, USA

Edited by:
Loreto Holuigue, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Steven H. Spoel, University of Edinburgh, UK
Jens Staal, Ghent University, Belgium

***Correspondence:**
Daniel F. Klessig, Boyce Thompson Institute for Plant Research, Cornell University, 533 Tower Road, Ithaca, NY 14853, USA
e-mail: dfk8@cornell.edu

†Present address:
Miaoying Tian, Department of Plant and Environmental Protection Sciences, University of Hawaii at Manoa, Honolulu, USA;
Magali Moreau, Department of Chemistry and Chemical Biology, Cornell University, Ithaca, USA;
Sang-Wook Park, Department of Entomology and Plant Pathology, Auburn University, Auburn, USA;
Muhammed Asif, Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan;
Caroline C. von Dahl, Karlsruhe Institute of Technology, Karlsruhe, Germany;
Kai Shi, Department of Horticulture, Zhejiang University, Hangzhou, China;
Inish O'Doherty, Pfizer Inc., San Diego, USA

‡*These authors have contributed equally to this work.*

Salicylic acid (SA) is an important hormone involved in many diverse plant processes, including floral induction, stomatal closure, seed germination, adventitious root initiation, and thermogenesis. It also plays critical functions during responses to abiotic and biotic stresses. The role(s) of SA in signaling disease resistance is by far the best studied process, although it is still only partially understood. To obtain insights into how SA carries out its varied functions, particularly in activating disease resistance, two new high throughput screens were developed to identify novel SA-binding proteins (SABPs). The first utilized crosslinking of the photo-reactive SA analog 4-AzidoSA (4AzSA) to proteins in an Arabidopsis leaf extract, followed by immuno-selection with anti-SA antibodies and then mass spectroscopy-based identification. The second utilized photo-affinity crosslinking of 4AzSA to proteins on a protein microarray (PMA) followed by detection with anti-SA antibodies. To determine whether the candidate SABPs (cSABPs) obtained from these screens were true SABPs, recombinantly-produced proteins were generated and tested for SA-inhibitable crosslinking to 4AzSA, which was monitored by immuno-blot analysis, SA-inhibitable binding of the SA derivative 3-aminoethylSA (3AESA), which was detected by a surface plasmon resonance (SPR) assay, or SA-inhibitable binding of [^{3}H]SA, which was detected by size exclusion chromatography. Based on our criteria that true SABPs must exhibit SA-binding activity in at least two of these assays, nine new SABPs are identified here; nine others were previously reported. Approximately 80 cSABPs await further assessment. In addition, the conflicting reports on whether NPR1 is an SABP were addressed by showing that it bound SA in all three of the above assays.

Keywords: salicylic acid, salicylic acid-binding proteins, salicylic acid signaling, plant immunity, disease resistance

INTRODUCTION

Salicylic acid (SA) and its derivatives, collectively termed salicylates, have been the focus of the medical community since the discovery in 1828/1829 by German and French pharmacologists that the active ingredient in willow bark, which relieves pain and fever, is salicin, a glycoside of SA. Salicin is rapidly converted into SA in the gastrointestinal tract. Over the next decade, German and French chemists produced synthetic SA, which greatly reduced SA's cost and widened its use. The synthesis of acetylSA (aspirin), which causes less stomach irritation than SA but is comparably efficacious, enabled this compound to become the most widely used drug worldwide (natural or synthetic; reviewed in Weissmann, 1991; Wick, 2012).

Efforts to characterize SA's role in plants span a much more recent history. For centuries, SA and other phenolic compounds synthesized by plants were thought to be non-essential for critical processes and thus called secondary metabolites (Hadacek et al., 2011). Three discoveries changed this view. First, Cleland and Ajami (1974) identified SA in the phloem sap of flowering *Xanthium strumarium*. Since this sap induced flowering in *Lemna gibba*, they suggested that SA is an endogenous signal. Second, Raskin and colleagues (reviewed in Raskin, 1992) demonstrated that a dramatic rise in SA levels preceded thermogenesis in the central column of the inflorescence of *Sauromatum guttatum*. Moreover, application of exogenous SA caused elevated temperatures in this organ, suggesting that SA is an important signaling molecule for thermogenesis in some plants. Third, analyses of disease resistance in the tobacco-tobacco mosaic virus pathosystem (White, 1979; Malamy et al., 1990) and the cucumber-tobacco necrosis virus/*Colletotrichum lagenarium* pathosystems (Meìtraux et al., 1990), followed by many studies over the following two decades (reviewed in Vlot et al., 2009), demonstrated that SA is a critical signaling hormone for the activation of several levels of immunity in response to biotrophic pathogens, including effector-trigger immunity (also called *R* gene-mediated resistance), Microbe-Associated Molecular Pattern (MAMP)-triggered immunity, and systemic acquired resistance. Thousands of papers documenting SA's involvement in plant disease resistance have been published over the past half century; this extensive research has revealed a complex signaling network of upstream and downstream components (reviewed in Vlot et al., 2009; Dempsey et al., 2011). In addition to its many roles in immunity and its involvement in thermogenesis and flowering, SA has been shown to play an important role(s) in responding to abiotic stresses, such as heat, chilling, drought, osmotic stress, and heavy metal toxicity. SA also regulates biochemical and physiological processes throughout a plant's life span, including seed germination, photosynthesis, respiration, growth, and senescence (reviewed in Rivas-San Vicente and Plasencia, 2011).

Several general approaches have been used to decipher how SA modulates the plant immune system. The first involved the isolation of mutants, primarily in Arabidopsis, that exhibited altered defenses-related responses following exogenous SA treatment. The most notable success of this genetic approach was the identification of NPR1/NIM1/SAI1 by four independent research groups (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). The pioneering work of Dong and co-workers demonstrated that NPR1 is a transcriptional co-factor that plays a critical role in positively regulating SA-induced immune responses (for review Spoel and Dong, 2012). The second approach utilized classical biochemical methods to identify proteins that bound radio-labeled SA in protein extracts prepared primarily from tobacco leaves. This approach yielded several SA-binding proteins (SABPs), all of which are enzymes. They include catalase and ascorbate peroxidase, which are the two major H_2O_2-scavenging enzymes, as well as carbonic anhydrase (named SABP3), and methyl salicylate esterase (named SABP2), which is involved in systemic acquired resistance (Chen et al., 1993; Durner and Klessig, 1995; Slaymaker et al., 2002; Kumar and Klessig, 2003; Park et al., 2007). The third approach used genetic and biochemical methods to assess whether SA directly/physically interacts with NPR1 and/or its paralogs NPR3 and NPR4. Fu et al. (2012) reported that while NPR1 did not bind SA, NPR3 and NPR4 did, and therefore concluded that NPR3 and NPR4 are receptors for SA. In contrast, Wu et al. (2012) demonstrated that NPR1 bound SA and thus concluded that it is an SA receptor.

While these efforts to identify SA receptors have provided important insights into SA's mechanisms of action during immune responses, many aspects of SA signaling remain unclear. Beyond determining whether NPR1, NPR3 and/or NPR4 function as SA receptors, some SA-induced defense responses are activated via an NPR1-independent pathway that is currently uncharacterized. Likewise, the mechanisms through which SA modulates many other NPR1-independent plant processes are unknown. To facilitate the identification of proteins through which SA mediates its effects on these processes, we developed two high-throughput strategies to identify putative/candidate SABPs (cSABPs) in Arabidopsis using biochemical and biophysical methods. The first relies on photo-affinity crosslinking to 4-Azido SA (4AzSA), followed by immuno-selection and mass spectroscopy-based identification (Tian et al., 2012), while the second utilizes 4AzSA crosslinking and immuno-detection of cSABPs on a protein microarray (PMA) (Moreau et al., 2013). Here, we report the identification of nine new SABPs, based on at least two independent assays, and provide a list of more than 100 cSABPs identified by these two high-throughput screens.

METHOD AND MATERIALS

PLANT GROWTH AND PATHOGEN INOCULATION

Arabidopsis thaliana plants were grown as described previously (Vlot et al., 2008). Pathogen inoculation and leaf harvest were performed as described previously (Tian et al., 2012).

PLASMID CONSTRUCTION, PROTEIN EXPRESSION AND PURIFICATION

cSABPs were selected for further analyses in part based on the absence of predicted trans-membrane domains by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). PCR amplified protein coding sequences from selected cSABPs were cloned into pET28a to generate recombinant proteins with an N-terminal His_6 tag. To increase the solubility of NPR1 and FBA5, N-terminal fusions to His_6-MBP (maltose binding protein) were generated in the pET-MALHT vector. The error-free clones were confirmed by sequencing and then transformed into either BL21 (DE3) or Rosetta2 (DE3) (Novagen) *E. coli* strains for protein expression. The bacteria were grown at 37°C in 2 liters of LB containing 50 μg/ml kanamycin for BL21 (DE3) or 50 μg/ml kanamycin and 34 μg/ml chloramphenicol for Rosetta 2(DE3) cells to an OD_{600} of 0.6, before addition of IPTG to a final concentration of 0.1–1 mM to induce gene expression. Induced cultures were incubated overnight at 20°C. The cells were then harvested by centrifugation and the pellet was resuspended into the lysis buffer (50 mM tris pH 7.4, 500 mM NaCl, 10% glycerol, 20 mM Imidazole, 0.5% triton X-100 and 1 mM PMSF). Resuspended cells were disrupted by sonication and cell debris was removed by centrifugation. The clarified supernatant was incubated with Ni-NTA His resin (Novagen)

for 1 h, then washed with approximately 40 bed volumes of lysis buffer containing increasing concentrations (20, 30, and 40 mM) of imidazole. The remaining proteins bound to the Ni-NTA resin were eluted in lysis buffer supplemented with 250 mM of imidazole. Eluted proteins were concentrated and subjected to gel filtration using a HiLoad 16/600 superdex 200 prep grade column (GE healthcare). Fractions containing the purified protein were collected, pooled, aliquoted, and stored at −80°C until use.

ISOLATION AND IDENTIFICATION OF 4AzSA-CROSSLINKED PROTEINS BY IMMUNO-SELECTION AND MASS SPECTROSCOPY

4AzSA-crosslinked proteins were isolated and identified as described previously (Tian et al., 2012).

IDENTIFICATION OF SA-BINDING PROTEINS VIA SA AFFINITY CHROMATOGRAPHY

SA-immobilized resin was prepared using a PharmaLink Immobilization Kit (Pierce), according to the manufacturer's instructions. The coupling with 0.5–1 mg SA typically resulted in ∼180 μg SA immobilized per mL resin. Protein extract from Arabidopsis leaves were suspended in loading buffer (50 mM KPO_4 (pH 8.0) containing 50 mM NaCl, a protease inhibitor cocktail (Sigma) and 0.1 % (v/v) Triton X-100) and loaded onto a column containing the SA-linked resin. The loaded column was washed with loading buffer without and then with 0.1–10 mM 4-HBA to remove non-specifically bound proteins. Column-retained proteins were eluted with loading buffer containing 5 mM SA, and analyzed by SDS-PAGE. Eluted proteins were identified by mass spectroscopy.

IDENTIFICATION OF 4AzSA-CROSSLINKED PROTEINS BY PMA

Arabidopsis TAP-tagged recombinant purified proteins were printed to produce high density Arabidopsis microarrays (Popescu et al., 2007). For identification of cSABPs, the Arabidopsis PMA chips, each containing 10,000 proteins printed in duplicate, were blocked using protein-free blocking buffer (PFBB; Thermo Fisher Scientific) for 1 h at 4°C. After applying PFBB containing or lacking 500 μM 4AzSA, the PMAs were incubated in the dark for 30 min at room temperature before irradiation with 250 mJ UV light using a GS GENE linker™ UV chamber (Bio-Rad). Irradiated PMAs were washed twice for 5 min with PFBB, twice for 5 min with PBS plus 0.1% Tween 20, and twice for 5 min with PFBB. The PMAs were then incubated at 4°C overnight with sheep α-SA antibody (1:2000 in PFBB; AbD Serotec) without shaking. For washing the PMAs were incubated twice for 5 min with PFBB, twice for 5 min with PBS plus 0.1% Tween 20, and twice for 5 min with PFBB. PMAs were then incubated with Dylight 649 conjugated donkey α-sheep secondary antibody (1:5000 in PFBB; Jackson ImmunoResearch) at RT for 1 h followed by six 5-min washing steps using PBS plus 0.1% Tween 20 and two 5-min steps with distilled water. Dried PMAs were scanned using a GenePix4000B scanner (Molecular Devices), and the data were collected using GenePix Pro 6.0 software (Molecular Devices).

STATISTICAL ANALYSIS FOR THE IDENTIFICATION OF 4AzSA-CROSSLINKED PROTEINS BY PMA

Microarray data were normalized using the quantile normalization method (Bolstad et al., 2003). Differential binding to the α-SA antibody of proteins with or without 4AzSAcrosslinking was determined with LIMMA (Smyth, 2004). Raw *p*-values of multiple tests were corrected using false discovery rate (FDR) (Benjamini and Hochberg, 1995). Proteins with FDR < 0.01 were identified as cSABPs.

ASSESSMENT OF 3AESA-BINDING ACTIVITY BY SPR

SPR analyses of 3AESA binding and competition by SA were performed with a Biacore 3000 instrument (GE Healthcare). Immobilization of 3AESA on the CM5 sensor chip was performed as described previously (Tian et al., 2012). Activation, deactivation, and preparation of the mock coupled flow cell were performed by using amine coupling kit using manufacturer guidelines (GE healthcare). Briefly, carboxyl group of CM5 sensor chip was activated by using a mixture of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and N—hydroxy-succinimide (NHS) for the period of 7 min at a flow rate of 5 μl/min. After activation of sensor chip, 10 mM of 3-AESA dissolved in 0.1 M borate buffer, pH 10 was passed over for the period of 30 min at a flow rate of 5 μl/min for immobilization.Next excess reactive groups was inactivated by flowing ethanolamine hydrochloride-NaOH pH 8.5 for the period of 7 min. at a flow rate of 5 μl/min. HBS-EP buffer (0.01 M HEPES, ph 7.4. 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20; GE healthcare) was used as a running buffer in all assays. To test SA binding of cSABPs, proteins were filtered and diluted in HBS-EP buffer with or without various concentrations of SA, and then flowed through the flow cell of sensor chip with 3-AESA immobilized or through the mock-coupled flow cell. The binding signal was generated by subtracting the signal generated by mock-coupled flow cells from that generated with the 3-AESA immobilized flow cell. The flow cells were regenerated by stripping off bound protein by flowing NaOH solution (pH12).

ASSESSMENT OF SA-BINDING ACTIVITY BY PHOTO-AFFINITY LABELING

SA-binding activity was assessed by photo-affinity labeling as described previously (Tian et al., 2012). Briefly, purified proteins (2 μg) were incubated 1 h on ice with 4AzSA (50 μM) in 40 μl 1X PBS without or with various concentrations of excess SA, followed by UV irradiation with 254 nm UV light at an energy level of 30 mJ using a GS GENE linker™ UV chamber (Bio-Rad). 10 μl of reaction mixture were subjected to SDS-PAGE followed by immuno-blotting with α-SA antibody (Novus Biologicals) to detect 4AzSA-crosslinked proteins.

[^{3}H]SA-BINDING ASSAYS AND DETERMINATION OF BINDING AFFINITY BETWEEN NPR1 AND SA

[^{3}H]SA-binding assays were performed using size exclusion chromatography as described previously (Chen and Klessig, 1991). Briefly, pre-equilibrate sephadex™ G-25 (GE healthcare) with PBS buffer containing 0.1% Tween-20 overnight at 4°C. Size-exclusion column was prepared using 1 ml syringe with glass wool

fiber as filter and packed with overnight equilibrated sephadex™ G-25; excess buffer was removed by centrifugation. Binding of [^{3}H]SA with His$_6$-MBP-tagged NPR1, His$_6$-MBP-tagged FER1, MBP and no protein control was performed in PBS buffer with 100 µl reaction volume in the absence or presence of excess unlabeled SA (10,000-fold). The reaction mix was incubated on ice for 1 h, and then loaded on the column and centrifuged. The flow through was collected and dissolved in scintillation liquid and radioactivity was measured by a scintillation counter (LS6500; Beckman Coulter, Pasadena, CA). The Kd value was determined by non-linear fitting model of Michaelis-Menten equation with [^{3}H]SA concentration from 5 to 640 nM using OriginPro 7.5 software (OriginLab, Northampton, MA).

RESULTS

IDENTIFICATION OF cSABPs BY IMMUNO-SELECTION OF 4AzSA CROSSLINKED PROTEINS AND AFFINITY CHROMATOGRAPHY WITH AN SA-LINKED MATRIX

Over the past several years, we have conducted four large-scale screens using a previously described strategy with 4AzSA (Tian et al., 2012), and three large-scale screens using SA linked to a matrix for affinity chromatography. It should be noted that 4AzSA is a biologically active SA analog which mimics SA function in plants and is bound by previously identified SABP such as MES9 (Tian et al., 2012). For the 4AzSA screen, soluble protein extracts prepared from Arabidopsis leaves were incubated with 4AzSA; UV irradiation was then used to covalently crosslink 4AzSA to the proteins binding it. The 4AzSA-crosslinked proteins were selected with antibodies directed against SA, and the selected proteins were identified by mass spectroscopy. The SA affinity chromatography selection was performed by loading soluble protein extracts prepared from Arabidopsis leaves onto a column containing SA immobilized on a matrix. After washing the column with the biologically inactive SA analog 4-hydroxy benzoic acid to remove non-specifically bound proteins, the remaining proteins bound to the SA matrix were eluted with 5 mM SA and identified by mass spectroscopy. Through these seven screens, 35 proteins were identified two or more times, including at least once via crosslinking to 4AzSA (**Table 1**). The proteins represent 26 different protein families, and include catalase and carbonic anhydrase, which were previously identified as SABPs in tobacco (Chen et al., 1993; Slaymaker et al., 2002). To determine whether these proteins represent true SABPs, the encoding genes for 19 SABPs were obtained and successfully expressed in *E. coli*. The His$_6$-tagged recombinant proteins, which were purified by affinity chromatography on a Ni matrix followed by size fractionation on a 16/600 superdex 200 column, were then tested for SA-binding activity, primarily by assessing SA-inhibitable binding to 4AzSA, which was detected with anti-SA antibodies using immuno-blotting, and by monitoring SA-inhibitable binding to a 3AESA-bound sensor chip, which was detected by SPR.

Here we report the analysis of 10 cSABPs: acyl-CoA oxidase 4 (ACX4), aldolase superfamily protein (FBA5), glutamine synthetase 2 (GS2), lipoxygenase 2 (LOX2), patellin 1, photosystem II subunit P-1, serine hydroxymethyltransferase 4 (SHM4), thioredoxin-m1 (TRX-m1), thioredoxin superfamily protein BAS1, and tripeptidyl peptidase II (TPPII). The results for nine more cSABPs, including α-ketoglutarate dehydrogenase, the glutathione S-transferase PHI family (Tian et al., 2012) and glyceraldehyde 3-phosphate dehydrogenase family, are included in **Table 1** but their characterization was reported previously (Tian et al., in press). Using 3AESA-linked sensor chips, dose-dependent SPR responses were detected for TRX-m1, TPPII, SHMT4, LOX2, and ACX4, and binding to the 3AESA-linked chip was competed with increasing concentrations of SA (**Figures 1A–E**). These five proteins also bound and crosslinked to 4AzSA, and, for all but ACX4, this binding was suppressed by increasing amounts of SA (**Figures 2A–E**). The demonstration that SA competes with 3AESA and 4AzSA for binding by TRX-m1, TPPII, SHM4, and LOX2 argues that these proteins exhibit authentic SA-binding activity, even though SA binding by LOX2 was relatively weak. Based on our criterion that a protein must exhibit SA-binding activity in at least two independent, different assays to be considered a true SABP, the remaining six cSABPs analyzed in this study, including ACX4, aldolase superfamily protein (At4g26530.1), glutamine synthetase 2, patellin 1, photosystem II subunit P-1, and thioredoxin superfamily protein BAS1, do not qualify.

TRX-m1 is a member of a large family of thiol:disulfide oxidoreductases; these proteins facilitate the oxidation, reduction, and/or isomerization of disulfide bonds in target proteins. This protein family includes eight cytosolic thioredoxins (designated h-type) and three types of chloroplastic thioredoxins, including one x-type, two f -type, and four m-type. Interestingly, Tada et al. (2008) identified TRX-h3 and TRX-h5 in a screen for NPR1-interacting partners and demonstrated their participation in SA-induced conversion of disulfide-linked NPR1 oligomers to NPR1 monomers.

TPPII is a serine protease belonging to the subtilisin superfamily (Book et al., 2005). This exopeptidase breaks down fragments of proteins generated by the ubiquitin—26S proteasome system (Tomkinson, 1999). The two purported SA receptors NRP3 and NPR4 have been shown to regulate the level of NPR1 via the ubiquitin—26S proteasome system (Fu et al., 2012). In animals, TPPII, together with endopeptidases like thimet oligopeptidase (TOP), appears to be essential for amino acid recycling (Tomkinson, 1999; Saric et al., 2004). Notably, Moreau et al. (2013) showed that TOP1 of Arabidopsis binds SA, resulting in suppression of its peptidase activity. Genetically altering *TOP1* expression was found to affect immunity. Other proteases also have been shown to participate in plant immune responses (van der Hoorn, 2008). Whether TPPII participates in immunity and whether SA binding modulates its function in this or other physiological processes is unknown.

SHMs, together with several other enzymes including glutamine synthetase (see below), are important components of photorespiration, which is initiated when ribulose-1,5-bisphosphate carboxylase/oxygenase (RBC) catalyzes oxygenation rather than carboxylation of ribulose 1,5 bisphosphate to generate 3-phosphoglycerate and 2-phosphoglycolate. 2-phosphoglycolate is recycled (and its products returned to the Calvin cycle) through a series of reactions, which include conversion of glycine to serine in the mitochondria by glycine decarboxylase

Table 1 | List of cSABPs identified by photo-activated crosslinking to 4AzSA and immuno-selection.

Locus ID	Protein name	SA binding assays			Altered enzymatic activity	Failed to purify	References
		SPR with 3AESA	Crosslinked to 4AzSA	[3H]SA binding			
At4g35830.1	Aconitase 1 (ACO1)						
At3g51840.1	Acyl-CoA oxidase 4 (ACX4)	Yes#	Yes				
At2g13360.1	Alanine:glyoxylate aminotransferase						
At2g36460.1	Aldolase superfamily protein						
At4g26530.1	Aldolase superfamily protein (FBA5)	No					
At5g55070.1	Alpha-ketoglutarate dehyrogenase E2 subunit (αKGDHE2)	Yes#	Yes#				Tian et al., 2012
At4g33090.1	Aminopeptidase M1						
At3g01500.3	Carbonic anhydrase 1			Yes			Slaymaker et al., 2002 (in tobacco)
At4g35090.1	Catalase 2			Yes	Yes		Chen et al., 1993 (in tobacco)
At3g13930.1	dihydrolipoamide acetyltransferase, long form protein					Yes	
At5g25980.1	Glucoside glucohydrolase 2					Yes	
At5g35630.1	Glutamine synthetase 2 (GS2)	No					
At4g02520.1	Glutathione S-transferase PHI 2 (GSTF2)	Yes#	Yes#				Tian et al., 2012
At2g47730.1	Glutathione S-transferase PHI 8 (GSTF8)	Yes#	Yes#		Yes		Tian et al., 2012
At2g30860.1	Glutathione S-transferase PHI 9 (GSTF9)	No	No				Tian et al., 2012
At3g26650.1	Glyceraldehyde 3-phosphate dehydrogenase A subunit 1 (GAPDHA1)	Yes#	Yes#				Tian et al., in press
At1g12900.2	Glyceraldehyde 3-phosphate dehydrogenase A subunit 2 (GAPDHA2)	Yes#	Yes#				Tian et al., in press
At1g42970.1	Glyceraldehyde 3-phosphate dehydrogenase B subunit (GAPDHB)		Yes				Tian et al., in press
At3g04120.1	Glyceraldehyde 3-phosphate dehydrogenase C subunit 1(GAPDHC1)	Yes#	Yes*				Tian et al., in press
At1g13440.1	Glyceraldehyde 3-phosphate dehydrogenase C subunit 2 (GAPDHC2)	Yes#	Yes*				Tian et al., in press
At2g01210.1	Leucine-rich repeat protein kinase family protein						
At3g45140.1	Lipoxygenase 2 (LOX2)	Yes# (weak)	Yes#				
At5g63310.1	Nucleoside diphosphate kinase 2					Yes	
At4g09320.1	Nucleoside diphosphate kinase family protein						
At1g72150.1	Patellin 1	No					
At1g06680.1	Photosystem II subunit P-1	No					
At5g38430.1	Ribulose bisphosphate small subunit 1B (RBCS1B)						
At3g62030.1	Rotamase CYP 4						
At3g55800.1	Sedoheptulose-bisphosphatase						
At4g13930.1	Serine hydroxymethyltransferase 4 (SHM4)	Yes#	Yes#				
At1g03680.1	Thioredoxin M-type 1 (TRX-m1)	Yes#	Yes#				
At3g11630.1	Thioredoxin superfamily protein (BAS1)	No					
At3g13300.1	Transducin/WD40 repeat-like superfamily protein						
At4g20850.1	Tripeptidyl peptidaseII (TPP2)	Yes#	Yes#				
At1g16880	Uridyl trnasferase-related						

Proteins listed here were identified two or more times in seven large scale screens involving crosslinking to 4AzSA followed by immune-selection or using affinity chromatography with an SA-linked matrix.

Competable with SA.

* *Enhanced by SA.*

Chracterized in this study.

Previousaly reported cSABPs/SABPs.

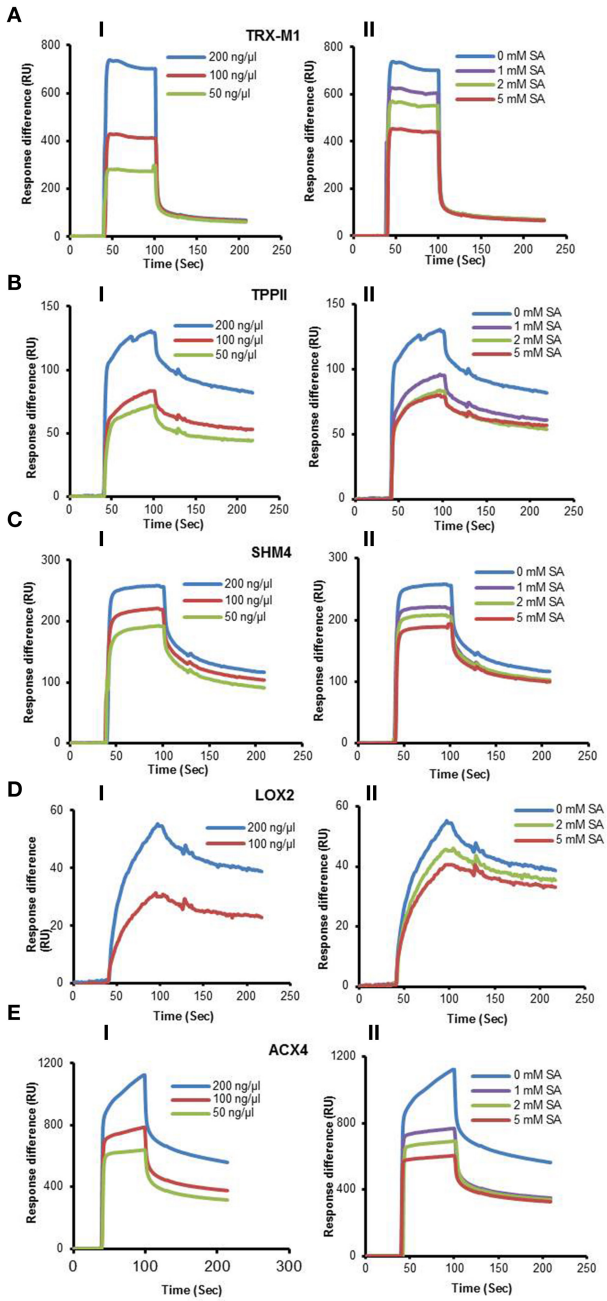


FIGURE 1 | SPR analyses of cSABPs identified by immuno-selection screens of 4AzSA crosslinked proteins. (i) Sensorgrams obtained with three concentrations (50, 100 or 200 ng/µl) of each recombinant, purified cSABP on a 3AESA-immobilized sensor chip for **(A)** TRX-m1, **(B)** TPPII, (**C**) SHM4, **(D)** LOX2, and **(E)** ACX4. (ii) Sensorgrams for each cSABP (200 ng/µl) in the absence (0 mM) or presence of three concentrations of SA (1, 2 or 5 mM) using a 3AESA-immobilized chip. The signals detected from a mock-coupled control chip were subtracted.

and SHM, concomitant with the production of ammonia and CO_2. Since re-assimilation of ammonia by the glutamine synthetase/glutamate synthase system and CO_2 by RBC consumes both ATP and reducing power, photorespiration reduces photosynthetic efficiency (Zhu et al., 2008). However, this process plays an important role in suppressing the production of reactive oxygen species (ROS), which would otherwise be generated by the excess light energy captured in chloroplasts (Kozaki and Takeba, 1996). Highly elevated levels of ROS cause photoinhibition and cellular damage, whereas at lower levels they act as defense signals, facilitating programmed cell death during the hypersensitive response and strengthening the cell wall, which provides a physical barrier to pathogen ingress (Mittler et al., 2004; Gechev et al., 2006; O'Brien et al., 2012). Perhaps SA binding to SHM4 and/or glutamine synthetase R2 (GSR2; see below) alters their enzymatic activity and thereby helps to modulate ROS levels.

Lipoxygenases catalyze the oxygenation of polyunsaturated fatty acids. This is the first step in the biosynthesis of oxylipins, a large group of biologically active fatty acid metabolites that includes jasmonates. The first step in the synthesis of jasmonic acid (JA) is the LOX2-mediated oxygenation of linolenic acid (Bannenberg et al., 2009). Interestingly, the enzyme responsible for catalyzing the next step, allene oxide synthase (AOS), is competitively inhibited by SA (Pan et al., 1998). Perhaps SA targets both of these JA biosynthetic enzymes as part of the well-established antagonism between these two critical defense signaling hormones (Pieterse et al., 2009; Vlot et al., 2009; Robert-Seilaniantz et al., 2011).

IDENTIFICATION OF cSABPs USING PROTEIN MICROARRAYS

We also have developed a second screen for identifying cSABPs that utilizes PMAs. To reduce non-specific interactions with the test reagents, the PMAs were treated with blocking buffer before incubation with buffer lacking (the control) or containing 4AzSA, followed by UV-induced crosslinking of 4AzSA to the bound proteins. The 4AzSA-crosslinked proteins were then detected using an α-SA antibody. This strategy was used previously to screen a PMA containing 5000 Arabidopsis proteins, from which TOP1 was identified as an SABP (Moreau et al., 2013). To further enhance the detection of proteins crosslinked to 4AzSA, the incubation/reaction and washing conditions were optimized and new PMAs, containing 10,000 additional Arabidopsis proteins printed in duplicate, were screened. The results from five replicate arrays reacted with 4AzSA and five control arrays not treated with 4AzSA were used for downstream analysis. The correlation coefficient among the replicates was high (**Table 2**), indicating high reproducibility of the arrays. Using a cutoff of FDR $<$ 0.01 and signal/control ratio of $>$1.5, 77 cSABPs were identified (**Table 3**). Twenty-seven cSABPs with FDR values ranging from 0.0018 to 0.0098 were selected for further characterization. Eight were successfully expressed in *E. coli* and purified by affinity chromatography and size fractionation. Five of these eight, including glutathione peroxidase 2 (GPX2), GSR2, hydroxypyruvate reductase 2 (HPR2), UDP-glucose 4-epimerase 2 (UGE2), and RBC small subunit 1A (RBCS1A), bound 3AESA and crosslinked to 4AzSA in an SA-inhibitable manner, indicating that they are SABPs (**Figures 3**, **4**). Analysis of protein phosphatase 2A (PP2A), GS2, and an α/β hydrolase superfamily member failed to meet the criteria for designation as SABPs (data not shown).

GPXs, like the SAPBs catalase and ascorbate peroxidase (Chen et al., 1993; Durner and Klessig, 1995), help regulate cellular

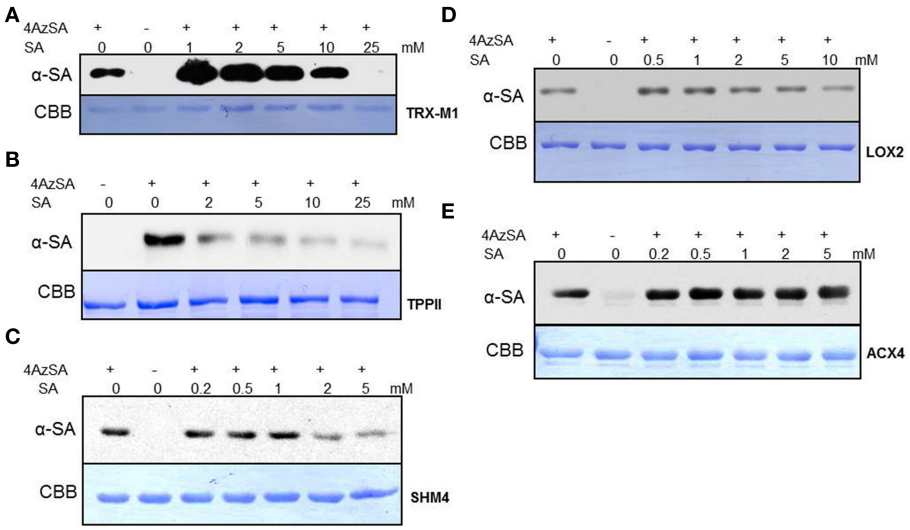


FIGURE 2 | Immuno-blot analyses monitoring SA competition of 4AzSA crosslinking to recombinant cSABPs identified by the 4AzSA/immuno-selection screen. Photo-activated crosslinking of 50 ng/μl of the indicated recombinant purified proteins: **(A)** TRX-m1, **(B)** TPPII, **(C)** SHM4, **(D)** LOX2, and **(E)** ACX4 to 4AzSA (50 μM) in the absence or presence of increasing amounts of SA was detected by immuno-blotting using an α-SA antibody. Reactions without 4AzSA served as controls. Note that controls with 4AzSA but without photo-activation were previously shown to give similar results (Tian et al., 2012). Proteins stained with Coomassie brilliant blue (CBB) served as the loading control.

Table 2 | Correlation of protein microarrays.

Microarray	+4AzSA#1	+4AzSA#2	+4AzsA#3	+4AzSA#4	+4AzsA#5	−4AzsA#1	−4AzsA#2	−4AzsA#3	−4AzsA#4	−4AzsA#5
+4AzSA#1	1	1	1	1	0.9	0.9	1	0.9	0.9	0.9
+4AzSA#2	1	1	1	1	0.9	0.9	1	1	0.9	0.9
+4AzSA#3	1	1	1	1	0.9	0.9	0.9	0.9	0.9	0.9
+4AzSA#4	1	1	1	1	0.9	0.9	1	0.9	0.9	0.9
+4AzSA#5	0.9	0.9	0.9	0.9	1	0.8	0.9	0.9	0.9	0.9
−4AzSA#1	0.9	0.9	0.9	0.9	0.8	1	0.9	0.9	0.9	0.9
−4AzSA#2	1	1	0.9	1	0.9	0.9	1	1	1	1
−4AzSA#3	0.9	1	0.9	0.9	0.9	0.9	1	1	1	1
−4AzSA#4	0.9	0.9	0.9	0.9	0.9	0.9	1	1	1	1
−4AzSA#5	0.9	0.9	0.9	0.9	0.9	0.9	1	1	1	1

Pearson correlation coefficient were calculated among the 10 protein microarrays. Five replicated microarrays with 4AzSA:+4AzSA#1, +4AzSA#2, +4AzSA#3, +4AzSA#4 and +4AzsA#5; five replicated microarrays without 4AzSA: −4AzSA#1, −4AzSA#2, −4AzSA#3, −4AZSA#4, and −4AzSA#5.

redox by scavenging H_2O_2. In Arabidopsis, GPX2 is located in the cytoplasm and its expression is induced by abiotic stresses, including salt, osmotic stress, and heavy metals. It also is induced by SA, but not by other hormones such as JA, abscisic acid or indole acetic acid (Milla et al., 2003). Whether SA inhibits GPX2 activity like it does for the other two H_2O_2-scavenging enzymes is not known.

Glutamine synthetases play key roles in nitrogen metabolism, including the assimilation of inorganic nitrogen via conversion of ammonia into glutamine. Two glutamine synthetases were identified in the PMA screen: GSR2 and GS2 (**Table 3**). GS2 was also identified by immuno-selection of 4AzSA-crosslinked proteins (**Table 1**). However, while GS2 failed to exhibit binding to 3AESA in the SPR assay, GSR2 was found to be an SABP (**Figures 3B**, **4B**). GS2 is located in chloroplasts, where it plays an important role in the reassimilation of ammonia released during photorespiration (Wallgrove et al., 1987). Whether GSR2 also plays a role during photorespiration is unknown.

HPR2 is another enzyme involved in photorespiration. It, like the peroxisome-localized HPR1, converts hydroxypruvate to glycerate, which, upon phosphorylation, is returned to the Calvin cycle as an intermediate. HPR2 is a cytosolic enzyme (Timm et al., 2008).

UGEs interconvert UDP-glucose and UDP-galactose. Arabidopsis contains five UGE isoforms, which are divided into two clades; one clade contains UGE1 and UGE3, and the other contains UGE2, UGE4, and UGE5. UGE 2 and UGE5 were both identified in the PMA screen (**Table 3**), and further analyses confirmed that UGE2 is an SABP (**Figures 3D**, **4D**). UGE2 and UGE4 are reported to cooperate in providing UDP-galactose for

Table 3 | List of cSABPs identified using protein microarrays.

Locus ID	Protein name	Signal ratio	FDR; adjusted *P*-Value	SA binding assays			Failed to purify	References
				SPR with 3AESA	Crosslinked to 4AzSA	[3H]SA binding		
At3g03900	Adenosine-5′-phosphosulfate kinase 3 (APK3)	3.2	0.004					
At4g37470	Alpha/Beta fold hydrolase, Karrikin insensitive 2 (KAI2)	2.60	0.005					
At5g19050	Alpha/Beta-hydrolases superfamily protein	2.17/2.31	0.002/0.007	Yes (Weak)	Yes			
Ag2g30200	[acyl-carrier-protein] S-malonyltransferases	2.05/2.00	0.005/0.0001					
At1g08250	Arogenate dehydratase (ADT6)	4.24/3.61	0.0002/0.0003				Yes	
At3g57510	(α) polygalacturonase protein (ADPG1)	1.87	0.004					
At4g22820	A20/AN1-like zinc finger family protein;	1.87	0.008					
At2g43360	Biotin auxotroph 2 (BIO2/BIOB)	3.29	0.009					
At3g01500	Carbonic anhydrase 1	1.94	0.004			Yes		Slaymaker et al., 2002 (in tobacco)
At5g12850	CCCH-type zinc finger protein with ARM repeat domain	1.81	0.007					
At1g08640	Chloroplast J-like domain 1 (CJD1)	1.92	0.007					
At5g54340	C2H2 and C2HC zinc fingers superfamily protein	2.39	0.008				Yes	
At2g44350	Citrate synthase 4 (CSY4)	1.67	0.007					
At1g33330	Class I peptide chain release factor	2.47/2.39	0.002/0.002				Yes	
At3g28760	Contains 3-dehydroquinate synthase domain	2.18	0.007				Yes	
At1g54220	Dihydrolipoamide acetyltransferase, long form protein	2.09	0.008				Yes	
At5g61410	D-ribulose-5-phosphate-3-epimerase (RPE)	2.55/2.37	0.002				Yes	
At1g17940	Endosomal targeting BRO1-like domain-containing protein	2	0.003					
At1g28570	(ERMO3);(MVP1); nuclear cage (NUC)	2.04	0.007					
At4g14630	Germin-like protein (GLP9)	5.81/3.99	0.001/0.004				Yes	
At5g39190	Germin-like protein (GLP2a, GER2)	1.76	0.008				Yes	
At1g17890	Germin-like protein (GER2)	1.95	0.007					
At1g06130	Glyoxalase 2–4 (GLX2-4)	2.41	0.0008				Yes	
At1g42970	Glyceraldehyde-3-phosphate dehydrogenase B subunit (GAPDHB)	2.90/2.53	0.0008/0.005		Yes			Tian et al., in press
At1g66200	Glutamine synthetase (GSR2)	2.49	0.003	Yes[#]	Yes[#]			
At5g35630	Glutamine synthetase (GS2)	1.89	0.008	No				
At2g31570	Glutathione peroxidase (GPX2)	2.64/2.17	0.003/0.007	Yes[#]	Yes[#]			
At1g53280	Homolog of animal DJ-1 superfamily protein (DJ1B)	2.61	0.003					
At1g79870	Hydroxypyruvate 2 (HPR2)	2.41	0.008	Yes[#]	Yes[#]			
At3g43270	Invertase/Pectin methylesterase inhibitor superfamily;	3.50/2.50	0.002/0.006				Yes	
At3g49220	Invertase/Pectin methylesterase inhibitor superfamily	3.37/3.22	0.0001/0.0003					
At3g10720	Invertase/Pectin methylesterase inhibitor superfamily	2.35	0.003					
At2g46110	Ketopentoate hydroxymethyltransferase 1 (KPGHMT1)	2.84	0.002288148					
At5g10920	L-Aspartase-like family protein; argininosuccinate lyase activity	2.18	0.007					

(Continued)

Table 3 | Continued

Locus ID	Protein name	Signal ratio	FDR; adjusted *P*-Value	SA binding assays			Failed to purify	References
				SPR with 3AESA	Crosslinked to 4AzSA	[3H]SA binding		
At4g09300	LisH and RanBPM domains containing protein	1.77	0.007					
At2g22370	Mediator 18 (MED18)	1.99	0.007					
At1g53240	Mitochrondrial malate dehydrogenase (MMDH1)	1.98	0.007					
At1g04410	NAD-dependent malate dehydrogenase 1 (C-NAD-MDH1)	2.33	0.006					
At5g12040	Nitrilase/Cyanide hydratase and apolipoprotein N-acyltransferase family protein	2.24/2.19	0.005/0.005				Yes	
At3g56520	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein	1.75	0.007					
At5g18900	2-oxoglutarate (2OG)/Fe(II)-dependent oxygenase	3.03	0.0003					
At1g10640	Pectin lyase-like protein	2.55/2.44	0.004/0.005					
At4g33220	Pectin methylesterase 44 (PME44)	1.87	0.008					
At3g59480	pfkB-like carbohydrate kinase	3.65	0.007				Yes	
At4g35110	Phospholipase-like protein 4 (PEARLI 4)	1.78	0.008					
At1g25490	Phosphoprotein phosphatase 2A (PP2A)	3.82/3.05	0.002/0.002	Yes (Weak)	Yes[#]			
At1g29410	Phosphoribosylanthranilate isomerase 3 (PAI3)	3.25/2.12	0.002/0.007				Yes	
At5g50850	Pyruvate dehydrogenase, MACCI-BOU (MAB1)	2.59	0.008					
At4g33070	Pyruvate decarboxylase 1 (PDC1)	2.21	0.006				Yes	
At1g80360	Pyridoxal phosphate (PLP)-dependent transferases (VAS1)	3.15/3.10	0.001/0.0004					
At3g25480	Rhodanese/Cell cycle control phosphatase	2.62/2.19	0.009/0.002					
At3g04790	Ribose 5-phosphate isomerase, type A protein	2.45/2.42	0.0006/0.004					
At1g67090	Ribulose biphosphate carboxylase small subunit 1A (RBCS1A)	4.60/3.89	0.002/0.002	Yes[#]	Yes[#]			
At5g38410	Ribulose bisphosphate small subunit 3B (RBCS3B)	3.92	0.0006					
At1g18980	RmlC-like cupins superfamily protein	1.70	0.007					
At1g21200	Sequence-specific DNA binding transcription factors	1.82	0.007					
At1g75980	Single hybrid motif superfamily protein	1.86	0.0098					
At5g43780	Sulfate adenylyltransferase, ATP sulfurylase (APS4)	2.37/2.13	0.002/0.004					
At2g37400	Tetratricopeptide repeat (TPR)-like superfamily protein	2.34	0.007				Yes	
At1g21400	Thiamin diphosphate-binding fold (THDP-binding) superfamily protein	2.27	0.0096				Yes	
At2g44790	Uclacyanin 2 (UCC2)	1.70	0.007					
At4g23920	UDP-D-glucose/UDP-D-galactose 4-epimerase 2 (UGE2)	3.39/3.02	0.0001/0.0001	Yes[#]	Yes[#]			
At4g10960	UDP-D-glucose/UDP-D-galactose 4-epimerase 5 (UGE5)	2.68/2.02	0.003/0.004					
At2g30140	UDP-Glucosyl transferase 87A2 (UGT87A2)	3.03	0.009					
At3g11340	UDP- Glycosyl transferase 76B1 (UGT76B1)	2.96	0.004					

(Continued)

Table 3 | Continued

Locus ID	Protein name	Signal ratio	FDR; adjusted *P*-Value	SA binding assays			Failed to purify	References
				SPR with 3AESA	Crosslinked to 4AzSA	[3H]SA binding		
At2g29740	UDP-glucosyl transferase 71C2 (UGT71C2)	1.94	0.007				Yes	
At5g59590	UDP-glucosyl transferase 76E2 (UGT76E2)	1.93	0.007					
At1g22400	UDP-glycosyl transferase 85A1 (UGT85A1)	1.70	0.007					
At2g34850	UDP-glucose 4-epimerase activity, MEE25)	2.60	0.004				Yes	
At4g38100	Unknown protein (CURT1D)	7.85/7.66	0.0001/0.0001				Yes	
At3g51090	Unknown function (DUF1640)	1.91	0.002					
At1g02540	Unknown protein	2.91/2.39	0.001/0.005				Yes	
At1g59710	Unknown function	1.75	0.007					
At1g68140	Unknown function	1.57	0.007					
At2g35900	Unknown protein	2.01	0.005					
At2g34470	Urease accessory protein G (UREG)	2.36	0.003					
At2g14620	Xyloglucan endotransglucosylase/hydrolase 10 (XTH10)	2.47	0.003					

FDR, false discovery rate.

Signal ratio corresponds to the ratio of fluorescent signal obtained on PMA incubated with 4AzSA divided by the signal obtained on PMA incubated without 4AzSA.

[#] *Competable with SA.*

Characterized in this study.

Previously reported cSABPs/SABPs.

cell wall biosynthesis and growth, while UGE5 may play a role during abiotic stress (Rosti et al., 2007).

Several members of the RBC small subunit (RBCS) family were identified in the PMA screen and/or at least once in the 4AzSA/immuno-selection screens. These include RBCS1A, RBCS1B, and RBCS3B (**Tables 1**, **2**). Initially we discounted the repeated identification of RBCS in our 4AzSA/immuno-selection screen, assuming that they were non-specifically selected because of their overwhelming abundance in soluble protein extracts. The likelihood that they were all false positives decreased significantly when RBCS1A and RBCS3B were identified repeatedly on the PMA screen, as protein abundance does not influence these results. Further characterization of RBCS1A confirmed that at least this RBCS is an SABP (**Figures 3E**, **4E**). Since SA is synthesized in chloroplast and has been linked to several metabolic processes, including redox homeostasis and photosynthesis (Mateo et al., 2006; Janda et al., 2014), the discovery that RBC, a central enzyme in photosynthesis, binds SA is perhaps not that surprising.

NPR1 IS AN SABP

Given the conflicting reports on whether NPR1 binds SA (Fu et al., 2012; Wu et al., 2012), we revisited this matter using the methods we have optimized/developed for identifying SABPs. Recombinant *Arabidopsis* NPR1 exhibited a dose-dependent SPR response on 3AESA-linked sensor chips, and NPR1 binding to 3AESA was competed by increasing concentrations of SA (**Figures 5A,B**). In addition, NPR1 bound and was crosslinked to 4AzSA; this crosslinking was suppressed by increasing levels of SA (**Figure 5C**). NPR1's SA binding ability was further confirmed using a classical method for identifying low molecular weight ligand binding proteins, namely size exclusion chromatography. NPR1 bound [^{3}H]SA, thereby excluding this ligand from entering the interior of the matrix bead. Furthermore, excess unlabeled SA competed with [^{3}H]SA for binding to NPR1 (**Figure 5D**). It binds SA with relatively high affinity with an apparent Kd on 191 $\pm$ 49 nM (**Figure 5E**). Therefore, based on these three independent assays, we conclude that NPR1 is an SABP.

DISCUSSION

In this study, we report the identification of nine new SABPs, as well as the generation of a large pool of cSABPs, many of whose SA-binding properties have yet to be tested. Most of these proteins were identified through recently developed high-throughput screens that utilize photo-activated crosslinking to stabilize the interaction between cellular proteins and 4AzSA. Biochemical and biological tests have previously demonstrated that 4AzSA mimics SA, as it induces expression of the prototypic SA-responsive *PR-1* gene and competes with [^{3}H]SA for binding to a known SABP, AtMES9 (Tian et al., 2012). For all nine SABPs, their ability to crosslink to 4AzSA in the initial screens was subsequently shown to represent authentic SA binding since (i) this crosslinking was suppressed in the presence of SA, and (ii) these proteins exhibited SA-inhibitable binding to 3AESA, which was covalently linked to an SPR sensor chip through an amide bond.

SPR is a highly sensitive method for identifying interactions that are weak and/or transient, quantitatively measuring interactions in real time. Photo-affinity labeling with 4AzSA also is well suited for identification of interactions that are weak and/or transient since 4AzSA binding is captured by photo-activated

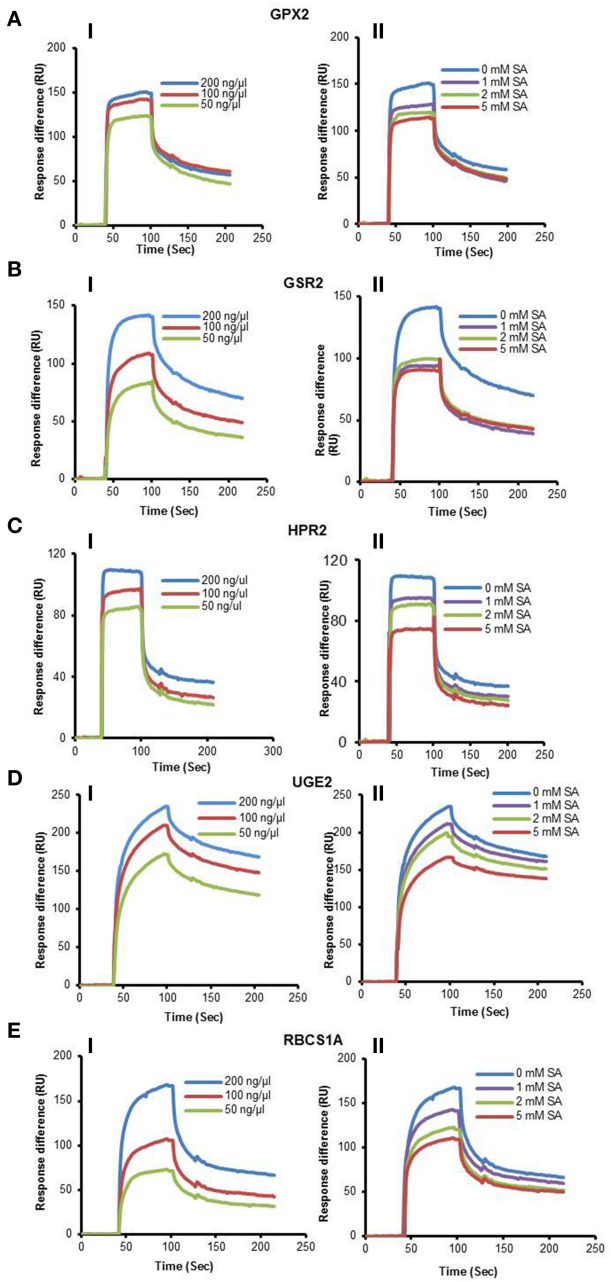


FIGURE 3 | SPR analyses of cSABPs identified by the PMA screen. (i) Sensorgrams obtained with three concentrations (50, 100 or 200 ng/µl) of each recombinant, purified cSABP using a 3AESA-immobilized sensor chip for **(A)** GPX2, **(B)** GSR2, **(C)** HPR2, **(D)** UGE2, and **(E)** RBCS1A. (ii) Sensorgrams for each cSABP (200 ng/µl) in the absence (0 mM) or presence of three concentrations of SA (1, 2 or 5 mM) using a 3AESA-immobilized chip. The signals detected from a mock-coupled control chip were subtracted.

crosslinking. However, a common problem with photo-affinity labeling is non-specific labeling, which can lead to many false positives (Kotzyba-Hilbert et al., 1995). Non-specificity and the resulting high number of false positives are a general problem with high-throughput screens, including those employing PMAs. This problem was particularly severe in our initial screen for cSABPs using SA linked to a PharmaLink matrix. Despite our attempts to remove proteins non-specifically bound to the matrix via excessive washing of the SA-linked matrix with 4-hydroxy benzoic acid, a biologically inactive SA analog, a large portion of the proteins subsequently eluted with SA were found to be false positives upon further characterization. This setback prompted us to develop new screens that rely upon stabilizing the interaction between cellular proteins and 4AzSA through photo-activated crosslinking. Of the 35 cSABPs identified in the 4AzSA/immunoselection screen, 19 were further analyzed to varying degrees in this and previous reports (**Table 1**; Tian et al., 2012, in press). Eleven of these 19 proteins met the criteria for designation as a true SABP, as they exhibited SA binding in at least two different assays. The nearly 60% success rate for this screen is somewhat misleading, since six of the 11 were members of just two protein families - GST and GAPDH. However, of the 16 cSABPs yet to be characterized, catalase and carbonic anhydrase are highly likely to be SABPs, given that their tobacco orthologs are SABPs (Chen et al., 1993; Durner and Klessig, 1995; Slaymaker et al., 2002). The results from our PMA screening strategy also appear promising, as 77 cSABPs were identified in a screen of 10,000 proteins. Only a small portion of these cSABPs have been characterized further, due to the recent optimization of this screen and technical difficulties generating the recombinant proteins. However, of the eight cSABPs analyzed thus far, five met the criteria to be designated as SABPs. Together, these findings suggest that both the 4AzSA/immuno-selection and the PMA screening strategies will yield very workable numbers of candidates that have a significant probability of being SABPs.

It is interesting to note that four of the nine newly identified SABPs are associated with redox regulation. The interplay between SA and redox homeostasis was first revealed with the discovery that SA inhibits the activity of two major H_2O_2-scavenging enzymes in tobacco: catalase, which is the first SABP identified (Chen et al., 1993), and ascorbate peroxidase (Durner and Klessig, 1995). Further linking SA and redox status was the discovery by Dong and coworkers that translocation of NPR1 from the cytoplasm to the nucleus, which is required for NPR1 to play its central positive role in SA-mediated immunity, is redox regulated (Mou et al., 2003). Many subsequent studies have documented the interplay among SA, ROS, redox homeostasis, and the activation of immune responses (Mateo et al., 2006; Dat et al., 2007; Vlot et al., 2009; Xu and Brosche, 2014). Of the four redox-associated SABPs, GPX2 is an H_2O_2 scavenger, while TRX-m1 is an oxidoreductase that regulates disulfide bond formation/deformation in target proteins. SHM4 and GSR2 function in photorespiration, which plays a critical role in preventing cellular damage due to overproduction of ROS generated by excess light energy. Over production of ROS also causes photoinhibition due to damage to the photosynthetic apparatus, particularly to photosystem II. Notably RBCS1A of the key photosynthesis enzyme RBC was found to bind SA.

Unlike animal systems, relatively few plant hormones have been identified with each mediating multiple biochemical and physiological responses. Our understanding of the biochemical and molecular mechanisms of phytohormone perception and

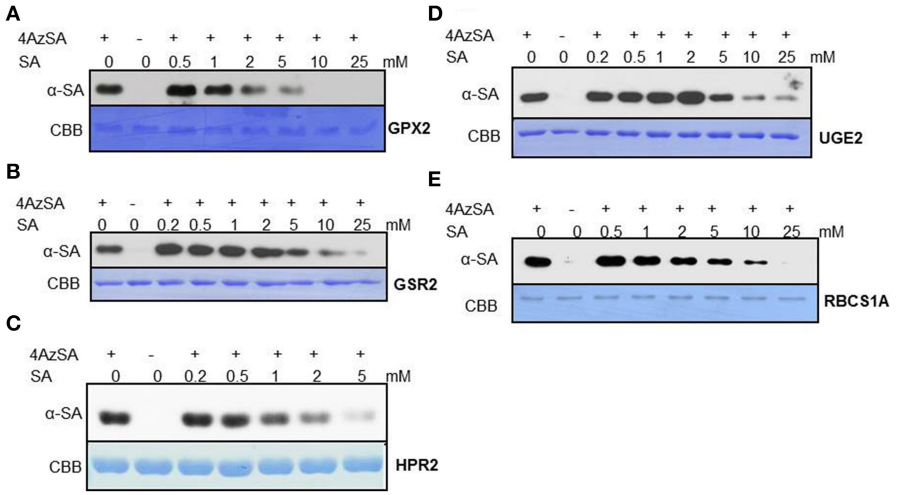


FIGURE 4 | Immuno-blot analyses monitoring SA competition of 4AzSA crosslinking to recombinant cSABPs identified by the PMA screen. Photo-activated crosslinking of 50 ng/μl of recombinant, purified **(A)** GPX2, **(B)** GSR2, **(C)** HPR2, **(D)** UGE2, or **(E)** RBCS1A to 4AzSA (50 μM) in the absence or presence of increasing amounts of SA was detected by immuno-blotting using an α-SA antibody. Reactions without 4AzSA served as controls. Proteins stained with Coomassie brilliant blue (CBB) served as the loading control.

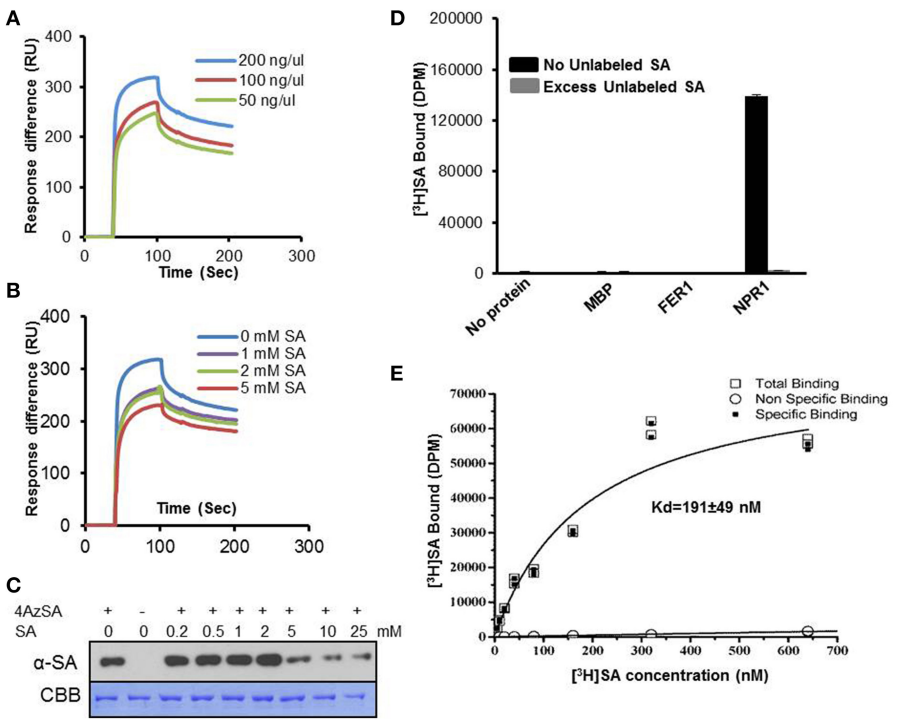


FIGURE 5 | SA-binding activity of NPR1 detected by SPR, photo-activated crosslinking to 4AzSA, and binding of [^{3}H]SA. (A) Sensorgrams obtained with three concentrations of recombinant, purified NPR1 (50, 100 or 200 ng/μl) using a 3AESA-immobilized sensor chip. **(B)** Sensograms for NPR1 (200 ng/μl) in the absence (0 mM) or presence of three concentrations of SA (1, 2 or 5 mM) on a 3AESA-immobilized chip. The signals detected from a mock-coupled control chip were subtracted. **(C)** Photo-activated crosslinking of 50 ng/μl of NPR1 to 4AzSA (50 μM) in the absence or presence of increasing amounts of SA was detected by immuno-blotting using an α-SA antibody. Reactions without 4AzSA served as controls. Proteins stained with Coomassie brilliant blue (CBB) served as the loading control. **(D)** Binding of [^{3}H]SA (200 nM) by 0.20 μg/μl His_6-MBP-tagged NPR1 in the absence or presence of a 10,000-fold excess of unlabeled SA was determined by size-exclusion chromatography. Chromatography reactions with [^{3}H]SA with no protein, with MBP, or with His_6-MBP-tagged ferretin 1 (FER1), which does not bind SA, served as negative controls. Error bars represent SE values calculated from three replications of a single experiment. The [^{3}H]SA binding assay with NPR1 was repeated at least four times with similar results. **(E)** Dissociation constant (Kd) of 0.125 μg/μl NPR1 binding to SA was determined by size-exclusion chromatography with different concentrations of [^{3}H]SA. No protein with different concentrations of [^{3}H]SA was used as non-specific binding control. Two replicates in a single experiment were used to calculate Kd; the experiment was done twice.

signaling also is relatively rudimentary in comparison to what is known in animals. While receptors for SA have recently been reported (Fu et al., 2012; Wu et al., 2012), the results from our studies suggest that many of SA's effects are mediated though a large number of SABPs whose biochemical/enzymatic activities are altered by SA binding. Classical receptors have been discovered for most phytohormones over the past several decades. However, we suspect that some of these phytohormones, like SA, utilize additional protein targets either in conjunction with or instead of their known receptors to mediate some of their myriad effects. Since the approaches and methods developed/optimized for the identification of SABPs are applicable to the identification of proteins that bind other low molecular weight compounds/ligands, such as other plant (or animal) hormones, their future use should clarify whether the SA signaling network serves as a paradigm for other phytohormones. In fact, we have used these approaches/methods to identify several novel human targets of the most used drug worldwide, namely aspirin, which is rapidly metabolized to SA. Natural derivatives of SA are found in several medicinal plants, which are used extensively in traditional medicine.

AUTHOR CONTRIBUTIONS

Daniel F. Klessig, Miaoying Tian, Murli Manohar, Sang-Wook Park, and Magali Moreau designed the research; Murli Manohar, Miaoying Tian, Magali Moreau, Sang-Wook Park, Hyong Woo Choi, Muhammed Asif, Patricia Manosalva, Caroline C. von Dahl, Kai Shi, and Inish O'Doherty performed SA-binding analyses; Giulia Friso and Klass J. van Wijk performed the mass spectroscopy analyses; Shisong M and Savithramma P. Dinesh-Kumar provided the protein microarrays; Murli Manohar, Miaoying Tian, Magali Moreau, Sang-Wook Park, Giulia Friso, Zhangjun Fei, Hyong Woo Choi, Frank C. Schroeder, and Daniel F. Klessig analyzed the data; and Daniel F. Klessig, Murli Manohar, Miaoying Tian, and Magali Moreau wrote the paper.

ACKNOWLEDGMENTS

We thank D'Maris Dempsey for assistance in writing the paper and Brian Crane for expression vector pET-MALHT. This work was supported by grant IOS-0820405 from the United States National Science Foundation to Daniel F. Klessig and Klass J. van Wijk.

REFERENCES

Bannenberg, G., Martinez, M., Hamburg, M., and Castresana, C. (2009). Diversity of the enzymatic activity in the lipoxygenase gene family of *Arabidopsis thaliana*. *Lipids* 44, 85–95. doi: 10.1007/s11745-008-3245-7

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. Ser. B* 57, 289–300.

Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003). A comparison of normalization methods for high-density oligonucleotide array data based on bias and variance. *Bioinformatics* 19, 185–193. doi: 10.1093/bioinformatics/19.2.185

Book, A. J., Yang, P., Scalf, M., Smith, L. M., and Vierstra, R. D. (2005). Tripeptidyl peptidase II. An oligomeric protease complex from *Arabidopsis*. *Plant Physiol.* 138, 1046–1057. doi: 10.1104/pp.104.057406

Cao, H., Bowling, S. A., Gordon, S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 6, 1583–1592. doi: 10.1105/tpc.6.11.1583

Chen, Z., and Klessig, D. F. (1991). Identification of a soluble salicylic acid-binding protein that may function in the plant disease-resistance response. *Proc. Natl. Acad. Sci. U.S.A.* 88, 8179–8193. doi: 10.1073/pnas.88.18.8179

Chen, Z., Silva, H., and Klessig, D. F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262, 1883–1886. doi: 10.1126/science.8266079

Cleland, C. F., and Ajami, A. (1974). Identification of the flower-inducing factor isolated from aphid honeydew as being salicylic acid. *Plant Physiol.* 54, 904–906. doi: 10.1104/pp.54.6.904

Dat, J. F., Capelli, N., and Van Breusegem, F. (2007). "The interplay between salicylic acid and reactive oxygen species during cell death in plants," in *Salicylic Acid—A Plant Hormone*, eds S. Hayat and A. Ahmad (New York, NY: Springer), 247–276.

Delaney, T. P., Friedrich, L., and Ryals, J. A. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6602–6606. doi: 10.1073/pnas.92.14.6602

Dempsey, D., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arabidopsis Book Am. Soc. Plant Biologist*. 9:e0156. doi: 10.1199/tab.0156

Durner, J., and Klessig, D. F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2, 6-dichloroisonicotinic acid, two inducers of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 92, 11312–11316. doi: 10.1073/pnas.92.24.11312

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I., and Laloi, C. (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Biol. Essays* 28, 1091–1101. doi: 10.1002/bies.20493

Glazebrook, J., Rogers, E. E., and Ausubel, F. M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 143, 973–982.

Hadacek, F., Bachmann, G., Engelmeier, D., and Chobot, V. (2011). Hormesis and a chemical raison d'être for secondary plant metabolites. *Dose Response* 9, 79–116. doi: 10.2203/dose-response.09-028.Hadacek

Janda, T., Gondor, O. K., Yordanova, R., Szalai, G., and Pal, M. (2014). Salicylic acid and photosynthesis: signaling and effects. *Acta. Physiol. Plant* 36, 2537–2546. doi: 10.1007/s11738-014-1620-y

Kotzyba-Hilbert, F., Kapfer, I., and Goeldner, M. (1995). Recent trends in photoaffinity labeling. *Angew. Chem. Int. Ed. Engl.* 34, 1296–1312. doi: 10.1002/anie.199512961

Kozaki, A., and Takeba, G. (1996). Photorespiration protects C3 plants from photooxidation. *Nature* 384, 557–560. doi: 10.1038/384557a0

Kumar, D., and Klessig, D. F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc Natl. Acad. Sci. U.S.A.* 100, 16101–16106. doi: 10.1073/pnas.0307162100

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of Tobacco to viral infection. *Science* 250, 1002–1004. doi: 10.1126/science.250.4983.1002

Mateo, A., Funck, D., Muhlenbock, P., Kular, B., Mullineaux, P. M., and Karpinski, S. (2006). Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. *J. Exp. Bot.* 57, 1795–1807. doi: 10.1093/jxb/erj196

Meìtraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006. doi: 10.1126/science.250.4983.1004

Milla, M. R., Maurer, A., Huerte, A. R., and Gustafson, J. P. (2003). Glutathione peroxidase genes in *Arabidopsis* are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. *Plant J.* 36, 602–615. doi: 10.1046/j.1365-313X.2003.01901.x

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490–498. doi: 10.1016/j.tplants.2004.08.009

Moreau, M., Westlake, T., Zampogna, G., Popescu, G., Tian, M., Noutsos, C., et al. (2013). The *Arabidopsis* oligopeptidases TOP1 and TOP2 are salicylic acid targets that modulate SA-mediated signaling and the immune response. *Plant J.* 76, 603–614. doi: 10.1111/tpj.12320

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

O'Brien, J. A., Daudi, A., Butt, V. S., and Bolwell, G. P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta* 236, 765–779. doi: 10.1007/s00425-012-1696-9

Pan, Z., Camara, B., Gardner, W. H., and Backhaus, R. A. (1998). Aspirin inhibition and acetylaton of the plant cytochrome P450, Allene oxide synthase, resembles that of animal prostaglandin endoperoxide H synthase. *J. Biol. Chem.* 273, 18139–18145. doi: 10.1074/jbc.273.29.18139

Park, S.-W., Kaiyomo, E., Kumar, D., Mosher, S. L., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Pieterse, M. J. C., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Seay, M., Gerstein, M., et al. (2007). Different binding of calmodulin-related proteins to their targets revealed through high-density *Arabidopsis* protein microarrays. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4730–4735. doi: 10.1073/pnas.0611615104

Raskin, I. (1992). Role of salicylic acid in plants. *Ann. Rev. Plant Physiol.* 43, 439–463. doi: 10.1146/annurev.pp.43.060192.002255

Rivas-San Vicente, M., and Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *J. Exp. Bot.* 62, 3321–3338. doi: 10.1093/jxb/err031

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Rosti, J., Barton, C. J., Albrecht, S., Dupree, P., Pauly, M., Findlay, K., et al. (2007). UDP-Glucose 4-epimerase isoforms UGE2 and UGE4 cooperate in providing UDP-galactose for cell wall biosynthesis and growth of *Arabidopsis thaliana*. *Plant Cell* 19, 1565–1579. doi: 10.1105/tpc.106.049619

Saric, T., Graf, C., and Goldberg, A. (2004). Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases. *J. Biol. Chem.* 279, 46723–46732. doi: 10.1074/jbc.M406537200

Shah, J., Tsui, F., and Klessig, D. F. (1997). Characterization of a salicylic acid-insensitive mutant (sail) of *Arabidopsis thaliana* identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. *Mol. Plant Microbe. Interact.* 10, 69–78. doi: 10.1094/MPMI.1997.10.1.69

Slaymaker, D. H., Navarre, D. A., Clark, D., del Pozo, O., Martin, G. B., and Klessig, D. F. (2002). The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11640–11645. doi: 10.1073/pnas.182427699

Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3:3. doi: 10.2202/1544-6115.1027

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Tada, Y., Spoel, S. H., Pajerowsaka-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant Immunity requires conformational changes of NPR1 via s-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Tian, M., Dahl, C., Liu, P., Friso, G., Wijk, K., and Klessig, D. F. (2012). The combined use of photoaffinity labeling and surface plasmon resonance-based technology identifies multiple salicylic acid-binding proteins. *Plant J.* 72, 1027–1038. doi: 10.1111/tpj.12016

Tian, M., Sasvari, Z., Friso, G., Rowland, E., Liu, X.-M., van Wijk, K., et al. (in press). Salicylic acid inhibits the replication of Tomato Bushy Stunt Virus by directly targeting a host component in the replication complex. *Mol. Plant Microbe. Interact.*

Timm, S., Nunes-Nesi, T., Parnik, T., Morgenthal, K., Wienkoop, S., Keerberg, O., et al. (2008). A cytosolic pathway for the conversion of hydroxypyruvate to glycerate during photorespiration in *Arabidopsis*. *Plant Cell* 20, 2848–2859. doi: 10.1105/tpc.108.062265

Tomkinson, B. (1999). Tripeptidyl peptidases: enzymes that count. *Trends Biochem. Sci.* 24, 355–359. doi: 10.1016/S0968-0004(99)01435-8

van der Hoorn, R. A. (2008). Plant proteases: from phenotypes to molecular mechanisms. *Annu. Rev. Plant Biol.* 59, 191–223. doi: 10.1146/annurev.arplant.59.032607.092835

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Vlot, A. C., Liu, P. P., Cameron, R. K., Park, S. W., Yang, Y., Kumar, D., et al. (2008). Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic resistance in *Arabidopsis thaliana*. *Plant J.* 56, 445–456. doi: 10.1111/j.1365-313X.2008.03618.x

Wallgrove, R. M., Turner, J. C., Hall, N. P., Kendall, A. C., and Bright, S. W. (1987). Barley mutants lacking chloroplast glutamine synthetase-biochemical and genetic analysis. *Plant Physiol.* 83, 155–158. doi: 10.1104/pp.83.1.155

Weissmann, G. (1991). Aspirin. *Sci. Am.* 264, 84–90. doi: 10.1038/scientific american0191-84

White, R. F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* 99, 410–412. doi: 10.1016/0042-6822(79)90019-9

Wick, J. Y. (2012). Aspirin: a history, a love story. *Consult. Pharm.* 27, 322–329. doi: 10.4140/TCP.n.2012.322

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Xu, E., and Brosche, M. (2014). Salicylic acid signaling inhibits apoplastic reactive oxygen species signaling. *BMC Plant Biol.* 14:155. doi: 10.1186/1471-2229-14-155

Zhu, X. G., Long, S. P., and Ort, D. R. (2008). What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? *Curr. Opin. Biotechnol.* 19, 153–159. doi: 10.1016/j.copbio.2008.02.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 October 2014; paper pending published: 14 November 2014; accepted: 15 December 2014; published online: 12 January 2015.

*Citation: Manohar M, Tian M, Moreau M, Park S-W, Choi HW, Fei Z, Friso G, Asif M, Manosalva P, von Dahl CC, Shi K, Ma S, Dinesh-Kumar SP, O'Doherty I, Schroeder FC, van Wijk KJ and Klessig DF (2015) Identification of multiple salicylic acid-binding proteins using two high throughput screens. Front. Plant Sci. **5**:777. doi: 10.3389/fpls.2014.00777*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in Plant Science

REVIEW
published: 25 March 2015
doi: 10.3389/fpls.2015.00170

How salicylic acid takes transcriptional control over jasmonic acid signaling

*Lotte Caarls, Corné M. J. Pieterse and Saskia C. M. Van Wees**

Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, Utrecht, Netherlands

Transcriptional regulation is a central process in plant immunity. The induction or repression of defense genes is orchestrated by signaling networks that are directed by plant hormones of which salicylic acid (SA) and jasmonic acid (JA) are the major players. Extensive cross-communication between the hormone signaling pathways allows for fine tuning of transcriptional programs, determining resistance to invaders and trade-offs with plant development. Here, we give an overview of how SA can control transcriptional reprogramming of JA-induced genes in *Arabidopsis thaliana*. SA can influence activity and/or localization of transcriptional regulators by post-translational modifications of transcription factors and co-regulators. SA-induced redox changes, mediated by thioredoxins and glutaredoxins, modify transcriptional regulators that are involved in suppression of JA-dependent genes, such as NPR1 and TGA transcription factors, which affects their localization or DNA binding activity. Furthermore, SA can mediate sequestering of JA-responsive transcription factors away from their target genes by stalling them in the cytosol or in complexes with repressor proteins in the nucleus. SA also affects JA-induced transcription by inducing degradation of transcription factors with an activating role in JA signaling, as was shown for the ERF transcription factor ORA59. Additionally, SA can induce negative regulators, among which WRKY transcription factors, that can directly or indirectly inhibit JA-responsive gene expression. Finally, at the DNA level, modification of histones by SA-dependent factors can result in repression of JA-responsive genes. These diverse and complex regulatory mechanisms affect important signaling hubs in the integration of hormone signaling networks. Some pathogens have evolved effectors that highjack hormone crosstalk mechanisms for their own good, which are described in this review as well.

Keywords: hormone crosstalk, transcription factors, regulation of gene expression, plant immunity, post-translational modifications

OPEN ACCESS

Edited by:
Jean Toby Greenberg, The University of Chicago, USA

Reviewed by:
Selena Gimenez-Ibanez, Centro Nacional de Biotecnologia, Spain
Zhixiang Chen, Purdue University, USA

****Correspondence:***
Saskia C. M. Van Wees, Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, Netherlands
s.vanwees@uu.nl

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *16 January 2015*
Accepted: *03 March 2015*
Published: *25 March 2015*

Citation:
Caarls L, Pieterse CMJ and Van Wees SCM (2015) How salicylic acid takes transcriptional control over jasmonic acid signaling. Front. Plant Sci. 6:170. doi: 10.3389/fpls.2015.00170

Introduction

The activation of inducible immune responses in the plant is tightly regulated, ensuring an effective and cost-efficient response to pathogenic microbes and herbivorous insects (Vos et al., 2013a). Recognition of an attacker leads to accumulation of signaling molecules like the plant hormones salicylic acid (SA) and jasmonic acid (JA) and its derivatives, which play major roles in the activation of downstream defense responses (reviewed by Pieterse et al., 2012). Generally speaking, SA activates resistance against biotrophic pathogens, while JA is critical for activation of defense

against herbivorous insects and necrotrophic pathogens. The SA- and JA-responsive signaling pathways are interdependent and act in complex networks. Other hormones participate in these defense signaling networks as well and can consequently modulate the outcome of the activated defense arsenal. Abscisic acid (ABA) and ethylene can act synergistically with distinct JA-regulated responses, while they generally antagonize SA responses. Auxin, gibberellins, and cytokinins can repress defense-related processes to prioritize growth of the plant, and vice versa their action can be suppressed by SA or JA leading to activation of defense at the expense of plant growth (Pieterse et al., 2012).

Most knowledge on hormone signaling pathways stems from work on the molecular genetic model plant *Arabidopsis thaliana*. Consequently, this review is based primarily on research with *Arabidopsis*, but we are aware that other plant species may regulate the interplay between hormone signaling pathways differently. We aim to focus on general mechanisms affecting transcriptional regulation that could also apply to other plant species. Hormone-modulated regulation of disease resistance is primarily achieved through effects on gene transcription. Activation or repression of target genes is accomplished by physical interaction between *trans*-acting proteins, such as transcription factors, and *cis*-acting DNA elements. Transcription factors and co-regulators can themselves be controlled at the transcriptional level, but they are also subject to post-translational modification through reduction or oxidation, sequestration, phosphorylation, degradation, or interaction with other transcription factors or co-factors (Moore et al., 2011). Moreover, transcriptional activation is determined by the accessibility of *cis*-acting elements, which can be influenced by remodeling of chromatin through modifications of histones (Liu et al., 2014).

Transcriptional and post-translational regulatory mechanisms are important in both SA- and JA-controlled signaling pathways. In the SA pathway, activity of NPR1, which was identified as a master transcriptional co-regulator of SA-dependent genes, is tightly regulated by several SA-dependent modifications (reviewed by Fu and Dong, 2013). SA induces a biphasic fluctuation in the cellular redox state that can be sensed by NPR1, which then switches from an oligomer to monomer form by reduction of intermolecular disulfide bonds. Thioredoxins TRX-h5 and TRX-h3 catalyze the formation of NPR1 monomers, which translocate to the nucleus (**Figure 1A**). Regulation of NPR1 monomer levels in the nucleus is also dependent on SA. NPR1 and NPR1-homologs NPR3 and NPR4 were described to be SA-receptors (Fu et al., 2012; Wu et al., 2012). NPR3 and NPR4 act as CUL3 ligase adapter proteins in proteasome-mediated degradation of NPR1. NPR3 and NPR4 differ in both their binding affinity for SA and binding capacity to NPR1, so that SA levels determine when NPR1 is targeted for degradation. When SA levels are low, NPR4 interacts with NPR1, leading to its degradation, and in this way untimely transcriptional activation in absence of SA is prevented. High SA levels facilitate binding between NPR1 and NPR3, again leading to removal of NPR1 (Fu et al., 2012). This degradation of NPR1 is thought to help activate programmed cell death, of which NPR1 is a negative regulator. When SA levels are intermediate, interaction between NPR1 and NPR3 is prevented, allowing NPR1 to accumulate and activate SA-dependent defenses. By interacting with transcription factors of the TGA family, NPR1 acts as a co-activator of SA-induced gene transcription, activating SA marker genes such as *PR1*, but also several *WRKY* transcription factor genes, which then fine-tune and amplify downstream transcriptional responses (Wang et al., 2006; Eulgem and Somssich, 2007).

Master regulators of the JA signaling pathway are the F-box protein COI1 and the JAZ repressor proteins. In the absence of JA, JAZ repressor proteins associate with the co-repressor TPL via the adapter protein NINJA, or with HDA6, thereby repressing various transcription factors, among which MYC2, EIN3, and EIL1 (**Figure 1A**; reviewed by Song et al., 2014). COI1 binds to JA-Ile, the bioactive form of JA, which leads to targeting of JAZ repressor proteins for degradation by the proteasome. The successive release of transcriptional activators then leads to activation of JA-responsive genes (**Figure 1B**). Two branches are distinguished in JA-dependent signaling: (i) MYC2 is the master regulator of the MYC branch, which is co-regulated by JA and ABA, activating downstream marker genes *VSP2* and *LOX2* (Lorenzo et al., 2004; Vos et al., 2013b), while (ii) EIN3, EIL1, and ERF transcription factors like ERF1 and ORA59 regulate the ERF branch, which is co-regulated by JA and ET, activating the downstream marker gene *PDF1.2* (Zhu et al., 2011; Pieterse et al., 2012; Wasternack and Hause, 2013).

Recent work indicates that suppression of the JA-responsive pathway by SA (hereafter also referred to as SA/JA crosstalk) is predominantly regulated at the level of gene transcription (Van der Does et al., 2013). First, SA/JA crosstalk proved to be independent of downregulation of JA biosynthesis itself, as the SA-mediated suppression of MeJA-induced *PDF1.2* was intact in the JA biosynthesis mutant *aos/dde2* (Leon-Reyes et al., 2010b). Using the JA-receptor mutant *coi1-1* ectopically expressing *ERF1* to constitutively express downstream JA-responsive genes, Van der Does et al. (2013) further demonstrated that SA can suppress ERF1-activated *PDF1.2* independently of COI1. Moreover, using GCC:GUS reporter lines, the GCC-box, which is a crucial *cis*-element in the regulation of *PDF1.2* expression, was shown to be sufficient for SA/JA crosstalk. This indicates that SA antagonizes JA signaling downstream of COI1, possibly by interfering with JA-regulated transcription factors. The ERF transcription factor ORA59 was then demonstrated to be degraded by SA. At the SA signaling side, using mutant *npr1-1*, master regulator NPR1 was previously shown to be essential for suppression of JA-responsive gene expression (Spoel et al., 2003). Further, several WRKY and TGA transcription factors have been shown to be important for SA/JA crosstalk (Pieterse et al., 2012; Gimenez-Ibanez and Solano, 2013). However, the ways by which these transcriptional regulators down-regulate JA signaling in the presence of SA are largely unknown. In this review, we discuss the regulatory mechanisms that SA employs to repress JA-regulated transcriptional activity. Where relevant, examples of how other hormones interfere with hormone-dependent transcriptional regulation will be given.

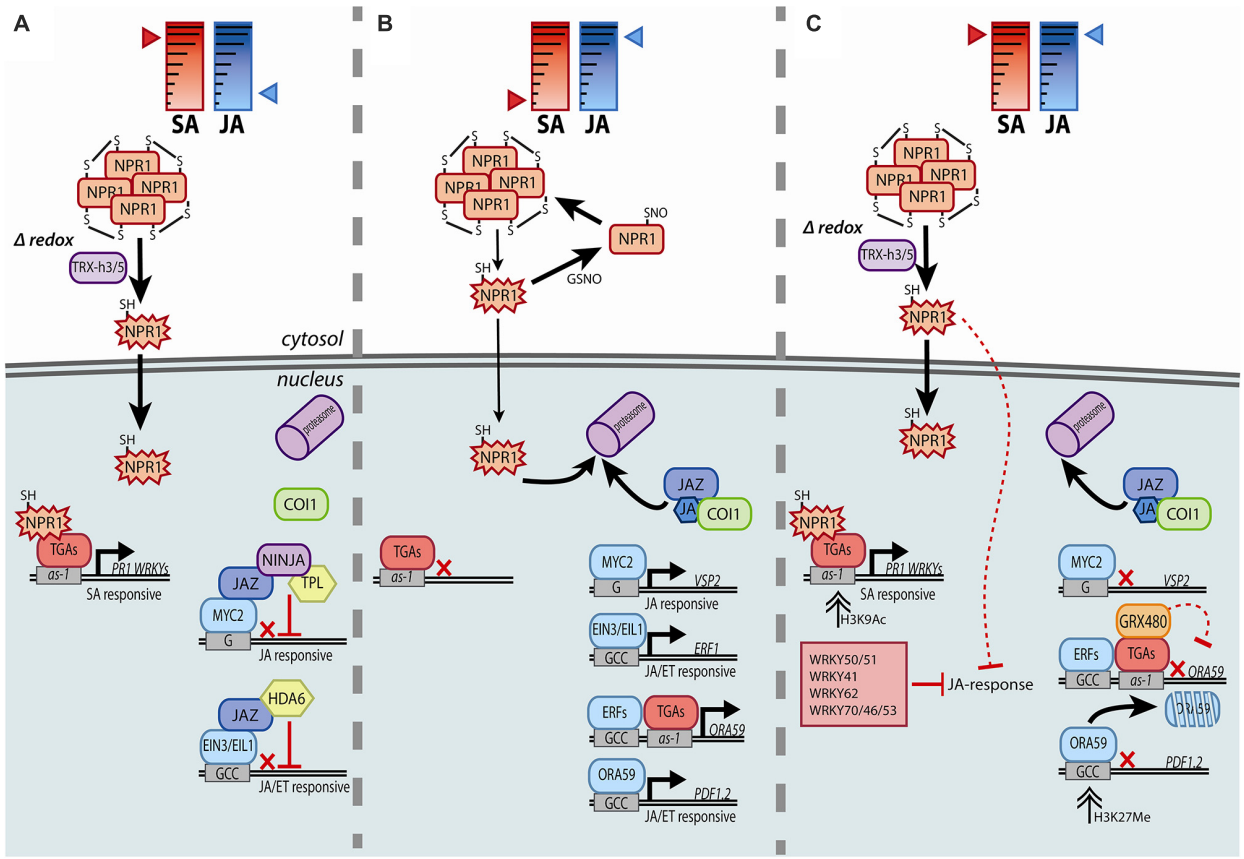


FIGURE 1 | Simplified model of the molecular machinery involved in the transcriptional regulation of the SA signaling pathway (A), the JA signaling pathway (B), or the antagonism of SA on the JA signaling pathway (C). By inducing reduction and monomerization of NPR1, SA activates NPR1 (star-shaped), which then triggers gene expression in the nucleus. JA-responsive genes are kept in check by JAZ repressors in the absence of JA. In the presence of JA, MYC or ERF transcription factors activate JA-responsive genes, but only if SA is absent. Activation of both the SA and JA signaling pathways leads to antagonism of JA-responsive gene expression by SA. There are indications for roles in SA/JA crosstalk for cytosolic NPR1, and nuclear localized TGAs, GRX480, and WRKYs. See text for details on the molecular processes underlying the transcriptional control, like redox signaling, sequestration, degradation, phosphorylation, and chromatin modification. Solid lines indicate established (in)activities and dashed lines hypothesized (in)activities, where black arrows specify activation and red blocks suppression. Red crosses indicate that gene transcription is hampered.

SA-Mediated Effects on Activity or Localization of Transcription Factors

SA-Induced Modification of Transcriptional Regulators via Redox Signaling

The activation of the immune response in plants is associated with rapid production of reactive oxygen intermediates (ROI) and increased levels of nitric oxide (NO). Redox-sensing small-molecule couples, such as reduced and oxidized glutathione, can limit damage from these redox active molecules. Moreover, these redox sensors transduce changes in ROI and NO levels into posttranslational modifications by reduction or oxidation of cysteine residues of transcriptional regulators, causing changes in transcriptional activity (Frederickson and Loake, 2014). Redox signaling is important in SA signaling and moreover, SA-induced redox changes are associated with the suppression of JA responses as well.

Role of Reduction of Transcriptional Regulators in SA Signaling

In SA signaling, master regulator NPR1 is subject to several redox-dependent modifications. It sequesters in the cytoplasm as an oligomer, formed by intermolecular disulfide bonds, which are facilitated by *S*-nitrosylation of cysteine residues via NO donor *S*-nitrosoglutathione (GSNO; **Figure 1B**). SA triggers cycles of cellular reduction and oxidation, measurable for example by enhanced total glutathione levels and a higher ratio of reduced to oxidized glutathione after SA treatment (Spoel and Loake, 2011). In response to activation of the SA pathway, thioredoxins catalyze the reduction of intermolecular disulphide bonds, causing a conformational change of NPR1 to its monomeric form. As a monomer, NPR1 is able to translocate from the cytosol to the nucleus and activate downstream signaling (**Figure 1A**) (Mou et al., 2003; Koornneef et al., 2008a; Tada et al., 2008). Other transcriptional regulators functioning in the SA pathway are also redox controlled. Transcription factor TGA1 contains

intramolecular disulfide bonds that prevent its interaction with NPR1. Only after reduction of these bonds under high SA conditions, TGA1 is able to interact with NPR1. Further *S*-nitrosylation and *S*-glutathionylation of the cysteine residues of TGA1 result in enhanced binding to DNA and activation of transcription (**Figure 1A**; Després et al., 2003; Lindermayr et al., 2010).

Role of the Redox State in SA/JA Crosstalk Signaling

Redox-mediated reduction of transcriptional regulators is not only essential for SA signaling, but is also implicated in SA/JA crosstalk. The enhancement in glutathione levels after SA treatment was shown to coincide exactly with the window of opportunity in which SA could suppress JA-induced *PDF1.2* expression, i.e., within 30 h after application of SA. In addition, treatment with glutathione synthesis inhibitor BSO blocked SA-mediated antagonism of *PDF1.2* expression (Koornneef et al., 2008a). Interestingly, JA can also influence the redox state of cells, but, in contrast to SA, it decreases the total amount of glutathione, and shifts the ratio between reduced and oxidized glutathione toward the oxidized state (Spoel and Loake, 2011). When SA and JA were applied simultaneously, the pattern of glutathione increase was the same as after treatment with SA alone, suggesting a role for redox regulation in prioritization of the SA pathway over the JA pathway (Koornneef et al., 2008a). So far, it is unclear how the SA-induced cellular reduction can influence JA-inducible responses.

Master regulator NPR1 is essential for SA/JA crosstalk and, therefore, the importance of SA-induced redox changes in SA/JA crosstalk could be related to reduction and translocation of NPR1 to the nucleus. However, the nuclear localization of NPR1 that follows SA-induced monomerization is, although essential for SA-responsive gene expression, not needed for SA-mediated suppression of JA-dependent genes (Spoel et al., 2003; Leon-Reyes et al., 2009). This was shown with *Arabidopsis* plants that overexpress a fusion protein of NPR1 that was retained in the cytosol: stimulation of the SA pathway in these plants resulted in a wild-type level of suppression of JA-induced *PDF1.2* (Spoel et al., 2003). The role of NPR1 in the cytoplasm for SA/JA crosstalk was confirmed in rice (*Oryza sativa*), where overexpression of OsNPR1 suppressed JA-responsive gene expression and defense against insects. However, when a mutated form of OsNPR1 was overexpressed that was constitutively present in the nucleus, herbivore resistance and expression of a JA-responsive gene were not affected (Yuan et al., 2007). Although NPR1 is exclusively needed in the cytosol for SA/JA crosstalk, it is still possible that redox-mediated modification of NPR1 is important in SA/JA crosstalk, for example if there is a role for the monomeric form of NPR1 in the cytosol to suppress JA signaling (Spoel et al., 2003; Beckers and Spoel, 2006). Alternatively, redox signaling may be important for post-translational modification of other factors with a role in SA/JA crosstalk, as described below.

The importance of redox regulation in SA/JA crosstalk is supported by the role of glutaredoxins (GRXs) in this phenomenon. GRXs are small ubiquitous redox enzymes that use glutathione to reduce their targets (Ndamukong et al., 2007; Ströher and Millar, 2012). SA is known to induce the expression of at least two GRXs, namely GRX480 and GRXS13, which are members of the group III class of GRXs in *Arabidopsis*. Overexpression of GRX480 blocks the induction of *PDF1.2* by JA, and overexpression of GRXS13 makes plants more susceptible to the necrotrophic fungus *Botrytis cinerea*, suggesting a role for both GRXs in suppression of JA signaling (Ndamukong et al., 2007; Camera et al., 2011). In fact, 10 more group III GRXs, which are also called ROXYs, are able to suppress activation of the *ORA59* promoter and are thus potentially involved in suppression of the JA pathway (Zander et al., 2012). Their antagonistic action on JA responses is likely downstream of NPR1, because expression of *GRX480* is reduced in the *npr1-1* mutant and overexpression of GRX480 in the *npr1-1* background still results in suppression of *PDF1.2* expression (Zander et al., 2012; Herrera-Vásquez et al., 2014). TGA transcription factors that are implicated in different hormonal signaling pathways and in SA/JA crosstalk (described more in-depth in SA-Inducible Expression of Transcription Factor Genes that Suppress JA Responses) are possible targets of group III GRXs, as they are shown to interact with each other (**Figure 1C**). Moreover, JA-induced *PDF1.2* expression is not impaired when GRX480 is overexpressed in the triple mutant *tga2/tga5/tga6* background, showing that the function of this GRX in suppression of JA-responses is dependent on these TGA transcription factors (Ndamukong et al., 2007; Zander et al., 2012).

Sequestration and Degradation of Transcription Factors by SA

Salicylic acid could antagonize JA signaling by preventing accessibility of JA-responsive transcriptional regulators to their target genes. This could be achieved by sequestering transcription factors in inactive complexes or by degradation of positive regulators.

Sequestering Transcriptional Regulators by Complexation

By directing transcription factors to the cytosol, the possibility to activate transcription is obviously obstructed. In addition, transcription factors can be kept in check in the nuclear compartment as well, by inducing complex formation with other proteins that inhibit binding to the DNA, resulting in reduced transcription. There are no examples yet of SA-mediated sequestration of transcription factors leading to antagonism of JA signaling. However, some other plant hormone signaling interactions have been reported to be partly regulated via this mechanism, of which an example is the interaction between the SA and the ABA signaling pathways. The transcription factor WRKY40 is induced by SA and suppresses expression of the ABA-responsive genes *ABI4* and *ABI5*. After ABA treatment, the ABA receptor ABAR interacts with WRKY40, which is then recruited to the cytosol. By this recruitment, binding of WRKY40 to ABA responsive promoters is inhibited and repression of ABA responsive genes is lifted (Shang et al., 2010; Liu et al., 2012).

In animal cells, cytosolic sequestration of a transcriptional regulator was shown to control the antagonistic interaction

between SA and prostaglandin signaling, which shares several aspects with SA/JA crosstalk in plants. SA and aspirin block the formation of prostaglandins in animal cells, which are considered structural analogs of JA in plants. SA induces retention of transcription factor NF-κB in the cytoplasm by enforcing its interaction with IκB. In response to stress, IκB kinase is activated and degrades IκB, leading to nuclear localization of NF-κB, which then activates gene expression, necessary for the production of prostaglandins. In cells that are exposed to SA, degradation of IκB is inhibited, which prevents the nuclear translocation of NF-κB. Interestingly, IκB in animals has structural similarity with NPR1 (reviewed by Spoel and Dong, 2012). In plants, the cytosolic location of NPR1 is important for SA-mediated antagonism of JA-responsive gene expression (Spoel et al., 2003; Stein et al., 2008). One possible function for cytosolic NPR1 is that it may sequester JA-regulated transcriptional activators in the cytoplasm, thereby preventing them from moving to the nucleus and activating transcription. However, whether SA can interfere with translocation of JA-responsive transcription factors to the nucleus remains to be demonstrated.

In the nucleus, transcription factors can be prevented from binding DNA and thus activating gene expression by interacting with repressor proteins, which have been reported to function as important regulators in several hormone signaling pathways (Robert-Seilaniantz et al., 2011). JAZ proteins in the JA pathway are examples of such repressors. JA-induced ubiquitination of JAZ proteins mediates their degradation via the 26S proteasome, which releases their repressive effect on positive transcriptional regulators. By increasing the stability of repressor proteins, hormones can antagonize another hormone's action. An example of this crosstalk mechanism is found in the SA-auxin interaction. Parallel to JAZ repressor proteins in the JA pathway, AUX-IAA proteins are the negative regulators that bind and inactivate activators of auxin signaling. Binding of auxin to F-box proteins TIR1 and TIR1-related proteins, which act as auxin receptors, leads to degradation of AUX-IAA repressors. SA was shown to inhibit the auxin signaling pathway through stabilization of AUX/IAA repressor proteins, probably indirectly through repression of TIR1. In this way, SA could lift the disease promoting effect of auxin in the infection of *Arabidopsis* by *Pseudomonas syringae* (Wang et al., 2007). Also crosstalk between JA and GA pathways is regulated through interaction with their key repressor proteins, JAZs and DELLAs, respectively. In the absence of GA, stabilized DELLA can interact with JAZ proteins, thus reducing the repressive effect of JAZ on JA-responsive gene expression. DELLAs are degraded when GA levels rise, leading to enhanced suppression of JA signaling by JAZs (Hou et al., 2010; Pieterse et al., 2014). On the other hand, JA delays GA-mediated degradation of DELLAs, which is associated with a reduction in growth, suggesting that the trade-off between JA-dependent defense and GA-dependent growth can be regulated by the DELLA-JAZ signaling module (Yang et al., 2012). There is no evidence, however, that SA interferes with the stability of JAZs to antagonize JA signaling. First, JAZ1 and JAZ9, two of the most important JAZ proteins, are still degraded in JA-treated *Arabidopsis* when plants are additionally treated with SA. Second, SA was shown to antagonize the JA signaling pathway downstream of COI1, the F-box protein that interacts with JAZ repressor proteins to target them for ubiquitination (Van der Does et al., 2013).

SA-Mediated Degradation of JA-Regulated Transcription Factors

Salicylic acid-induced degradation of activating transcription factors of JA signaling could contribute to the repression of JA-responsive genes. Recently, SA was shown to lead to degradation of ORA59, a positive regulator in the ERF branch of the JA pathway. A whole-genome expression profiling analysis showed that the GCC-box was overrepresented in MeJA-induced genes that were antagonized by SA at 24 h after treatment with a combination of the hormones. The GCC-box was subsequently shown to be sufficient for suppression by SA (Van der Does et al., 2013). Similarly, the GCC-box was enriched in promoters of ethylene-induced genes that were suppressed by SA (Zander et al., 2014). The GCC-box is an essential promoter element for activation of *PDF1.2* expression and ERF transcription factor ORA59 is an important regulator in this activation (Zarei et al., 2011). Van der Does et al. (2013) suggested that downregulation of transcription of *ORA59* is not essential for SA/JA crosstalk, but showed that protein levels of ORA59 diminished after SA treatment, suggesting that SA could target positive regulators in the JA pathway for degradation. So far, degradation of other positive regulators of JA signaling has not been reported. The degradation rate of MYC2, master regulator of the MYC branch in the JA pathway, is likely not influenced by SA (Chico et al., 2014).

Phosphorylation of Transcription Factors Influences Transcription

Perception of pathogenic microbes by the plant leads to activation of mitogen-activated protein kinases (MPKs) that can subsequently phosphorylate transcriptional regulators. Phosphorylation of transcription factors influences gene transcription by changing the binding strength to DNA, or affecting sequestration or stability (Tena et al., 2011). In particular MPK3, MPK4, and MPK6, which act at the last step of MAPK signaling cascades, are known to phosphorylate transcription factors and have been implicated in immune signaling (Meng and Zhang, 2013). For example, phosphorylation of WRKY33 by MPK3 and MPK6 is likely responsible for the WRKY33-mediated induction of the *WRKY33* gene itself and of *PAD3*, which is a camalexin biosynthesis gene (Mao et al., 2011). It has also been suggested that WRKY33 is controlled by sequestration in a complex with MKS1 and MPK4. Upon bacterial pathogen attack the activated MAPK signaling cascade phosphorylates MKS1, which leads to disassociation from MPK4 so that WRKY33 is released from the complex and could bind to the promoter of *PAD3* (Qiu et al., 2008).

There is not much known about the role of MAPK cascades in the interplay between different hormone pathways. MAPK cascades are important in the JA pathway, so inhibition of MAPK cascades by SA could be an effective way to antagonize JA

signaling. For example, JA activates MPK6 and many AP2/ERFs transcription factors are phosphorylated and activated by MPK6, among which positive regulators ERF6 and ERF104 (Takahashi et al., 2007; Bethke et al., 2009; Popescu et al., 2009; Meng et al., 2013). It is not known if SA can prevent this phosphorylation to inhibit activation of the JA-regulated AP2/ERF transcription factors. MPK4 was thought to function as an integrator of SA and JA signaling as the mutant *mpk4* constitutively expresses SA-inducible *PR* genes and fails to express *PDF1.2*, which correlates with enhanced resistance to biotrophic pathogens and increased susceptibility to necrotrophic pathogens (Petersen et al., 2000; Brodersen et al., 2006). However, recently it was suggested that MPK4 is guarded by the R protein SUMM2. Reduction of the kinase activity of MPK4 by the bacterial effector HopAI1 is monitored by SUMM2, and leads to activation of SA-dependent defense responses (Zhang et al., 2012b). The effects of MPK4 on SA signaling are thus indirect, and this makes a role for MPK4 as an integrator of SA and JA signaling unlikely. However, whether MPK4's role in JA signaling is a direct or indirect one needs to be studied further.

SA-Inducible Expression of Transcription Factor Genes that Suppress JA Responses

Salicylic acid may also antagonize JA-inducible gene transcription by inducing the expression of genes encoding transcriptional regulators that interfere with JA signaling. These SA-induced regulators could inhibit a positive regulator of JA-inducible gene expression by interacting with it, as is described for the GRX480-TGA interaction in "Role of Reduction of Transcriptional Regulators in SA Signaling." Alternatively, SA could induce transcription of suppressive transcription factors that directly bind to the promoter of JA responsive genes to repress their expression. Examples of TGA, ERF, WRKY, and bHLH transcription factors that are induced by SA and inhibit JA-dependent transcription are reviewed below.

TGA Transcription Factor Family

TGA transcription factors have a role in various hormone-regulated transcriptional responses. They can generally activate SA-dependent gene expression, but are also known to have both positive and negative effects on JA/ethylene-dependent responses. TGA transcription factors are a class of bZIP transcription factors that bind to the *as-1* element (TGACG) in promoters. In *Arabidopsis*, 10 TGAs exist of which several have been shown to interact with NPR1 (reviewed by Gatz, 2013). The *PR1* promoter contains an *as-1* element, and the triple mutant *tga2/tga5/tga6* is, like *npr1*, compromised in SAR and does not express *PR1* upon treatment with the SA-mimic INA (Zhang et al., 2003). In response to SA, a ternary complex of TGA, NPR1, and DNA is formed that can activate transcription of *PR1* (**Figure 1A**). In non-induced conditions, suppression of *PR1* by TGAs has also been reported (Rochon et al., 2006; Pape et al., 2010). TGAs are important for activation of JA/ethylene-dependent genes as well. Although mutant *tga2/tga5/tga6* adult plants responded with *PDF1.2* induction upon treatment with JA, they did not express *PDF1.2* in response to ethylene or *B. cinerea* infection (Zander et al., 2010).

In addition, TGAs can be essential for suppression of JA responsive genes by SA, as JA-induced *PDF1.2* is not suppressed after a combination treatment with SA in mutant *tga2/tga3/tga5/tga6* (Leon-Reyes et al., 2010a). Microarray analysis comparing wild-type and *tga2/tga5/tga6* mutant plants showed that after treatment with ethylene precursor ACC, 374 genes were induced in wild-type plants, of which 136 were dependent on TGA2/TGA5/TGA6. Half of these ACC-inducible TGA-dependent genes were, in wild-type plants, suppressed by SA after a combination treatment of ACC with SA. This suggests a role for TGAs in both activation of ethylene-responsive genes and SA-mediated repression of these genes (Zander et al., 2014). The *PDF1.2* promoter contains an *as-1* element, but this was shown not to be important for the antagonistic effect on JA-induced *PDF1.2* expression by SA (Spoel et al., 2003). However, Zander et al. (2014) showed that the TGAs directly target the *as-1* element in the promoter of *ORA59* and could regulate both induction of *ORA59* by ACC treatment and suppression of *ORA59* by SA (**Figure 1C**). Transcriptional regulation of *ORA59* by TGAs is in line with the observation that the GCC-box is enriched in the promoter elements of ACC-induced, SA-suppressed genes. How can TGA factors act as both activators and repressors in different hormone signaling pathways? Possibly, different co-factors can be recruited to TGA factors depending on both the promoter context and the hormonal context. In the case of activation of transcription by SA, TGAs have been shown to interact with transcriptional activators NPR1 and GRAS protein SLC14 (Rochon et al., 2006; Fode et al., 2008). Upon JA accumulation, TGAs may interact with so-far unknown JA signaling regulators to promote JA responsive gene expression. When SA/JA crosstalk is activated, SA induces GRXs, which could interact with TGAs on the *ORA59* promoter leading to repression of JA-inducible genes (**Figure 1C**). GRXs were shown to down-regulate *ORA59* expression in a TGA-dependent manner, as discussed in "Role of Reduction of Transcriptional Regulators in SA Signaling" (Zander et al., 2012).

Both Zander et al. (2014) and Van der Does et al. (2013) point to ORA59 as a major target of antagonism by SA. However, while the first show that SA targets expression of *ORA59*, the protein levels of ORA59 were shown to be influenced by SA by the latter. The apparent discrepancy between these two studies could partly be explained by the different combination of hormones that both groups studied, SA-ethylene or SA–JA, respectively. Support for differences in crosstalk mechanisms depending on hormonal context comes from the observation that in an ethylene-rich environment the SA-antagonized expression of JA-inducible *PDF1.2* became independent of NPR1 (Leon-Reyes et al., 2009) or was even completely impaired when plant tissue was exposed to high levels of ethylene prior to treatment with SA (Leon-Reyes et al., 2010a). However, it is very well possible that ORA59 is regulated by SA at both the transcriptional and post-translational level, and that both mechanisms complement each other (**Figure 1C**).

ERF Transcription Factor Family

Transcription factors of the ERF subfamily of AP2/ERF family of transcription factors can bind the GCC-box and can act as

activators, such as ORA59, but also as repressors of transcription. Fourteen of the 122 ERFs in *Arabidopsis* contain an EAR domain, which is an active repressor domain that interacts with the general co-repressor TPL (Nakano et al., 2006). EAR-domain-containing ERF4 and ERF9 were shown to be able to suppress *PDF1.2* expression (McGrath et al., 2005; Maruyama et al., 2009). Because of the importance of the GCC-box in SA/JA crosstalk, the suppression of JA-responsive genes may, besides through negative regulation of ORA59 by SA (covered in SA-Mediated Degradation of JA-Regulated Transcription Factors and TGA Transcription Factor Family), in part be regulated by suppressive SA-induced ERFs. This hypothesis has up to now not been tested.

WRKY Transcription Factor Family

WRKY transcription factors are foremost known for their inducibility by SA and pathogens, and their role in regulating SA-dependent gene expression. There are, however, also examples of WRKYs that positively regulate other hormone-regulated genes, including JA-responsive defense genes (Journot-Catalino et al., 2006; Xu et al., 2006). The W-box (C/TTGACC/T) is a DNA element that is bound by WRKY transcription factors (Eulgem and Somssich, 2007). Importantly, the W-box motif was reported to be enriched in JA-responsive genes that were antagonized by SA (Van der Does et al., 2013), suggesting the involvement of WRKYs in SA/JA crosstalk as well. Indeed, several WRKYs have been implicated in suppression of JA-induced *PDF1.2* expression (**Figure 1C**). Overexpression of SA-induced WRKY70 suppressed MeJA-induced *PDF1.2* expression (Li et al., 2004, 2006). However, in a *wrky70* mutant, JA-dependent genes were induced by JA and suppressed by the combination treatment, indicating that WRKY70 is sufficient but not required for SA/JA crosstalk (Ren et al., 2008; Leon-Reyes et al., 2010a). Redundancy of different WRKYs could possibly explain the lack of a crosstalk phenotype of the single *wrky70* mutant, as double and triple mutants of *wrky70* with *wrky46* and *wrky53* did show enhanced *PDF1.2* expression after MeJA treatment (Hu et al., 2012). Overexpression of the transcription factor MYB44 also led to suppression of the JA marker genes *VSP1* and *PDF1.2*, which is likely established through activation of *WRKY70*. MYB44 is inducible by SA and binds to the *WRKY70* promoter leading to its expression (Shim et al., 2013; Zou et al., 2013). Furthermore, WRKY62 was suggested to function in suppression of JA responses, because a *wrky62* mutant displayed enhanced expression of JA responsive genes, while an overexpressor exhibited reduced expression. *WRKY62* is induced by SA and was suggested to act downstream of cytosolic NPR1 (Mao et al., 2007). To end, WRKY41 has been implicated in suppression of JA responsiveness, since overexpression of WRKY41 led to increased *PR5* and reduced *PDF1.2* expression. However, in contrast to the aforementioned *WRKY* genes, *WRKY41* is likely not a direct target of NPR1 and SA only slightly induces *WRKY41* expression (Higashi et al., 2008).

Studies on the *ssi2* mutant revealed two other WRKYs that are involved in SA/JA crosstalk. The *ssi2* mutant was initially identified in a screen for *npr1* suppressors and displays high SA responses while JA responses are repressed (Shah et al., 2001). The increased SA levels were not needed for the repression of JA responses, but instead lowered levels of 18:1 fatty acids appeared to regulate the repression of JA signaling (Kachroo et al., 2001, 2003; Nandi et al., 2005). In *ssi2* mutants, 19 *WRKYs* were induced, of which five in a SA-independent manner. Double mutants of *ssi2* with *wrky50* or *wrky51* restored the induction of *PDF1.2* and resistance against *B. cinerea* without altering the 18:1 fatty acid levels. WRKY50 and WRKY51 thus negatively regulate JA responses under low 18:1 conditions. Single and double mutants of *wrky50* and *wrky51* also failed to suppress *PDF1.2* and *VSP2* after a combination treatment with SA and JA (Gao et al., 2011). Therefore, these two WRKYs seem to play important roles in the suppression of JA responses.

How can WRKY transcription factors repress JA responses? After their induction by SA, they could bind to W-boxes in JA-responsive genes to inhibit their expression directly or indirectly (Van der Does et al., 2013). There is no experimental proof of this repressive mechanism under the influence of SA yet, but recently WRKY51 has been reported to interact with JAV1, a VQ-motif containing protein that negatively regulates JA responses and acts in the nucleus (Hu et al., 2013).

bHLH Transcription Factor Family

Transcription factors of the bHLH family, including MYC2, play crucial roles in the JA signaling pathway. MYC2 is a master regulator of JA responses (reviewed by Kazan and Manners, 2013). The last 2 years have witnessed an boost in bHLHs that function as negative regulators in the JA signaling pathway (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013; Fonseca et al., 2014). Whether these repressive bHLHs are manipulated by SA to establish SA/JA crosstalk is currently unknown, but they are not obviously regulated at the transcription level by SA (BAR public database).

SA/JA Crosstalk could be Enforced by Chromatin Modification at Target Genes

Salicylic acid can further control gene expression by remodeling of chromatin around target genes. Chromatin is the complex of DNA and histones and its condensed structure can reduce accessibility of DNA and thus inhibit transcription. Modifications of chromatin can result in local loosening of this structure, which creates access for transcriptional machinery and regulatory proteins to the DNA. Chromatin modifications include methylation, acetylation, phosphorylation, ubiquitination, or sumoylation of histones (Iwasaki and Paszkowski, 2014). Acetylation of histones is associated with activation of genes, while deacetylation of histones is correlated with gene repression. Enzymes called histone acyltransferases and histone deacetylases (HDA) can carry out these respective histone modifications (Liu et al., 2014). HDA6 and HDA19 were described to interfere with JA signaling. HDA6 interacts with JAZ1, JAZ3, and JAZ9 and is recruited to repress EIN3/EIL1-dependent transcription (Zhu et al., 2011). In contrast, HDA19 was reported to have a positive role in the ERF branch and in defense against *Alternaria brassicicola* (Zhou et al., 2005). HDA19 also targets SA signaling by binding to the *PR1* and

PR2 promoters leading to their repression (Choi et al., 2012), and by reducing transcriptional activity of WRKY38 and WRKY62 (Kim et al., 2008). Since chromatin remodeling plays an important role in SA and JA signaling, it could also well be manipulated by SA to antagonize JA signaling. However, Koornneef et al. (2008b) showed that at the *PDF1.2* promoter there was no change in acetylation of histones after exogenous application of a combination of SA and MeJA.

Chromatin modifications are also described to be an important mechanism to prime plants for enhanced defense (Conrath, 2011). Interestingly, it was suggested that priming and SA/JA crosstalk could be carried over to offspring through acetylation and methylation of histones as well. Luna et al. (2012) showed that *Arabidopsis* plants that were inoculated with the bacterial pathogen *P. syringae* in the first generation, were more resistant to *P. syringae* and the oomycete pathogen *Hyaloperonospora arabidopsidis* in the next generation, and more susceptible to the necrotrophic pathogen *A. brassicicola*. This correlated with increased *PR1* expression and reduced *VSP2* and *PDF1.2* expression in the second generation and was dependent on NPR1. Acetylation of histone H3 at Lys-9 (H3K9) at the *PR1* promoter, which is associated with increased transcription, was enhanced in these plants. Conversely, tri-methylation of H3K27, which is associated with transcriptional silencing, was enriched at the *PDF1.2* promoter (**Figure 1C**), suggesting that histone modifications were responsible for the observed increased or decreased transcription (Luna et al., 2012). It is not clear yet how these changes can be transmitted to offspring, since there is no evidence that histone modifications are inherited. DNA methylation, which is often associated with histone modifications, is a possible modification that could be passed on to next generations. DNA methylation was shown to have an effect on SA- and JA-regulated responses: epiRIL lines, which are identical at the DNA sequence level but highly variable at the level of DNA methylation, showed differences in responsiveness to both treatments (Latzel et al., 2012).

Rewiring of Hormone-Regulated Transcriptional by Pathogens

In the evolutionary arms race, pathogens have evolved effectors that are secreted into plant cells upon infection to reduce disease resistance or increase plant susceptibility (reviewed by Kazan and Lyons, 2014). Interestingly, several pathogen effectors can highjack a plant's intricate hormonal crosstalk mechanism for their own good, resulting in lower induction of effective defenses. Some effectors are hormones themselves or are hormone-mimics that disturb the hormone balance in plants. The most famous example of such an effector is the JA-mimic coronatine, that is secreted by *Pseudomonas* pathogens and suppresses SA signaling (Zheng et al., 2012). More recently, effectors that interfere with signaling hubs in transcriptional regulation of JA signaling, such as JAZs, have been discovered. Effectors HopZ1a and HopX1 of two different *Pseudomonas* pathogen strains bind to and degrade JAZ repressor proteins, leading to activation of JA signaling and concomitant suppression of SA-regulated defense signaling (Jiang et al., 2013; Gimenez-Ibanez et al., 2014).

Other effectors can establish antagonism of SA signaling by manipulating the plant transcriptional machinery via interference with Mediator subunits. Mediator is a multi-protein transcriptional co-activator complex, which functions as a bridge between transcription factors and RNA polymerase II. Mediator recruits RNA polymerase II to promoters in response to different signals and controls the polymerase activity during transcription initiation and elongation (Conaway and Conaway, 2011). Several Mediator subunits have been implicated in SA- and/or JA-dependent gene expression. Mediator subunit MED16 was shown was shown to be important in defense against both biotrophic and necrotrophic pathogens by regulating SA- and JA/ethylene-responsive transcription and could therefore be viewed as a node of convergence between SA- and JA/ethylene-dependent pathways (Wathugala et al., 2012; Zhang et al., 2012a). Subunit MED25 was shown to be important for activation of JA-dependent genes, and likely acts through interaction with JA-responsive transcription factors, including ERF1, ORA59, and MYC2 (Çevik et al., 2012). The subunit MED19 positively regulates SA-dependent resistance that is effective against *H. arabidopsidis*. MED19 was shown to be targeted for degradation by the *H. arabidopsidis* effector HaRxL44. Expression of HaRxL44 in plants led to induction of JA-responsive genes, a response that is observed in *med19* plants as well (Caillaud et al., 2013). These data suggest that HaRxL44 induces degradation of MED19 to rewire transcription from SA-responsive to JA-responsive, leading to enhanced infection by *H. arabidopsidis*. This example illustrates the highly sophisticated manner in which effectors manipulate the plant transcriptional machinery to influence hormonal signaling.

Conclusion and Perspectives

In the last years, knowledge on the interplay between different plant hormone signaling pathways has vastly increased. In this review we focused on the molecular mechanisms (potentially) underlying antagonistic effects of SA on JA-mediated transcriptional responses and highlighted several transcriptional regulators (like NPR1, TGA, WRKY, and ORA59) as signal integrators. However, there is still much unknown about hormonal crosstalk mechanisms. The use of whole-transcriptome sequencing techniques after combinatorial hormone treatment or pathogen infection will aid in the identification and characterization of additional transcriptional regulators that can act as nodes of convergence in multiple signaling pathways (Van Verk et al., 2013). Combining transcriptome data with ChIP-seq or DNase-seq studies, which can identify DNA sites occupied by transcription factors, can provide more detailed knowledge on the mechanisms by which these crosstalk transcriptional regulators rewire hormonal signaling. In addition, more intensive proteomic studies are necessary to get a full scale picture of the posttranslational modifications that influence the action of key transcriptional regulators. The knowledge gained from pharmacological experiments, in which combinations of hormones are applied

exogenously, should be corroborated under biological conditions that trigger hormonal crosstalk, like (combinatorial) pathogen infection. Insights into the crosstalk signaling hubs that function in complex hormonal signaling networks will not only increase our fundamental knowledge on plant immune signaling but can also provide leads to develop crops with multi-attacker resistance and optimal growth.

Acknowledgments

The authors of this review are supported by VIDI grant no. 11281 of the Dutch Technology Foundation STW, which is part of the Netherlands Organization of Scientific Research, and ERC Advanced Investigator Grant no. 269072 of the European Research Council.

References

Beckers, G. J. M., and Spoel, S. H. (2006). Fine-tuning plant defence signalling: salicylate versus jasmonate. *Plant Biol.* 8, 1–10. doi: 10.1055/s-2005-872705

Bethke, G., Unthan, T., Uhrig, J. F., Poschl, Y., Gust, A. A., Scheel, D., et al. (2009). Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in *Arabidopsis thaliana* via ethylene signaling. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8067–8072. doi: 10.1073/pnas.0810206106

Brodersen, P., Petersen, M., Bjorn Nielsen, H., Zhu, S., Newman, M.-A., Shokat, K. M., et al. (2006). Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *Plant J.* 47, 532–546. doi: 10.1111/j.1365-313X.2006.02806.x

Caillaud, M.-C., Asai, S., Rallapalli, G., Piquerez, S., Fabro, G., and Jones, J. D. G. (2013). A downy mildew effector attenuates salicylic acid-triggered immunity in Arabidopsis by interacting with the host mediator complex. *PLoS Biol.* 12:e1001919. doi: 10.1371/journal.pbio.1001732

Camera, S. L., L'Haridon, F., Astier, J., Zander, M., Abou-Mansour, E., Page, G., et al. (2011). The glutaredoxin ATGRXS13 is required to facilitate *Botrytis cinerea* infection of *Arabidopsis thaliana* plants. *Plant J.* 68, 507–519. doi: 10.1111/j.1365-313X.2011.04706.x

Çevik, V., Kidd, B. N., Zhang, P., Hill, C., Kiddle, S., Denby, K. J., et al. (2012). MEDIATOR25 acts as an integrative hub for the regulation of jasmonate-responsive gene expression in *Arabidopsis*. *Plant Physiol.* 160, 541–555. doi: 10.1104/pp.112.202697

Chico, J.-M., Fernández-Barbero, G., Chini, A., Fernández-Calvo, P., Díez-Díaz, M., and Solano, R. (2014). Repression of jasmonate-dependent defenses by shade involves differential regulation of protein stability of MYC transcription factors and their JAZ repressors in *Arabidopsis*. *Plant Cell* 26, 1967–1980. doi: 10.1105/tpc.114.125047

Choi, S.-M., Song, H.-R., Han, S.-K., Han, M., Kim, C.-Y., Park, J., et al. (2012). HDA19 is required for the repression of salicylic acid biosynthesis and salicylic acid-mediated defense responses in Arabidopsis. *Plant J.* 71, 135–146. doi: 10.1111/j.1365-313X.2012.04977.x

Conaway, R. C., and Conaway, J. W. (2011). Function and regulation of the Mediator complex. *Curr. Opin. Genet. Dev.* 21, 225–230. doi: 10.1016/j.gde.2011.01.013

Conrath, U. (2011). Molecular aspects of defence priming. *Trends Plant Sci.* 16, 524–531. doi: 10.1016/j.tplants.2011.06.004

Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., et al. (2003). The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. *Plant Cell* 15, 2181–2191. doi: 10.1105/tpc.012849

Eulgem, T., and Somssich, I. E. (2007). Networks of WRKY transcription factors in defense signaling. *Curr. Opin. Plant Biol.* 10, 366–371. doi: 10.1016/j.pbi.2007.04.020

Fode, B., Siemsen, T., Thurow, C., Weigel, R., and Gatz, C. (2008). The *Arabidopsis* GRAS protein SCL14 interacts with class II TGA transcription factors and is essential for the activation of stress-inducible promoters. *Plant Cell* 20, 3122–3135. doi: 10.1105/tpc.108.058974

Fonseca, S., Fernández-Calvo, P., Fernández, G. M., Díez-Díaz, M., Gimenez-Ibanez, S., López-Vidriero, I., et al. (2014). bHLH003, bHLH013 and bHLH017 are new targets of JAZ repressors negatively regulating JA responses. *PLoS ONE* 9:e86182. doi: 10.1371/journal.pone.0086182

Frederickson, D. E., and Loake, G. J. (2014). Redox regulation in plant immune function. *Antioxid. Redox Signal.* 21, 1373–1388. doi: 10.1089/ars.2013.5679

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gao, Q.-M., Venugopal, S., Navarre, D., and Kachroo, A. (2011). Low oleic acid-derived repression of jasmonic acid-inducible defense responses requires the WRKY50 and WRKY51 proteins. *Plant Physiol.* 155, 464–476. doi: 10.1104/pp.110.166876

Gatz, C. (2013). From pioneers to team players: TGA transcription factors provide a molecular link between different stress pathways. *Mol. Plant Microbe Interact.* 26, 151–159. doi: 10.1094/MPMI-04-12-0078-IA

Gimenez-Ibanez, S., Boter, M., Fernandez-Barbero, G., Chini, A., Rathjen, J. P., and Solano, R. (2014). The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in *Arabidopsis*. *PLoS Biol.* 12:e1001792. doi: 10.1371/journal.pbio.1001792

Gimenez-Ibanez, S., and Solano, R. (2013). Nuclear jasmonate and salicylate signaling and crosstalk in defense against pathogens. *Front. Plant Sci.* 4:72. doi: 10.3389/fpls.2013.00072

Herrera-Vásquez, A., Carvallo, L., Blanco, F., Tobar, M., Villarroel-Candia, E., Vicente-Carbajosa, J., et al. (2014). Transcriptional control of glutaredoxin *GRXC9* expression by a salicylic acid-dependent and NPR1-independent pathway in *Arabidopsis*. *Plant Mol. Biol. Rep.* doi: 10.1007/s11105-014-0782-5

Higashi, K., Ishiga, Y., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. (2008). Modulation of defense signal transduction by flagellin-induced WRKY41 transcription factor in *Arabidopsis thaliana*. *Mol. Genet. Genomics* 279, 303–312. doi: 10.1007/s00438-007-0315-0

Hou, X., Lee, L. Y. C., Xia, K., Yen, Y., and Yu, H. (2010). DELLAs modulate jasmonate signaling via competitive binding to JAZs. *Dev. Cell* 19, 884–894. doi: 10.1016/j.devcel.2010.10.024

Hu, P., Zhou, W., Cheng, Z., Fan, M., Wang, L., and Xie, D. (2013). *JAV1* controls jasmonate-regulated plant defense. *Mol. Cell* 50, 504–515. doi: 10.1016/j.molcel.2013.04.027

Hu, Y., Dong, Q., and Yu, D. (2012). *Arabidopsis* WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*. *Plant Sci.* 185, 288–297. doi: 10.1016/j.plantsci.2011.12.003

Iwasaki, M., and Paszkowski, J. (2014). Epigenetic memory in plants. *EMBO J.* 33, 1987–1998. doi: 10.15252/embj.201488883

Jiang, S., Yao, J., Ma, K.-W., Zhou, H., Song, J., He, S. Y., et al. (2013). Bacterial effector activates jasmonate signaling by directly targeting JAZ transcriptional repressors. *PLoS Pathog.* 9:e1003715. doi: 10.1371/journal.ppat.1003715

Journot-Catalino, N., Somssich, I. E., Roby, D., and Kroj, T. (2006). The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana*. *Plant Cell* 18, 3289–3302. doi: 10.1105/tpc.106.044149

Kachroo, P., Kachroo, A., Lapchyk, L., Hildebrand, D., and Klessig, D. F. (2003). Restoration of defective cross talk in ssi2 mutants: role of salicylic acid, jasmonic acid, and fatty acids in SSI2-mediated signaling. *Mol. Plant Microbe Interact.* 16, 1022–1029. doi: 10.1094/MPMI.2003.16.11.1022

Kachroo, P., Shanklin, J., Shah, J., Whittle, E. J., and Klessig, D. F. (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9448–9453. doi: 10.1073/pnas.151258398

Kazan, K., and Lyons, R. (2014). Intervention of phytohormone pathways by pathogen effectors. *Plant Cell* 26, 2285–2309. doi: 10.1105/tpc.114.125419

Kazan, K., and Manners, J. M. (2013). MYC2: the master in action. *Mol. Plant* 6, 686–703. doi: 10.1093/mp/sss128

Kim, K.-C., Lai, Z., Fan, B., and Chen, Z. (2008). *Arabidopsis* WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. *Plant Cell* 20, 2357–2371. doi: 10.1105/tpc.107.055566

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F. C., Van Loon, L. C., et al. (2008a). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiol.* 147, 1358–1368. doi: 10.1104/pp.108.121392

Koornneef, A., Rindermann, K., Gatz, C., and Pieterse, C. M. J. (2008b). Histone modifications do not play a major role in salicylate-mediated suppression of jasmonate-induced *PDF1.2* gene expression. *Commun. Integr. Biol.* 1, 143–145. doi: 10.4161/cib.1.2.6997

Latzel, V., Zhang, Y., Karlsson Moritz, K., Fischer, M., and Bossdorf, O. (2012). Epigenetic variation in plant responses to defence hormones. *Ann. Bot.* 110, 1423–1428. doi: 10.1093/aob/mcs088

Leon-Reyes, A., Du, Y., Koornneef, A., Proietti, S., Körbes, A. P., Memelink, J., et al. (2010a). Ethylene signaling renders the jasmonate response of *Arabidopsis* insensitive to future suppression by salicylic acid. *Mol. Plant Microbe Interact.* 23, 187–197. doi: 10.1094/MPMI-23-2-0187

Leon-Reyes, A., Van der Does, D., De Lange, E. S., Delker, C., Wasternack, C., Van Wees, S. C. M., et al. (2010b). Salicylate-mediated suppression of jasmonate-responsive gene expression in Arabidopsis is targeted downstream of the jasmonate biosynthesis pathway. *Planta* 232, 1423–1432. doi: 10.1007/s00425-010-1265-z

Leon-Reyes, A., Spoel, S. H., De Lange, E. S., Abe, H., Kobayashi, M., Tsuda, S., et al. (2009). Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. *Plant Physiol.* 149, 1797–1809. doi: 10.1104/pp.108.133926

Li, J., Brader, G., Kariola, T., and Palva, E. T. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J.* 46, 477–491. doi: 10.1111/j.1365-313X.2006.02712.x

Li, J., Brader, G., and Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16, 319–331. doi: 10.1105/tpc.016980

Lindermayr, C., Sell, S., Müller, B., Leister, D., and Durner, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *Plant Cell* 22, 2894–2907. doi: 10.1105/tpc.109.066464

Liu, X., Yang, S., Zhao, M., Luo, M., Yu, C.-W., Chen, C.-Y., et al. (2014). Transcriptional repression by histone deacetylases in plants. *Mol. Plant* 7, 764–772. doi: 10.1093/mp/ssu033

Liu, Z.-Q., Yan, L., Wu, Z., Mei, C., Lu, K., Yu, Y.-T., et al. (2012). Cooperation of three WRKY-domain transcription factors WRKY18, WRKY40, and WRKY60 in repressing two ABA-responsive genes *ABI4* and *ABI5* in *Arabidopsis*. *J. Exp. Bot.* 63, 6371–6392. doi: 10.1093/jxb/ers293

Lorenzo, O., Chico, J. M., Sanchez-Serrano, J. J., and Solano, R. (2004). *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *Plant Cell* 16, 1938–1950. doi: 10.1105/tpc.022319

Luna, E., Bruce, T. J. A., Roberts, M. R., Flors, V., and Ton, J. (2012). Next-generation systemic acquired resistance. *Plant Physiol.* 158, 844–853. doi: 10.1104/pp.111.187468

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., and Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in *Arabidopsis*. *Plant Cell* 23, 1639–1653. doi: 10.1105/tpc.111.084996

Mao, P., Duan, M., Wei, C., and Li, Y. (2007). WRKY62 transcription factor acts downstream of cytosolic NPR1 and negatively regulates jasmonate-responsive gene expression. *Plant Cell Physiol.* 48, 833–842. doi: 10.1093/pcp/pcm058

Maruyama, Y., Yamoto, N., Suzuki, Y., Chiba, Y., Yamazaki, K.-I., Sato, T., et al. (2009). The Arabidopsis transcriptional repressor ERF9 participates in resistance against necrotrophic fungi. *Plant Sci.* 213, 79–87. doi: 10.1016/j.plantsci.2013.08.008

McGrath, K. C., Dombrecht, B., Manners, J. M., Schenk, P. M., Edgar, C. I., Maclean, D. J., et al. (2005). Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. *Plant Physiol.* 139, 949–959. doi: 10.1104/pp.105.068544

Meng, X., Xu, J., He, Y., Yang, K.-Y., Mordorski, B., Liu, Y., et al. (2013). Phosphorylation of an ERF transcription factor by *Arabidopsis* MPK3/MPK6 regulates plant defense gene induction and fungal resistance. *Plant Cell* 25, 1126–1142. doi: 10.1105/tpc.112.109074

Meng, X., and Zhang, S. (2013). MAPK cascades in plant disease resistance signaling. *Annu. Rev. Phytopathol.* 51, 245–266. doi: 10.1146/annurev-phyto-082712-102314

Moore, J. W., Loake, G. J., and Spoel, S. H. (2011). Transcription dynamics in plant immunity. *Plant Cell* 23, 2809–2820. doi: 10.1105/tpc.111.087346

Mou, Z., Fan, W. H., and Dong, X. N. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Nakano, T., Suzuki, K., Fujimura, T., and Shinshi, H. (2006). Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol.* 140, 411–432. doi: 10.1104/pp.105.073783

Nakata, M., Mitsuda, N., Koo, M. H. A. J. K., Moreno, J. E., Suzuki, K., Howe, G. A., et al. (2013). A bHLH-type transcription factor, ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR/JA-ASSOCIATED MYC2-LIKE1, acts as a repressor to negatively regulate jasmonate signaling in *Arabidopsis*. *Plant Cell* 25, 1641–1656. doi: 10.1105/tpc.113.111112

Nandi, A., Moeder, W., Kachroo, P., Klessig, D. F., and Shah, J. (2005). *Arabidopsis ssi2*-conferred susceptibility to *Botrytis cinerea* is dependent on *EDS5* and *PAD4*. *Mol. Plant Microbe Interact.* 18, 363–370. doi: 10.1094/MPMI-18-0363

Ndamukong, I., Abdallat, A. A., Thurow, C., Fode, B., Zander, M., Weigel, R., et al. (2007). SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive *PDF1.2* transcription. *Plant J.* 50, 128–139. doi: 10.1111/j.1365-313X.2007.03039.x

Pape, S., Thurow, C., and Gatz, C. (2010). The Arabidopsis *PR-1* promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI. *Plant Physiol.* 154, 1805–1818. doi: 10.1104/pp.110.165563

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., et al. (2000). *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. *Cell* 103, 1111–1120. doi: 10.1016/S0092-8674(00)00213-0

Pieterse, C. M. J., Pierik, R., and Van Wees, S. C. M. (2014). Different shades of JAZ during plant growth and defense. *New Phytol.* 204, 261–264. doi: 10.1111/nph.13029

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., et al. (2009). MAPK target networks in *Arabidopsis thaliana* revealed using functional protein microarrays. *Genes Dev.* 23, 80–92. doi: 10.1101/gad.1740009

Qiu, J.-L., Fiil, B. K., Petersen, K., Nielsen, H. B., Botanga, C. J., Thorgrimsen, S., et al. (2008). *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO J.* 27, 2214–2221. doi: 10.1038/emboj.2008.147

Ren, C.-M., Zhu, Q., Gao, B.-D., Ke, S.-Y., Yu, W.-C., Xie, D.-X., et al. (2008). Transcription factor WRKY70 displays important but no indispensable roles in jasmonate and salicylic acid signaling. *J. Integr. Plant Biol.* 50, 630–637. doi: 10.1111/j.1744-7909.2008.00653.x

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Rochon, A., Boyle, P., Wignes, T., Fobert, P. R., and Després, C. (2006). The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *Plant Cell* 18, 3670–3685. doi: 10.1105/tpc.106.046953

Sasaki-Sekimoto, Y., Jikumaru, Y., Obayashi, T., Saito, H., Masuda, S., Kamiya, Y., et al. (2013). Basic Helix-Loop-Helix transcription factors JA-ASSOCIATED MYC2-LIKE 1 (JAM1), JAM2 and JAM3 are negative regulators of jasmonate responses in Arabidopsis. *Plant Physiol.* 163, 291–234. doi: 10.1104/pp.113.220129

Shah, J., Kachroo, P., Nandi, A., and Klessig, D. F. (2001). A recessive mutation in the *Arabidopsis SSI2* gene confers SA- and *NPR1*-independent expression of *PR* genes and resistance against bacterial and oomycete pathogens. *Plant J.* 25, 563–574. doi: 10.1046/j.1365-313x.2001.00992.x

Shang, Y., Lu, Y., Liu, Z.-Q., Cao, Z., Mei, C., Xin, Q., et al. (2010). The Mg-chelatase H subunit of *Arabidopsis* antagonizes a group of WRKY transcription

repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* 22, 1909–1935. doi: 10.1105/tpc.110.073874

Shim, J. S., Jung, C., Lee, S., Min, K., Lee, Y.-W., Choi, Y., et al. (2013). *AtMYB44* regulates *WRKY70* expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. *Plant J.* 73, 483–495. doi: 10.1111/tpj.12051

Song, S., Qi, T., Fan, M., Zhang, X., Gao, H., Huang, H., et al. (2013). The bHLH subgroup IIId factors negatively regulate jasmonate-mediated plant defense and development. *PLoS Genet.* 9:e1003653. doi: 10.1371/journal.pgen.1003653

Song, S., Qi, T., Wasternack, C., and Xie, D. (2014). Jasmonate signaling and crosstalk with gibberellin and ethylene. *Curr. Opin. Plant Biol.* 21, 112–119. doi: 10.1016/j.pbi.2014.07.005

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770. doi: 10.1105/tpc.009159

Spoel, S. H., and Loake, G. J. (2011). Redox-based protein modifications: the missing link in plant immune signalling. *Curr. Opin. Plant Biol.* 14, 358–364. doi: 10.1016/j.pbi.2011.03.007

Stein, E., Molitor, A., Kogel, K. H., and Waller, F. (2008). Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol.* 49, 1747–1751. doi: 10.1093/pcp/pcn147

Ströher, E., and Millar, A. H. (2012). The biological roles of glutaredoxins. *Biochem. J.* 446, 333–348. doi: 10.1042/BJ20112131

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Takahashi, F., Yoshida, R., Ichimura, K., Mizoguchi, T., Seoe, S., Yonezawac, M., et al. (2007). The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in *Arabidopsis*. *Plant Cell* 19, 805–818. doi: 10.1105/tpc.106.046581

Tena, G., Boudsocq, M., and Sheen, J. (2011). Protein kinase signaling networks in plant innate immunity. *Curr. Opin. Plant Biol.* 14, 519–529. doi: 10.1016/j.pbi.2011.05.006

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M. C., Rodenburg, N., Pauwels, L., et al. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCF^{COI1}-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* 25, 744–761. doi: 10.1105/tpc.112.108548

Van Verk, M. C., Hickman, R., Pieterse, C. M. J., and Van Wees, S. C. M. (2013). RNA-Seq: revelation of the messengers. *Trends Plant Sci.* 18, 175–179. doi: 10.1016/j.tplants.2013.02.001

Vos, I. A., Pieterse, C. M. J., and Van Wees, S. C. M. (2013a). Costs and benefits of hormone-regulated plant defences. *Plant Pathol.* 62, 43–55. doi: 10.1111/ppa.12105

Vos, I. A., Verhage, A., Schuurink, R. C., Watt, L. G., Pieterse, C. M. J., and Van Wees, S. C. M. (2013b). Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. *Front. Plant Sci.* 4:539. doi: 10.3389/fpls.2013.00539

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2:e123. doi: 10.1371/journal.ppat.0020123

Wang, D., Pajerowska-Mukhtar, K., Hendrickson Culler, A., and Dong, X. (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. *Curr. Biol.* 17, 1784–1790. doi: 10.1016/j.cub.2007.09.025

Wasternack, C., and Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Ann. Bot.* 111, 1021–1058. doi: 10.1093/aob/mct067

Wathugala, D. L., Hemsley, P. A., Moffat, C. S., Cremelie, P., Knight, M. R., and Knight, H. (2012). The Mediator subunit SFR6/MED16 controls defence gene expression mediated by salicylic acid and jasmonate responsive pathways. *New Phytol.* 195, 217–230. doi: 10.1111/j.1469-8137.2012.04138.x

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell* 18, 1310–1326. doi: 10.1105/tpc.105.037523

Yang, D. L., Yao, J., Mei, C. S., Tong, X. H., Zeng, L. J., Li, Q., et al. (2012). Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1192–E1200. doi: 10.1073/pnas.1201616109

Yuan, Y., Zhong, S., Li, Q., Zhu, Z., Lou, Y., Wang, L., et al. (2007). Functional analysis of rice *NPR1*-like genes reveals that *OsNPR1/NH1* is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. *Plant Biotechnol. J.* 5, 313–324. doi: 10.1111/j.1467-7652.2007.00243.x

Zander, M., Chen, S., Imkampe, J., Thurow, C., and Gatz, C. (2012). Repression of the *Arabidopsis thaliana* jasmonic acid/ethylene-induced defense pathway by TGA-interacting glutaredoxins depends on their C-terminal ALWL motif. *Mol. Plant* 5, 831–840. doi: 10.1093/mp/ssr113

Zander, M., La Camera, S., Lamotte, O., Métraux, J.-P., and Gatz, C. (2010). *Arabidopsis thaliana* class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. *Plant J.* 61, 200–210. doi: 10.1111/j.1365-313X.2009.04044.x

Zander, M., Thurow, C., and Gatz, C. (2014). TGA transcription factors activate the salicylic acid-suppressible branch of the ethylene-induced defense program by regulating *ORA59* expression. *Plant Physiol.* 165, 1671–1683. doi: 10.1104/pp.114.243360

Zarei, A., Korbes, A. P., Younessi, P., Montiel, G., Champion, A., and Memelink, J. (2011). Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the *PDF1.2* promoter in Arabidopsis. *Plant Mol. Biol.* 75, 321–331. doi: 10.1007/s11103-010-9728-y

Zhang, X., Wang, C., Zhang, Y., Sun, Y., and Mou, Z. (2012a). The *Arabidopsis* mediator complex subunit16 positively regulates salicylate-mediated systemic acquired resistance and jasmonate/ethylene-induced defense pathways. *Plant Cell* 24, 4294–4309. doi: 10.1105/tpc.112.103317

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., et al. (2012b). Disruption of PAMP-induced MAP kinase cascade by a *Pseudomonas syringae* effector activates plant immunity mediated by the NB-LRR protein SUMM2. *Cell Host Microbe* 11, 253–263. doi: 10.1016/j.chom.2012.01.015

Zhang, Y. L., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of Arabidopsis transcription factors *TGA2*, *TGA5*, and *TGA6* reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653. doi: 10.1105/tpc.014894

Zheng, X.-Y., Spivey, N. W., Zeng, W., Liu, P.-P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596. doi: 10.1016/j.chom.2012.04.014

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wu, K. (2005). HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. *Plant Cell* 17, 1196–1204. doi: 10.1105/tpc.104.028514

Zhu, Z., An, F., Feng, Y., Li, P., Xue, L., Mu, A., et al. (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12539–12544. doi: 10.1073/pnas.1103959108

Zou, B., Jia, Z., Tian, S., Wang, X., Gou, Z., Lü, B., et al. (2013). AtMYB44 positively modulates disease resistance to *Pseudomonas syringae* through the salicylic acid signalling pathway in *Arabidopsis*. *Funct. Plant Biol.* 40, 304–313. doi: 10.1071/FP12253

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 19 March 2015
doi: 10.3389/fpls.2015.00171

Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression

Ariel Herrera-Vásquez†, *Paula Salinas*† and *Loreto Holuigue* *

Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

It is well established that salicylic acid (SA) plays a critical role in the transcriptional reprograming that occurs during the plant defense response against biotic and abiotic stress. In the course of the defense response, the transcription of different sets of defense genes is controlled in a spatio-temporal manner via SA-mediated mechanisms. Interestingly, different lines of evidence indicate that SA interplays with reactive oxygen species (ROS) and glutathione (GSH) in stressed plants. In this review we focus on the evidence that links SA, ROS, and GSH signals to the transcriptional control of defense genes. We discuss how redox modifications of regulators and co-regulators involved in SA-mediated transcriptional responses control the temporal patterns of gene expression in response to stress. Finally, we examine how these redox sensors are coordinated with the dynamics of cellular redox changes occurring in the defense response to biotic and abiotic stress.

Keywords: glutathione, glutaredoxin GRXC9/GRX480, NPR1, reactive oxygen species, salicylic acid, thioredoxin TRXh5, TGA transcription factors

OPEN ACCESS

Edited by:
Pietro Daniele Spanu, Imperial College London, UK

Reviewed by:
Zuhua He, Shanghai Institute for Biological Sciences – Chinese Academy of Sciences, China
Gabor Jakab, University of Pécs, Hungary

***Correspondence:**
Loreto Holuigue, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda 340, Santiago 8320000, Chile
lholuigue@bio.puc.cl

†*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *19 December 2014*
Accepted: *03 March 2015*
Published: *19 March 2015*

Citation:
Herrera-Vásquez A, Salinas P and Holuigue L (2015) Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. Front. Plant Sci. 6:171. doi: 10.3389/fpls.2015.00171

Interplay between Salicylic Acid (SA) and Redox Signals in the Defense Response to Stress

A feed-forward loop between salicylic acid (SA) and reactive oxygen species (ROS) production in the defense response to stress was first reported at the early 1990s (Chen et al., 1993). This early report was followed by a controversy on whether H_2O_2 was downstream or upstream of SA in the pathway for induction of *Pathogenesis-Related 1* (*PR1*) expression (Neuenschwander et al., 1995; Chamnongpol et al., 1996). Later on, it was demonstrated that ROS signals are involved both upstream and downstream SA signaling in response to stress. Interestingly, the evidence indicates that SA does not only play a pro-oxidant role, but it also has an antioxidant role in concert with glutathione (GSH) in the response to stress. In this first section we present a comprehensive picture of the relationships between SA, ROS, and GSH in the response to stress signaling (**Figure 1**).

ROS Bursts Trigger SA Signaling

It is well known that activation of SA signaling in stressed plants is preceded by oxidative bursts originating in different cellular compartments (Wrzaczek et al., 2013). In the case of basal (PTI) and induced (ETI) defense responses against pathogens infection, it has been extensively

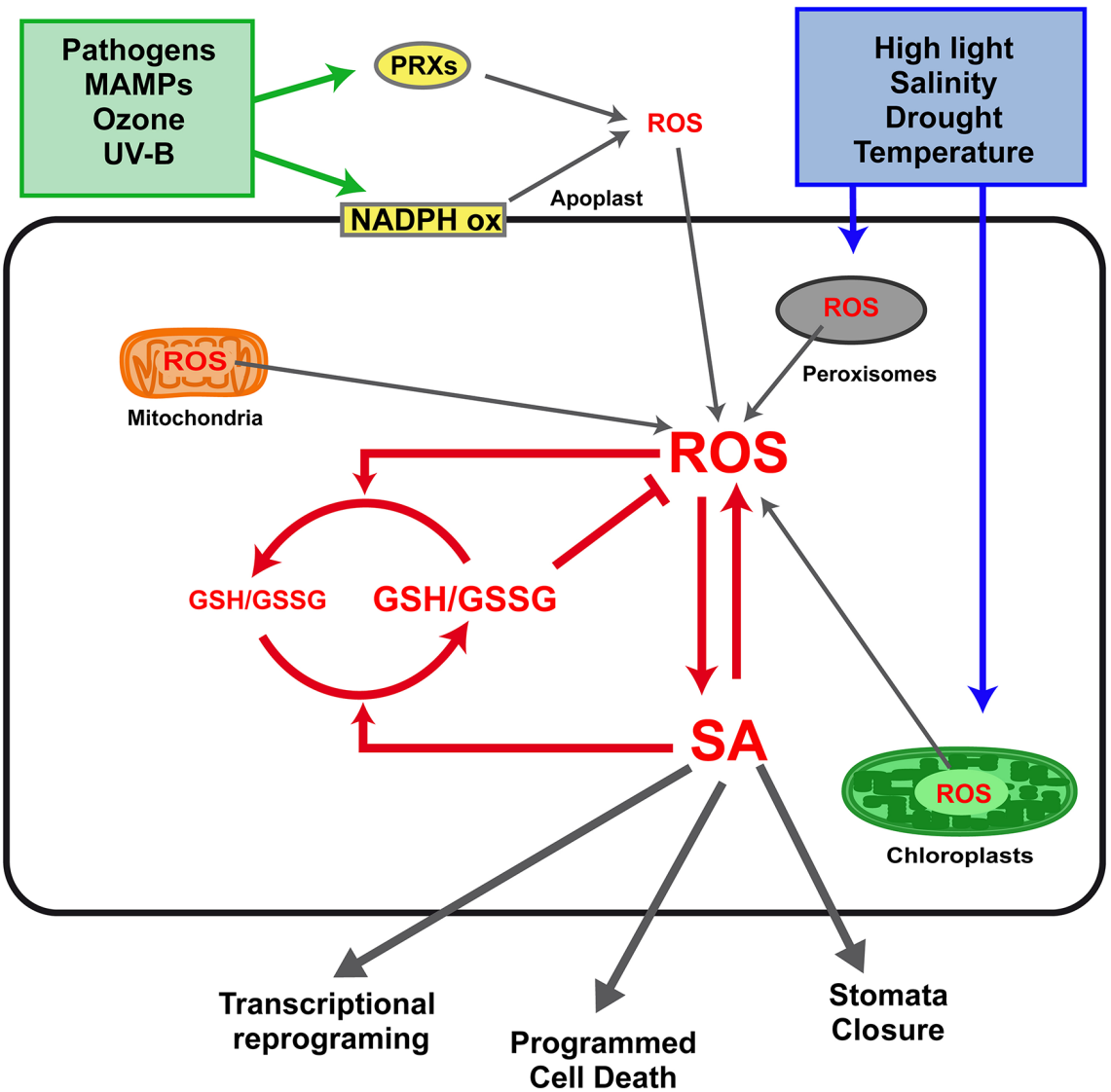


FIGURE 1 | Interplay between salicylic acid (SA), reactive oxygen species (ROS), and glutathione (GSH) in defense responses to biotic and abiotic stress. Stress conditions such as infection with pathogens, exposure to microbe-associated molecular patterns (MAMPs), ozone, and UV-B treatments, trigger ROS production mainly at the apoplast. This production of ROS is mediated by plasma membrane NADPH oxidases (NADPH ox) and cell wall peroxidases (PRXs). Other stresses, such as high light radiation, salinity, drought, and temperature, trigger ROS production mainly at the chloroplasts and peroxisomes. Mitochondria have been also described as an important source of ROS during defense responses (Lam et al., 2001). A feed-forward loop between H_2O_2 and SA synthesis occurs in response to stress, as described in the text. SA also has an antioxidant role, increasing GSH levels and reducing power, which in turn is involved in ROS scavenging. Finally, the interplay between intracellular levels of SA, H_2O_2, and GSH determines transcriptional reprogramming, programmed cell death, and stomata closure, the three main outputs of the defense responses.

reported that increases in SA levels are preceded by apoplastic H_2O_2 bursts mediated by NADPH oxidases and extracellular peroxidases (PRXs; Mackerness et al., 2001; Torres et al., 2002; Joo et al., 2005; Tsuda et al., 2008; O'Brien et al., 2012; Mammarella et al., 2014). Although PTI and ETI responses are activated in the plant by recognition of different pathogens molecules, they share several signals including ROS and SA. Differences in the timing and levels at which these signals are produced in PTI and ETI determine differences in the speed and strength at which these immune reactions are established to be effective in counteracting potential pathogens with low cost on fitness (Tsuda et al., 2008; Katagiri and Tsuda, 2010).

Apoplastic H_2O_2 bursts also precede SA signaling in plant responses to exposure to ozone and UV-B (Grant and Loake, 2000; Mackerness et al., 2001; Torres et al., 2002; Joo et al., 2005; Ogawa et al., 2007; Garcion et al., 2008; O'Brien et al., 2012). Pharmacological evidences supports that increases in apoplastic H_2O_2 levels after UV-B trigger SA biosynthesis (Mackerness et al., 2001). Noteworthy, in the case of ozone, ROS signaling starts at guard-cells chloroplasts and then it

propagates to the apoplast of neighbor cells (Joo et al., 2005).

Salicylic acid also functions as a signal of other types of abiotic stresses such as high light exposure, salinity, drought, and temperature (Mateo et al., 2006; Lee and Park, 2010; Wan et al., 2012; Miura and Tada, 2014). In contrast to the above mentioned stresses, these conditions generate ROS accumulation in chloroplasts and peroxisomes (Apel and Hirt, 2004; Holuigue et al., 2007). Although involvement of SA in these cases has been demonstrated in SA-deficient and overproducer plants (Mateo et al., 2006; Lee and Park, 2010; Wan et al., 2012; Miura and Tada, 2014), direct evidence of increased SA levels has been only reported in oat plants exposed to drought (Sánchez-Martín et al., 2014). Interestingly, increased levels of SA have been detected in plants with sustained ROS production in peroxisomes (catalase 2 knockout, *cat2*; Chaouch et al., 2010) and in chloroplast (thylakoidal ascorbate PRX gene silencing, *tAPX RNAi*; Maruta et al., 2012; Noshi et al., 2012). The evidence obtained using these models indicate that H_2O_2 originated in chloroplasts and peroxisomes triggers SA biosynthesis, which is essential for main outputs of the defense response: transcriptional reprogramming, cell death, and stomatal closure (**Figure 1**).

The mechanisms by which H_2O_2 generated in the apoplast, chloroplasts, and peroxisomes triggers SA biosynthesis remains unknown. *ICS1* and *ICS2* are the two *Arabidopsis* genes coding for isochorismate synthase, the key enzyme controlling SA biosynthesis (Garcion et al., 2008). *ICS1* upregulation was detected in the ETI response to pathogens, in response to UV-B, ozone, and drought stress (Wildermuth et al., 2001; Ogawa et al., 2007; Zhang et al., 2010; Wan et al., 2012), as well as in *cat2* plants (Chaouch et al., 2010). In contrast, upregulation of *ICS2* but not of *ICS1* was detected in *tAPX RNAi* plants (Noshi et al., 2012). Transcription factors that regulate *ICS1* expression, such as CBP60, SARD1, and WRKY8/28/48 (Zhang et al., 2010; van Verk et al., 2011; Gao et al., 2013), or upstream *PAD4/EDS1* genes expression, such as CAMTA3/SR1 and ZAT6 (Du et al., 2009; Shi et al., 2014) represent potential candidates for ROS-mediated regulation of SA biosynthesis.

Remarkably, it has recently been proposed that Ca^{+2} signaling regulate SA production (Seyfferth and Tsuda, 2014), based on evidence that the activity of CBP60, WRKY8/28/48, and CAMTA3/SR1 factors are modulated by calcium dependent protein kinases (CDPKs) and calmodulin (CaM; Du et al., 2009; Gao et al., 2013; Truman et al., 2013). Indeed, intracellular increase of cytosolic Ca^{+2} was first described as an upstream signal that controls apoplastic ROS production through the modification of NADPH oxidase by CDPKs (Dubiella et al., 2013; Gao et al., 2013). Recently, Ca^{+2} has been also proposed to act downstream ROS signaling (Wrzaczek et al., 2013), based on previous evidence that exogenous treatments with H_2O_2 promote Ca^{+2} influxes (Price et al., 1994; Pei et al., 2000). Therefore, the possibility that a Ca^{+2} signal mediates activation of SA production triggered by ROS, represents an interesting aspect to explore.

SA Modulates Redox Homeostasis

An ambivalent effect of SA in promoting ROS accumulation (prooxidant) and ROS scavenging (antioxidant), has being reported in several stress models, including the ETI response to pathogens and responses to high light, drought, salinity, and cold stress (Mou et al., 2003; Mateo et al., 2006; Miura and Tada, 2014). On one hand, SA promotes ROS production during early events of signaling, being these ROS essential for defense responses (Garreton et al., 2002; Lee et al., 2010; Khokon et al., 2011). Furthermore, high concentrations of SA (>100 μM) promote ROS production, inducing oxidative stress, and reducing tolerance to drought and salinity (Lee et al., 2010; Miura and Tada, 2014). How can SA promote ROS accumulation? Early reports showed SA-mediated inhibition of catalase and cytosolic ascorbate PRX, two main H_2O_2 detoxifying enzymes (Chen et al., 1993; Durner and Klessig, 1995). Then, SA-promoted production of ROS by extracellular PRXs was identified in stomatal closure control in drought response (Khokon et al., 2011; Miura et al., 2013).

On the contrary, the available evidence supports that SA promotes ROS scavenging being essential for the antioxidant response that constrains ROS bursts in responses to avirulent bacteria (Grant and Loake, 2000), high light (Mateo et al., 2006), ozone (Yoshida et al., 2009), salinity (Lee et al., 2010), and in *cat2* mutants (Chaouch et al., 2010). Recent studies show that SA and GSH interplay as redox signals, fostering a role for SA in the antioxidant response (Dubreuil-Maurizi et al., 2011; Foyer and Noctor, 2011; Dubreuil-Maurizi and Poinssot, 2012; Han et al., 2013). Plants that over accumulate SA show increased GSH levels and reducing power (ratio GSH/GSSG; Mateo et al., 2006) while abolishment of SA accumulation in a *cat2* background (*cat2 sid2*) reduces the GSH/GSSG ratio (Chaouch et al., 2010; Noctor et al., 2014). Conversely, plants deficient in GSH biosynthesis (phytoalexin-deficient mutant, *pad2-1*) have decreased levels of SA and *ICS1* transcripts (Dubreuil-Maurizi et al., 2011). This suggests that SA can play an antioxidant role by modulating GSH levels and reducing power (**Figure 1**), through still unknown mechanisms.

The dual redox effect of SA is reflected by a biphasic redox dynamics in plants treated with SA or INA (Mou et al., 2003; Mateo et al., 2006). A first oxidative phase, characterized by a transient increase in ROS levels and decline in GSH reducing power, is followed by a reductive phase characterized by an increase in GSH levels and reducing power. This temporal dynamics determines a sequential activation of the redox-regulated processes involved in the transcription of defense genes.

Redox-Modulated Processes in the SA-Mediated Control of Gene Expression

Salicylic acid plays a pivotal role in the genetic reprogramming, being responsible for transcriptional control of 100s of defense genes that are sequentially turned on/off (Maleck et al., 2000; Wang et al., 2006; Blanco et al., 2009). Interestingly, several redox-regulated processes have been discovered in the transcription of SA-regulated genes (Mou et al., 2003; Koornneef et al., 2008; Tada et al., 2008). The evidence suggests that

cellular redox changes occurring in response to stress are translated into transcriptional responses, through redox modifications of master regulators and co-regulators (Moore et al., 2011). Here, we focus in the redox-modulated processes mediated by SA that control the expression of three *Arabidopsis* model genes: *PR1*, *GRXC9* (*glutaredoxin C9* or *GRX480*), and *ORA59* (*Octadecanoid-Responsive AP2/ERF domain protein 59*; **Figure 2**). These genes have been studied in greater detail and they respond to SA with particular temporal patterns and mechanisms, being therefore good models for different classes of SA-regulated genes.

Members of the TGA and WRKY transcription factor families, that recognize the TGA box (TGACGTCA) and the W box (TTGACT), respectively, have been involved in SA-mediated

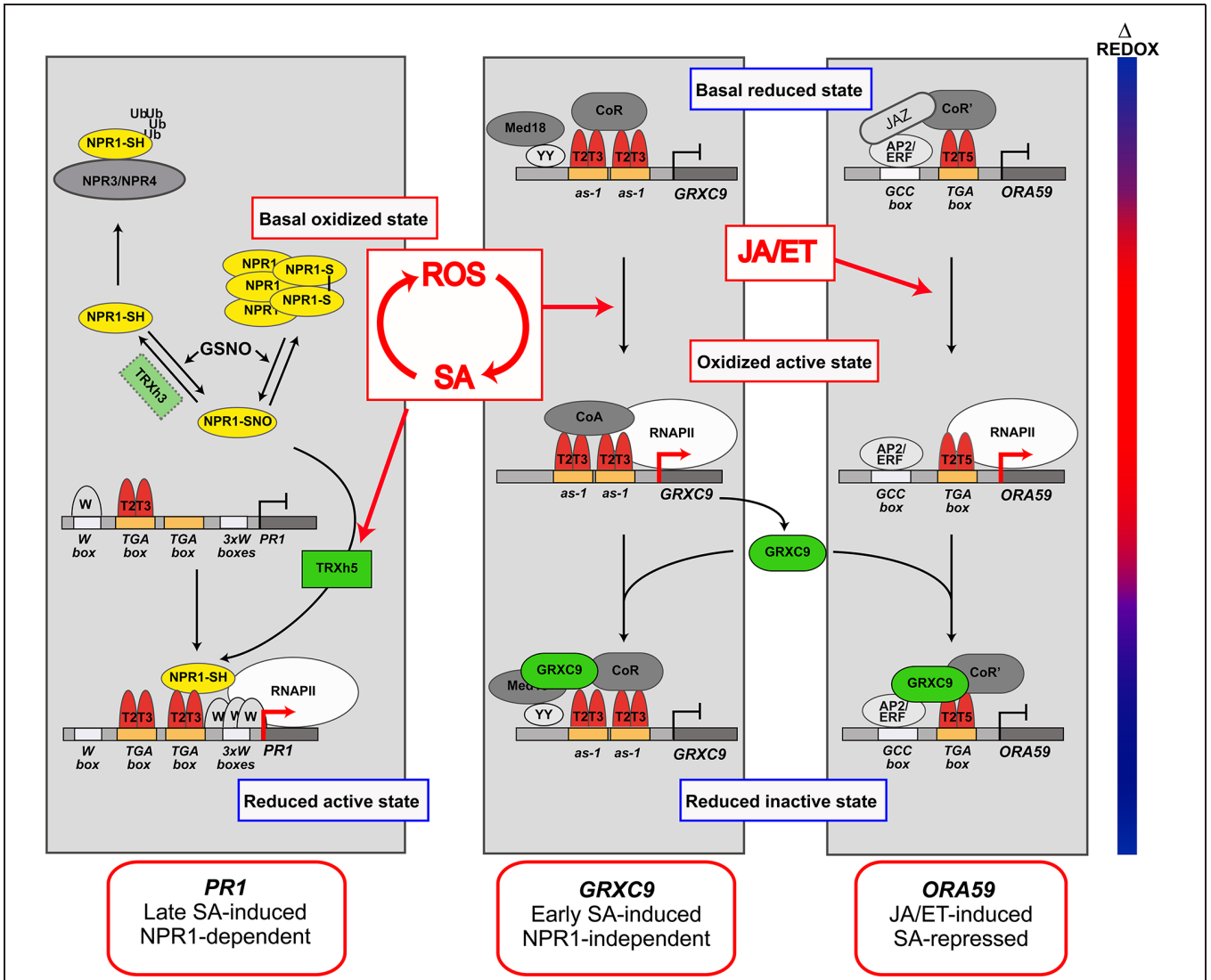


FIGURE 2 | Redox-modulated processes in the SA-mediated control of gene expression. Model for the transcriptional control of genes representing three main groups of SA-regulated genes: SA-induced non-expressor of pathogenesis-related (PR) genes 1 (NPR1) -dependent late genes (*PR1*, **Left**); SA-induced NPR1-independent early genes [*glutaredoxin C9* (*GRXC9*), **Medium**]; and JA/ET-induced SA-repressed genes [*Octadecanoid-Responsive AP2/ERF domain protein 59* (*ORA59*), **Right**]. The temporal dynamics of the redox changes (Δ Redox) occurring during the defense response to stress are represented by the bar at the left, where blue indicates reductive states and red indicates oxidative states. The temporal dynamics in the formation of transcriptionally active and inactive complexes in the promoter of *PR1*, *GRXC9*, and *ORA59*, according to redox changes dynamics, are included in each panel. The places where ROS/SA, and JA/ET signals act in these pathways, is indicated by red arrows. The components identified (or suspected) as redox sensors in these pathways, whose mechanisms of action are discussed in the text, are indicated in color. TGA factors (red) are involved in the three pathways. Homodimers or heterodimers of TGA2 and TGA3 (T2T3) or TGA2 and TGA5 (T2T5) factors act as platforms for the formation of transcriptionally inactive and active complexes. Active complexes promote recruitment of RNA polymerase II (RNAPII) and gene transcription (red arrows at promoters). NPR1 (yellow) is the master co-activator for SA-inducible NPR1-dependent pathway and is redox-regulated by oxido-reduction of Cys residues. TRXh5 and GRXC9 (green) are oxidoreductases coded by SA-inducible genes, which catalyze reduction of NPR1 and of a still unknown component in *GRXC9* and *ORA59* promoters. Other transcriptional factors and co-factors not directly involved in redox regulation are shown in gray tones.

transcriptional regulation (Pandey and Somssich, 2009; Gatz, 2013). Furthermore, co-regulators including non-expressor of *PR* genes 1 (NPR1), SCL14, and Med 18 control transcription of different groups of SA-regulated genes (Fu and Dong, 2013). In this second section we will focus our attention on NPR1, TGA factors, and two oxidoreductases, to discuss evidence that point them as redox sensors in the expression of SA-regulated genes (**Figure 2**).

Non-Expressor of *PR Genes* 1 (NPR1), a Master Redox Sensor

Non-Expressor of *PR* genes is the master co-activator for *PR1* and most SA-induced genes, and was the first redox sensor described for SA-regulated genes (Mou et al., 2003). Particularly at the *PR1* promoter, SA stimulates NPR1 interaction with TGA2 and TGA3, which enhances its binding to TGA boxes, forming a *trans*-activating complex for RNA polymerase II (RNAPII) recruitment (**Figure 2**, left panel; Lebel et al., 1998; Kesarwani et al., 2007; Pape et al., 2010). Current knowledge indicates that SA-promoted redox modification of Cys residues in NPR1 determines the levels of the active, reduced, and monomeric form of NPR1 in the nucleus (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2008; Lindermayr et al., 2010). The levels of nuclear NPR1 are also regulated by other SA-mediated mechanisms, such as proteasome-mediated degradation and phosphorylation (Pajerowska-Mukhtar et al., 2013).

Salicylic acid is essential for NPR1 redox modification, but how it controls this process is still not well understood. NPR1 reduction is catalyzed by thioredoxin TRXh5 (Tada et al., 2008; Kneeshaw et al., 2014), coded by the only member of *TRXh* gene class transcriptionally induced by SA and oxidative stress (Laloi et al., 2004; Tada et al., 2008; Belin et al., 2014). Whether NPR1 monomerization also occurs under oxidative stress, has not been explored yet. Furthermore, evidence indicates that both, oligomerization and monomerization of NPR1 involves *S*-nitrosoglutathione (GSNO) mediated *S*-nitrosylation (Feechan et al., 2005; Rusterucci et al., 2007; Lindermayr et al., 2010).

Non-Expressor of *PR* genes 1 reduction and therefore induction of NPR1-dependent genes, including *WRKYs* and *PR1*, correlate with the reductive phase of the defense response (Mou et al., 2003). Based on the evidence summarized here, we propose a model for SA-mediated NPR1 redox control and its influence on *PR1* induction (**Figure 2**, left panel).

Interestingly, the discovery of the direct binding of SA to NPR1 (Wu et al., 2012), and also to NPR3, and NPR4, which control NPR1 degradation (Fu et al., 2012), suggests the existence of a direct mechanism by which nuclear NPR1 levels and activity can be regulated according to the levels of SA, that in turn reflects the cellular redox state.

TGA Factors, a Potential Node for Integrative Cellular Redox Regulation?

TGA factors have been postulated as redox sensors (Spoel and Loake, 2011), based on evidence showing that modification of Cys residues in TGA1 and TGA4 modulate their binding to NPR1 and to DNA (Despres et al., 2003; Lindermayr et al., 2010). TGA1 and TGA4 compose class I TGA and their function is not critical for the expression of SA-regulated genes (Kesarwani et al., 2007; Shearer et al., 2012; Wang and Fobert, 2013; Herrera-Vásquez et al., 2014). In contrast, the evidence supports that class II TGAs (TGA2, TGA5, and TGA6), and to a lesser extent TGA3, are the essential factors for SA-regulated expression of defense genes (Johnson et al., 2003; Zhang et al., 2003; Kesarwani et al., 2007; Herrera-Vásquez et al., 2014). Intriguingly, there is still no direct evidence of regulation of these factors through redox modification. Nevertheless, a potential for TGA2/5/6 as a node for general redox regulation in response to stress, is supported by the evidence described below.

TGA2 represses *PR1* basal expression but can also activate it upon SA-mediated stress challenge by interacting with negative and positive TGA boxes at the *PR1* promoter (Johnson et al., 2003; Zhang et al., 2003; Kesarwani et al., 2007; Pape et al., 2010). The essential role of TGA2/5/6 in *PR1* expression can be extrapolated to the group of NPR1-dependent genes with overrepresentation of the TGA box (Maleck et al., 2000).

We have shown that TGA2/5/6 are also essential for early SA-dependent and NPR1-independent induction of a set of genes with antioxidant and detoxifying activities (Blanco et al., 2009). *GRXC9*, which codes for a glutaredoxin of the plant-specific CC subfamily, is used here as a model for this pathway (**Figure 2**, medium panel; Ndamukong et al., 2007; Blanco et al., 2009; Herrera-Vásquez et al., 2014). SA-induced expression of *GRXC9* requires two *as-1* promoter elements that constitutively bind TGA2 and TGA3 factors (Herrera-Vásquez et al., 2014). *as-1* elements, consisting of two TGA boxes separated by four base pairs (Krawczyk et al., 2002), confer early and transient induction by SA through ROS (Qin et al., 1994; Johnson et al., 2001; Garreton et al., 2002). Two *as-1* elements were also found in the *TRXh5* promoter, although its functionality has not been explored yet (Laloi et al., 2004). We propose that early induction of *GRXC9*, and probably of *TRXh5* also, occurs during the oxidative phase of the defense response mediated by ROS signals (**Figure 2**; Mou et al., 2003; Herrera-Vásquez et al., 2014). TGA2/5/6 are also essential for the induction of *as-1*-containing genes involved in chemical detoxification (Mueller et al., 2008; Stotz et al., 2013).

Furthermore, the well-recognized antagonistic effect of SA on JA/ET-mediated transcriptional responses (Pieterse et al., 2009), is also mediated by class II TGAs (Ndamukong et al., 2007; Zander et al., 2010). SA inhibits expression of a group of JA/ET-induced genes, including *PDF1.2*, through repression of *ORA59*, which codes for a master transcription factor from the AP2/ERF family (Zander et al., 2010; Van der Does et al., 2013). Interestingly, ACC-induced and SA-repressed *ORA59* expression depend of TGAs class II factors, through their binding to a TGA box present in the *ORA59* promoter (**Figure 2**, right panel; Zander et al., 2014). Kinetic and pharmacological studies indicate that SA suppresses JA-responsive genes only within a specific time frame requiring SA-mediated increase in GSH levels (Koornneef et al., 2008). Therefore, SA-mediated *ORA59* repression occurs in the reductive phase of the defense response, which is consistent with evidence indicating that NPR1 is required for SA-mediated repression of JA/ET-induced genes (Spoel, 2003).

Taken together, we can conclude that class II TGAs (particularly TGA2) are essential in different mechanisms of transcriptional control mediated by SA and ROS signals, which

operate at different times in the defense response to stress (**Figure 2**). Accordingly, a strong phenotype of stress sensitivity is detected in *tga2/5/6* triple mutant plants (Zhang et al., 2003; Mueller et al., 2008). The question is how TGA2 activity is controlled by SA and ROS signals? The only clue for a redox control of TGA2 is that it interacts with GRXC9 in the nucleus (Ndamukong et al., 2007). Interestingly, *GRXC9* overexpression represses the expression of its own gene and of *ORA59* while GRXC9 forms part of the complex bound to the *as-1*-containing region of the *GRXC9* promoter (Herrera-Vásquez et al., 2014; Zander et al., 2014). These findings are integrated in the model shown in **Figure 2**. This model shows that SA, by inducing expression of *GRXC9*, controls the expression of antioxidant genes and at the same time represses JA/ET-mediated responses. We speculate that GRXC9 catalyzes the reduction of a protein from the transactivating complex in both genes, triggering their inactivation. Although evidence for functional associations of TGA factors and CC-type GRXs suggests that TGAs can be redox-modified (Murmu et al., 2010), there is still no evidence of this modification.

Oxidoreductases as Redox Sensors in the SA-Mediated Control of Gene Expression

The involvement of TRX/GRX oxidoreductases in SA-mediated transcription was first proposed some years ago (Fobert and Despres, 2005). As described above, two Cys-containing oxidoreductases, TRXh5, and GRXC9, were later on recognized as important elements for redox control in SA-mediated transcriptional responses. *TRXh5* and *GRXC9* genes are induced by SA during the oxidative phase of the defense response. TRXh5 reduces NPR1, which is essential for NPR1-dependent transcriptional responses (Tada et al., 2008). Instead, GRXC9 probably reduces a still unknown protein that represses the expression of genes from SA-dependent NPR1-independent as well as JA/ET-dependent SA-repressed pathways. These processes occur during the reductive phase of the defense response (**Figure 2**). Considering that TRXh5 and GRXC9 are in turn reduced and regenerated at the expense of the reducing power of NADPH and GSH, respectively (Meyer et al., 2012), these enzymes become key redox sensors that coordinate transcription and the cellular redox state.

Conclusion and Future Directions

The evidence discussed here indicates that redox-modulated processes are critical for the fine-tune regulation of gene expression mediated by SA. These processes occur in a temporaly controlled manner, coordinated with the cellular redox changes occurring during the defense response. Although important advances have occurred during the last years, we still have a fragmented knowledge of the network of redox processes that allows a coordinated transcriptional response to stressful conditions. Focusing on SA–ROS interplay, one important challenge is to understand how ROS generated in different cell compartments and cell types triggers SA biosynthesis. Furthermore, considering that all stress conditions generate oxidative bursts, but not all lead to SA accumulation, how is the specificity of ROS signals for triggering SA biosynthesis established? A point of convergence of the responses to different stresses mediated by SA, such as the PAD4/EDS1/SAG101 complex located upstream in the SA-signaling pathway (Wiermer et al., 2005), can be explored as a node for redox regulation of SA biosynthesis in response to stress.

In relation to the redox mechanisms that control the SA-mediated transcriptional response, the evidence discussed here supports the involvement of NPR1, TGA factors, and the oxidoreductases TRXh5 and GRXC9 as redox sensors. Several intriguing aspects about these sensors are pending, such as the promiscuous and essential role of TGA2 in the control of genes that respond to oxidant and reducing cellular redox states. Whether TGA2 itself can be redox modified, particularly reduced by GRXC9 to trigger gene repression during the reductive phase of the defense response, is a critical point that still needs to be answered. In this context, an interesting target to explore for redox regulation is MED18. The MED18 protein is a member of the Mediator Complex that interacts with the Ying Yang 1 transcription factor (YY1; Lai et al., 2014). This complex co-represses three genes coding for oxidoreductases involved in defense: GRXC9, TRXh5, and GRXS13 (La Camera et al., 2011; Laporte et al., 2012).

Finally, an important challenge for the future is to incorporate the temporal and spatial perspective in the analysis of the redox processes associated to transcriptional activity. New technical approaches that allow to record cell-specific changes in ROS levels, the redox state of GSH and new markers for gene expression will help in unraveling the sequential events occurring in different groups of cells exposed to stress during the time course of the defense response.

Acknowledgments

This work was supported by the National Commission for Science and Technology CONICYT (FONDECYT grant N° 1141202) and the Millennium Science Initiative (Nucleus for Plant Functional Genomics, grant N° P10-062-F).

References

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399. doi: 10.1146/annurev.arplant.55.031903.141701

Belin, C., Bashandy, T., Cela, J., Delorme-Hinoux, V., Riondet, C., and Reichheld, J. P. (2014). A comprehensive study of thiol reduction gene expression under stress conditions in *Arabidopsis thaliana*. *Plant Cell Environ.* 38, 299–314. doi: 10.1111/pce.12276

Blanco, F., Salinas, P., Cecchini, N. M., Jordana, X., Van Hummelen, P., Alvarez, M. E., et al. (2009). Early genomic responses to salicylic acid in *Arabidopsis*. *Plant Mol. Biol.* 70, 79–102. doi: 10.1007/s11103-009-9458-9451

Chamnongpol, S., Willekens, H., Langebartels, C., Van Montagu, M., Inzé, D., and Van Camp, W. (1996). Transgenic tobacco with a reduced catalase activity

develops necrotic lesions and induces pathogenesis-related expression under high light. *Plant J.* 10, 491–503. doi: 10.1046/j.1365-313X.1996.10030491.x

Chaouch, S., Queval, G., Vanderauwera, S., Mhamdi, A., Vandorpe, M., Langlois-Meurinne, M., et al. (2010). Peroxisomal hydrogen peroxide is coupled to biotic defense responses by ISOCHORISMATE SYNTHASE1 in a daylength-related manner. *Plant Physiol.* 153, 1692–1705. doi: 10.1104/pp.110.153957

Chen, Z., Silva, H., and Klessig, D. F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262, 1883–1886. doi: 10.1126/science.8266079

Despres, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., et al. (2003). The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. *Plant Cell* 15, 2181–2191. doi: 10.1105/tpc.012849

Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S., et al. (2009). Ca(2+)/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* 457, 1154–1158. doi: 10.1038/nature07612

Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C. P., et al. (2013). Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8744–8749. doi: 10.1073/pnas.1221294110

Dubreuil-Maurizi, C., and Poinssot, B. (2012). Role of glutathione in plant signaling under biotic stress. *Plant Signal. Behav.* 7, 210–212. doi: 10.4161/psb.18831

Dubreuil-Maurizi, C., Vitecek, J., Marty, L., Branciard, L., Frettinger, P., Wendehenne, D., et al. (2011). Glutathione deficiency of the *Arabidopsis* mutant pad2-1 affects oxidative stress-related events, defense gene expression, and the hypersensitive response. *Plant Physiol.* 157, 2000–2012. doi: 10.1104/pp.111.182667

Durner, J., and Klessig, D. F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 92, 11312–11316.

Feechan, A., Kwon, E., Yun, B. W., Wang, Y., Pallas, J. A., and Loake, G. J. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8054–8059. doi: 10.1073/pnas.0501456102

Fobert, P. R., and Despres, C. (2005). Redox control of systemic acquired resistance. *Curr. Opin. Plant Biol.* 8, 378–382. doi: 10.1016/j.pbi.2005.05.003

Foyer, C. H., and Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* 155, 2–18. doi: 10.1104/pp.110.167569

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gao, X., Chen, X., Lin, W., Chen, S., Lu, D., Niu, Y., et al. (2013). Bifurcation of *Arabidopsis* NLR immune signaling via Ca(2)(+)-dependent protein kinases. *PLoS Pathog.* 9:e1003127. doi: 10.1371/journal.ppat.1003127

Garcion, C., Lohmann, A., Lamodiere, E., Catinot, J., Buchala, A., Doermann, P., et al. (2008). Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of *Arabidopsis*. *Plant Physiol.* 147, 1279–1287. doi: 10.1104/pp.108.119420

Garreton, V., Carpinelli, J., Jordana, X., and Holuigue, L. (2002). The as-1 promoter element is an oxidative stress-responsive element and salicylic acid activates it via oxidative species. *Plant Physiol.* 130, 1516–1526. doi: 10.1104/pp.009886

Gatz, C. (2013). From pioneers to team players: TGA transcription factors provide a molecular link between different stress pathways. *Mol. Plant Microbe Interact.* 26, 151–159. doi: 10.1094/MPMI-04-12-0078-IA

Grant, J. J., and Loake, G. J. (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol.* 124, 21–29. doi: 10.1104/pp.124.1.21

Han, Y., Chaouch, S., Mhamdi, A., Queval, G., Zechmann, B., and Noctor, G. (2013). Functional analysis of *Arabidopsis* mutants points to novel roles for glutathione in coupling H(2)O(2) to activation of salicylic acid accumulation and signaling. *Antioxid. Redox Signal.* 18, 2106–2121. doi: 10.1089/ars.2012.5052

Herrera-Vásquez, A., Carvallo, L., Blanco, F., Tobar, M., Villarroel-Candia, E., Vicente-Carbajosa, J., et al. (2014). Transcriptional control of glutaredoxin *GRXC9* expression by a salicylic acid-dependent and NPR1-independent pathway in *Arabidopsis*. *Plant Mol. Biol. Rep.* doi: 10.1007/s11105-014-0782-5

Holuigue, L., Salinas, P., Blanco, F., and Garreton, V. (2007). "Salicylic acid and reactive oxygen species in the activation of stress defense genes," in *Salicylic Acid – A Plant Hormone,* eds S. Hayat and A. Ahmad (Dordrecht: Springer Science & Business Media), 197–246.

Johnson, C., Boden, E., and Arias, J. (2003). Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in *Arabidopsis*. *Plant Cell* 15, 1846–1858. doi: 10.1105/tpc.012211

Johnson, C., Glover, G., and Arias, J. (2001). Regulation of DNA binding and trans-activation by a xenobiotic stress-activated plant transcription factor. *J. Biol. Chem.* 276, 172–178. doi: 10.1074/jbc.M005143200

Joo, J. H., Wang, S., Chen, J. G., Jones, A. M., and Fedoroff, N. V. (2005). Different signaling and cell death roles of heterotrimeric G protein alpha and beta subunits in the *Arabidopsis* oxidative stress response to ozone. *Plant Cell* 17, 957–970. doi: 10.1105/tpc.104.029603

Katagiri, F., and Tsuda, K. (2010). Understanding the plant immune system. *Mol. Plant Microbe Interact.* 23, 1531–1536. doi: 10.1094/MPMI-04-10-0099

Kesarwani, M., Yoo, J., and Dong, X. (2007). Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in *Arabidopsis*. *Plant Physiol.* 144, 336–346. doi: 10.1104/pp.106.095299

Khokon, A. R., Okuma, E., Hossain, M. A., Munemasa, S., Uraji, M., Nakamura, Y., et al. (2011). Involvement of extracellular oxidative burst in salicylic acid-induced stomatal closure in *Arabidopsis*. *Plant Cell Environ.* 34, 434–443. doi: 10.1111/j.1365-3040.2010.02253.x

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.1105/tpc.12.12.2339

Kneeshaw, S., Gelineau, S., Tada, Y., Loake, G. J., and Spoel, S. H. (2014). Selective protein denitrosylation activity of Thioredoxin-h5 modulates plant Immunity. *Mol. Cell* 56, 153–162. doi: 10.1016/j.molcel.2014.08.003

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F. C., Van Loon, L. C., et al. (2008). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiol.* 147, 1358–1368. doi: 10.1104/pp.108.121392

Krawczyk, S., Thurow, C., Niggeweg, R., and Gatz, C. (2002). Analysis of the spacing between the two palindromes of activation sequence-1 with respect to binding to different TGA factors and transcriptional activation potential. *Nucleic Acids Res.* 30, 775–781. doi: 10.1093/nar/30.3.775

La Camera, S., L'haridon, F., Astier, J., Zander, M., Abou-Mansour, E., Page, G., et al. (2011). The glutaredoxin ATGRXS13 is required to facilitate *Botrytis cinerea* infection of *Arabidopsis thaliana* plants. *Plant J.* 68, 507–519. doi: 10.1111/j.1365-313X.2011.04706.x

Lai, Z., Schluttenhofer, C. M., Bhide, K., Shreve, J., Thimmapuram, J., Lee, S. Y., et al. (2014). MED18 interaction with distinct transcription factors regulates multiple plant functions. *Nat. Commun.* 5, 3064. doi: 10.1038/ncomms4064

Laloi, C., Mestres-Ortega, D., Marco, Y., Meyer, Y., and Reichheld, J. P. (2004). The *Arabidopsis* cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. *Plant Physiol.* 134, 1006–1016. doi: 10.1104/pp.103.035782

Lam, E., Kato, N., and Lawton, M. (2001). Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* 411, 848–853. doi: 10.1038/35081184

Laporte, D., Olate, E., Salinas, P., Salazar, M., Jordana, X., and Holuigue, L. (2012). Glutaredoxin GRXS13 plays a key role in protection against photooxidative stress in *Arabidopsis*. *J. Exp. Bot.* 63, 503–515. doi: 10.1093/jxb/err301

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. (1998). Functional analysis of regulatory sequences controlling PR-1 gene expression in *Arabidopsis*. *Plant J.* 16, 223–233. doi: 10.1046/j.1365-313x.1998.00288.x

Lee, S., Kim, S. G., and Park, C. M. (2010). Salicylic acid promotes seed germination under high salinity by modulating antioxidant activity in *Arabidopsis*. *New Phytol.* 188, 626–637. doi: 10.1111/j.1469-8137.2010.03378.x

Lee, S., and Park, C. M. (2010). Modulation of reactive oxygen species by salicylic acid in *Arabidopsis* seed germination under high salinity. *Plant Signal. Behav.* 5, 1534–1536. doi: 10.4161/psb.5.12.13159

Lindermayr, C., Sell, S., Muller, B., Leister, D., and Durner, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *Plant Cell* 22, 2894–2907. doi: 10.1105/tpc.109.066464

Mackerness, S., John, C. F., Jordan, B., and Thomas, B. (2001). Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. *FEBS Lett.* 489, 237–242. doi: 10.1016/S0014-5793(01)02103-2

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K. A., et al. (2000). The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* 26, 403–410. doi: 10.1038/82521

Mammarella, N. D., Cheng, Z., Fu, Z. Q., Daudi, A., Bolwell, G. P., Dong, X., et al. (2014). Apoplastic peroxidases are required for salicylic acid-mediated defense against Pseudomonas syringae. *Phytochemistry* doi: 10.1016/j.phytochem.2014.07.010 [Epub ahead of print].

Maruta, T., Noshi, M., Tanouchi, A., Tamoi, M., Yabuta, Y., Yoshimura, K., et al. (2012). H_2O_2-triggered retrograde signaling from chloroplasts to nucleus plays specific role in response to stress. *J. Biol. Chem.* 287, 11717–11729. doi: 10.1074/jbc.M111.292847

Mateo, A., Funck, D., Muhlenbock, P., Kular, B., Mullineaux, P. M., and Karpinski, S. (2006). Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. *J. Exp. Bot.* 57, 1795–1807. doi: 10.1093/jxb/erj196

Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, J. P., and Riondet, C. (2012). Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. *Antioxid. Redox Signal.* 17, 1124–1160. doi: 10.1089/ars.2011.4327

Miura, K., Okamoto, H., Okuma, E., Shiba, H., Kamada, H., Hasegawa, P. M., et al. (2013). SIZ1 deficiency causes reduced stomatal aperture and enhanced drought tolerance via controlling salicylic acid-induced accumulation of reactive oxygen species in *Arabidopsis*. *Plant J.* 73, 91–104. doi: 10.1111/tpj.12014

Miura, K., and Tada, Y. (2014). Regulation of water, salinity, and cold stress responses by salicylic acid. *Front. Plant Sci.* 5:4. doi: 10.3389/fpls.2014.00004

Moore, J. W., Loake, G. J., and Spoel, S. H. (2011). Transcription dynamics in plant immunity. *Plant Cell* 23, 2809–2820. doi: 10.1105/tpc.111.087346

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/s0092-8674(03)00429-x

Mueller, S., Hilbert, B., Dueckershoff, K., Roitsch, T., Krischke, M., Mueller, M. J., et al. (2008). General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in *Arabidopsis*. *Plant Cell* 20, 768–785. doi: 10.1105/tpc.107.054809

Murmu, J., Bush, M. J., Delong, C., Li, S., Xu, M., Khan, M., et al. (2010). *Arabidopsis* basic leucine-zipper transcription factors TGA9 and TGA10 interact with floral glutaredoxins ROXY1 and ROXY2 and are redundantly required for anther development. *Plant Physiol.* 154, 1492–1504. doi: 10.1104/pp.110.159111

Ndamukong, I., Abdallat, A. A., Thurow, C., Fode, B., Zander, M., Weigel, R., et al. (2007). SA-inducible *Arabidopsis* glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. *Plant J.* 50, 128–139. doi: 10.1111/j.1365-313X.2007.03039.x

Neuenschwander, U., Vernooij, B., Friedrich, L., Uknes, S., Kessmann, H., and Ryals, J. (1995). Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? *Plant J.* 8, 227–233. doi: 10.1046/j.1365-313X.1995.08020227.x

Noctor, G., Lelarge-Trouverie, C., and Mhamdi, A. (2014). The metabolomics of oxidative stress. *Phytochemistry* doi: 10.1016/j.phytochem.2014.09.002 [Epub ahead of print].

Noshi, M., Maruta, T., and Shigeoka, S. (2012). Relationship between chloroplastic H2O2 and the salicylic acid response. *Plant Signal. Behav.* 7, 944–946. doi: 10.4161/psb.20906

O'Brien, J. A., Daudi, A., Finch, P., Butt, V. S., Whitelegge, J. P., Souda, P., et al. (2012). A peroxidase-dependent apoplastic oxidative burst in cultured *Arabidopsis* cells functions in MAMP-elicited defense. *Plant Physiol.* 158, 2013–2027. doi: 10.1104/pp.111.190140

Ogawa, D., Nakajima, N., Tamaoki, M., Aono, M., Kubo, A., Kamada, H., et al. (2007). The isochorismate pathway is negatively regulated by salicylic acid signaling in O3-exposed *Arabidopsis*. *Planta* 226, 1277–1285. doi: 10.1007/s00425-007-0556-555

Pajerowska-Mukhtar, K. M., Emerine, D. K., and Mukhtar, M. S. (2013). Tell me more: roles of NPRs in plant immunity. *Trends Plant Sci.* 18, 402–411. doi: 10.1016/j.tplants.2013.04.004

Pandey, S. P., and Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* 150, 1648–1655. doi: 10.1104/pp.109.138990

Pape, S., Thurow, C., and Gatz, C. (2010). The *Arabidopsis* PR-1 promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI1. *Plant Physiol.* 154, 1805–1818. doi: 10.1104/pp.110.165563

Pei, Z. M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G. J., et al. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406, 731–734. doi: 10.1038/35021067

Pieterse, C. M., Leon-Reyes, A., Van Der Ent, S., and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Price, A. H., Taylor, A., Ripley, S. J., Griffiths, A., Trewavas, A. J., and Knight, M. R. (1994). Oxidative signals in tobacco increase cytosolic calcium. *Plant Cell* 6, 1301–1310. doi: 10.1105/tpc.6.9.1301

Qin, X. F., Holuigue, L., Horvath, D. M., and Chua, N. H. (1994). Immediate early transcription activation by salicylic acid via the cauliflower mosaic virus as-1 element. *Plant Cell* 6, 863–874. doi: 10.1105/tpc.6.6.863

Rusterucci, C., Espunya, M. C., Diaz, M., Chabannes, M., and Martinez, M. C. (2007). S-nitrosoglutathione reductase affords protection against pathogens in *Arabidopsis*, both locally and systemically. *Plant Physiol.* 143, 1282–1292. doi: 10.1104/pp.106.091686

Sánchez-Martín, J., Heald, J. I. M., Kingston-Smith, A., Winters, A. N. A., Rubiales, D., Sanz, M., et al. (2014). A metabolomic study in oats (*Avena sativa*) highlights a drought tolerance mechanism based on salicylate signalling pathways and the modulation of carbon, antioxidant and photo-oxidative metabolism. *Plant Cell Environ.* doi: 10.1111/pce.12501 [Epub ahead of print].

Seyfferth, C., and Tsuda, K. (2014). Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. *Front. Plant Sci.* 5:697. doi: 10.3389/fpls.2014.00697

Shearer, H. L., Cheng, Y. T., Wang, L., Liu, J., Boyle, P., Despres, C., et al. (2012). *Arabidopsis* clade I TGA transcription factors regulate plant defenses in an NPR1-independent fashion. *Mol. Plant Microbe Interact.* 25, 1459–1468. doi: 10.1094/MPMI-09-11-0256

Shi, H., Wang, X., Ye, T., Chen, F., Deng, J., Yang, P., et al. (2014). The Cysteine2/Histidine2-Type Transcription Factor ZINC FINGER OF *Arabidopsis thaliana*6 Modulates Biotic and Abiotic Stress Responses by Activating Salicylic Acid-Related Genes and C-REPEAT-BINDING FACTOR Genes in *Arabidopsis*. *Plant Physiol.* 165, 1367–1379. doi: 10.1104/pp.114.242404

Spoel, S. H. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770. doi: 10.1105/tpc.009159

Spoel, S. H., and Loake, G. J. (2011). Redox-based protein modifications: the missing link in plant immune signalling. *Curr. Opin. Plant Biol.* 14, 358–364. doi: 10.1016/j.pbi.2011.03.007

Stotz, H. U., Mueller, S., Zoeller, M., Mueller, M. J., and Berger, S. (2013). TGA transcription factors and jasmonate-independent COI1 signalling regulate specific plant responses to reactive oxylipins. *J. Exp. Bot.* 64, 963–975. doi: 10.1093/jxb/ers389

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Torres, M. A., Dangl, J. L., and Jones, J. D. (2002). *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl. Acad. Sci. U.S.A.* 99, 517–522. doi: 10.1073/pnas.012452499

Truman, W., Sreekanta, S., Lu, Y., Bethke, G., Tsuda, K., Katagiri, F., et al. (2013). The CALMODULIN-BINDING PROTEIN60 family includes both negative and positive regulators of plant immunity. *Plant Physiol.* 163, 1741–1751. doi: 10.1104/pp.113.227108

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J.* 53, 763–775. doi: 10.1111/j.1365-313X.2007.03369.x

Van der Does, D., Leon-Reyes, A., Koornneef, A., van Verk, M. C., Rodenburg, N., Pauwels, L., et al. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* 25, 744–761. doi: 10.1105/tpc.112.108548

van Verk, M. C., Bol, J. F., and Linthorst, H. J. (2011). WRKY transcription factors involved in activation of SA biosynthesis genes. *BMC Plant Biol.* 11:89. doi: 10.1186/1471-2229-11-89

Wan, D., Li, R., Zou, B., Zhang, X., Cong, J., Wang, R., et al. (2012). Calmodulin-binding protein CBP60g is a positive regulator of both disease resistance and drought tolerance in *Arabidopsis*. *Plant Cell Rep.* 31, 1269–1281. doi: 10.1007/s00299-012-1247-7

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2:e123. doi: 10.1371/journal.ppat.0020123

Wang, L., and Fobert, P. R. (2013). *Arabidopsis* clade I TGA factors regulate apoplastic defences against the bacterial pathogen Pseudomonas syringae through endoplasmic reticulum-based processes. *PLoS ONE* 8:e77378. doi: 10.1371/journal.pone.0077378

Wiermer, M., Feys, B. J., and Parker, J. E. (2005). Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* 8, 383–389. doi: 10.1016/j.pbi.2005.05.010

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Wrzaczek, M., Brosche, M., and Kangasjarvi, J. (2013). ROS signaling loops – production, perception, regulation. *Curr. Opin. Plant Biol.* 16, 575–582. doi: 10.1016/j.pbi.2013.07.002

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Yoshida, S., Tamaoki, M., Ioki, M., Ogawa, D., Sato, Y., Aono, M., et al. (2009). Ethylene and salicylic acid control glutathione biosynthesis in ozone-exposed *Arabidopsis thaliana*. *Physiol. Plant.* 136, 284–298. doi: 10.1111/j.1399-3054.2009.01220.x

Zander, M., La Camera, S., Lamotte, O., Metraux, J. P., and Gatz, C. (2010). *Arabidopsis thaliana* class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. *Plant J.* 61, 200–210. doi: 10.1111/j.1365-313X.2009.04044.x

Zander, M., Thurow, C., and Gatz, C. (2014). TGA transcription factors activate the salicylic acid-suppressible branch of the ethylene-induced defense program by regulating ORA59 expression. *Plant Physiol.* 165, 1671–1683. doi: 10.1104/pp.114.243360

Zhang, Y., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653. doi: 10.1105/tpc.014894

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y. T., He, J., et al. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18220–18225. doi: 10.1073/pnas.1005225107

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 18 May 2015
doi: 10.3389/fpls.2015.00327

Dimerization and thiol sensitivity of the salicylic acid binding thimet oligopeptidases TOP1 and TOP2 define their functions in *redox*-sensitive cellular pathways

Timothy J. Westlake[1,2], *William A. Ricci*[3], *George V. Popescu*[1,4*] and *Sorina C. Popescu*[1,2*]

[1] The Boyce Thompson Institute for Plant Research, Ithaca, NY, USA, [2] Department of Plant Pathology and Plant Microbe Biology, Cornell University, Ithaca, NY, USA, [3] Department of Biology, West Chester University of Pennsylvania, West Chester, PA, USA, [4] National Institute for Lasers, Plasma and Radiation Physics, Bucharest, Romania

OPEN ACCESS

Edited by:
Hua Lu,
University of Maryland Baltimore, USA

Reviewed by:
Graham Noctor,
Université Paris-Sud, France
Zhonglin Mou,
University of Florida, USA

***Correspondence:**
George V. Popescu,
National Institute for Lasers, Plasma and Radiation Physics, Str. Atomistilor, Nr. 409, 077125 Magurele, Romania
george.popescu@inflpr.ro;
Sorina C. Popescu,
Boyce Thompson Institute for Plant Research, Tower Rd., Ithaca, NY 14830, USA
scp78@cornell.edu

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: 18 December 2014
Accepted: 27 April 2015
Published: 18 May 2015

Citation:
Westlake TJ, Ricci WA, Popescu GV and Popescu SC (2015) Dimerization and thiol sensitivity of the salicylic acid binding thimet oligopeptidases TOP1 and TOP2 define their functions in redox-sensitive cellular pathways. Front. Plant Sci. 6:327. doi: 10.3389/fpls.2015.00327

A long-term goal in plant research is to understand how plants integrate signals from multiple environmental stressors. The importance of salicylic acid (SA) in plant response to biotic and abiotic stress is known, yet the molecular details of the SA-mediated pathways are insufficiently understood. Our recent work identified the peptidases *TOP1* and *TOP2* as critical components in plant response to pathogens and programmed cell death (PCD). In this study, we investigated the characteristics of *TOPs* related to the regulation of their enzymatic activity and function in oxidative stress response. We determined that TOP1 and TOP2 interact with themselves and each other and their ability to associate in dimers is influenced by SA and the thiol-based reductant DTT. Biochemical characterization of TOP1 and TOP2 indicated distinct sensitivities to DTT and similarly robust activity under a range of pH values. Treatments of *top* mutants with Methyl Viologen (MV) revealed *TOP1* and *TOP2* as a modulators of the plant tolerance to MV, and that exogenous SA alleviates the toxicity of MV in *top* background. Finally, we generated a TOP-centered computational model of a plant cell whose simulation outputs replicate experimental findings and predict novel functions of TOP1 and TOP2. Altogether, our work indicates that *TOP1* and *TOP2* mediate plant responses to oxidative stress through spatially separated pathways and positions proteolysis in a network for plant response to diverse stressors.

Keywords: thimet oligopeptidase, salicylic acid, redox potential, systems model, oxidative stress

Introduction

Plants are dynamic living systems wherein external and internal signals induce changes over time. Plant cells decode signals from varied and often concurrent stressors in order to mount appropriate defenses. Salicylic acid (SA) is a small phenolic molecule with hormonal properties that plays critical roles in plant stress response to biotic and abiotic factors (Rivas-San Vicente and Plasencia, 2011; Denancé et al., 2013). The discovery of SA-binding proteins revealed that SA-mediated signaling and perception involves interactions of SA with multiple protein

partners (An and Mou, 2011; Moreau et al., 2013). The apparent complexity of SA-mediated immune pathways in regards to the number and regulatory mechanisms of participating components, is evidenced by the diversity of cellular and plant-level physiological outcomes that include oxidative bursts, programmed cell death (PCD), and local and systemic pathogen resistance. Therefore, integrative approaches that merge experimental and analytical approaches applied to the study of SA cellular pathways would be invaluable in uncovering both the mechanistic details of cellular elements or processes under SA regulation and the general rules that govern the functioning of immune pathways and plants response to environment.

Previously, we used a protein microarray screen to identify two SA-binding proteins—the thimet oligopeptidases TOP1 and TOP2 classified in as putative zinc- and thiol-dependent endopeptidases based on homology to the metazoan counterpart (Moreau et al., 2013). Biochemical assays verified that both TOP1 and TOP2 bind SA with distinct affinities and revealed that SA inhibits non-competitively TOP1 and TOP2's peptidase activities (Moreau et al., 2013). *In vitro* biochemical evidence suggests that TOP1 is a component of organellar proteolytic machinery; TOP1 is predicted to participate in the degradation of imported proteins' signal sequences and potentially to play a broader role in general organellar peptide degradation (Kmiec et al., 2013; Moreau et al., 2013). Conversely, *TOP2* encodes a cytosolic peptidase; biochemical evidence implicates TOP2 in the proteolytic machinery downstream of the 26S proteasome and TOP2 was hypothesized to prevent the accumulation of free peptides generated from oxidative stress (Polge et al., 2009). The potential functions of plant TOPs in the regulated proteolysis mirrors those of the metazoan TOP which plays active roles in controlling the accumulation of bioactive neuropeptides, hormones, and antigenic peptides (Chu and Orlowski, 1985; York et al., 2003; Shivakumar et al., 2005). Our previous work determined that *TOP1* and *TOP2* are components of the immune response (Moreau et al., 2013). Altered expression of *TOPs* inhibited plant response to pathogens that induce effector-triggered immunity and the development of pathogen-triggered PCD. Further exploration into stress-related functions of *TOPs* established that *TOP1* and *TOP2* are necessary for plant response to high concentrations of exogenous SA (Moreau et al., 2013), and brought forth new questions about TOPs enzymatic characteristics and their specific roles in the oxidative stress response.

Controlled oxidative bursts—characterized by rapid accumulation of reactive oxygen species (ROS) in the apoplast, cytosol, and organelles—are a common characteristic of cellular stress caused by biotic and some abiotic factors (Wrzaczek et al., 2013). In both dicots and monocots, SA influences the accumulation of ROS during stress response and consequently, cell survival. SA-mediated oxidative and reductive bursts can lead to *redox*-based modifications of sensors which are proteins with higher chemical reactivity whose location and ionic state render them sensitive to oxidation by ROS (Mou et al., 2003). Interestingly, metazoan TOPs are described as thiol-dependent peptidases since their activity is markedly altered by thiols such as dithiothreitol (DTT) (Tisljar and Barrett, 1990) and are considered as likely participants in the cellular *redox* reactions where thiols are part of the antioxidant defense and signaling processes (Ferreira et al., 2013). Currently, the identity of plant *redox* sensors and the mechanistic basis of the complex relationship that exists between SA and ROS homeostasis remain largely unknown (Foyer and Noctor, 2013).

To understand how multiple cellular components cooperate and influence each other to generate appropriate physiological outputs to pathogen infection or environmental stress, it is critical to establish a platform for the system-level study of plant stress response. Computational models that represent the known structure and dynamics of stress-related pathways could uncover novel relationships and facilitate a predictive understanding of plants at molecular level. The complementarity of *TOP1*-/*TOP2*-mediated stress responses, their distinct spatial localization, levels of expression, and enzymatic activity, makes the computational study of their system-level dynamics necessary and potentially insightful for unraveling the complexity of SA pathways.

In this study, we describe novel functions of *TOP1* and *TOP2* and characterize aspects of TOP peptidases that may relate to their cellular regulation by SA and the reduction-oxidation cellular environment in the context of plant response to oxidative stress.

Results

TOP1 and TOP2 Form Dimers in Plant Cells

We examined the propensity of TOP1 and TOP2 to assemble in homo- and hetero-dimers using two distinct methods. First, we utilized a split-luciferase complementation assay (SLCA) that allowed for observation of protein-protein interactions within the context of the plant cell environment (Fujikawa and Kato, 2007). *Arabidopsis* protoplasts were prepared and co-transformed with pairs of plasmid constructs containing the coding sequences of *TOP1* or *TOP2*, cloned in frame with either the N-terminal or the C-terminal halves of the *Renilla* luciferase coding sequence (TOP1- or TOP2-NLuc and TOP1- or TOP2-CLuc). Interactions between TOPs-Luc fusions were detected by measuring luminescence released upon the restoration of luciferase enzymatic activity. Protoplasts expressing luciferase terminal fusions with known interactors (MKK5 and HOPF2) (Wang et al., 2010), constituted the positive interaction control, while protoplasts expressing TOP1-Luc or TOP2-Luc in pairs with non-interacting proteins constituted the negative controls (**Figures 1A–D**). We found that the protoplasts expressing various combinations of TOP1-Luc and TOP2-Luc fusions exhibited significantly greater luminescence intensities than those of the negative control; furthermore, the luminescence intensity of the TOP2-TOP2 interaction was significantly greater (3.4-fold higher in average) than that of TOP1-TOP1 or TOP1-TOP2—both of which showed similar levels of luminescence intensity (**Figure 1E**). The ability of TOP2 and TOP1 to bind to each other suggests that the binding sites of the two peptidases are conserved. TOP1 and TOP2 are homologs with a high degree of similarity; although their N-termini differ with

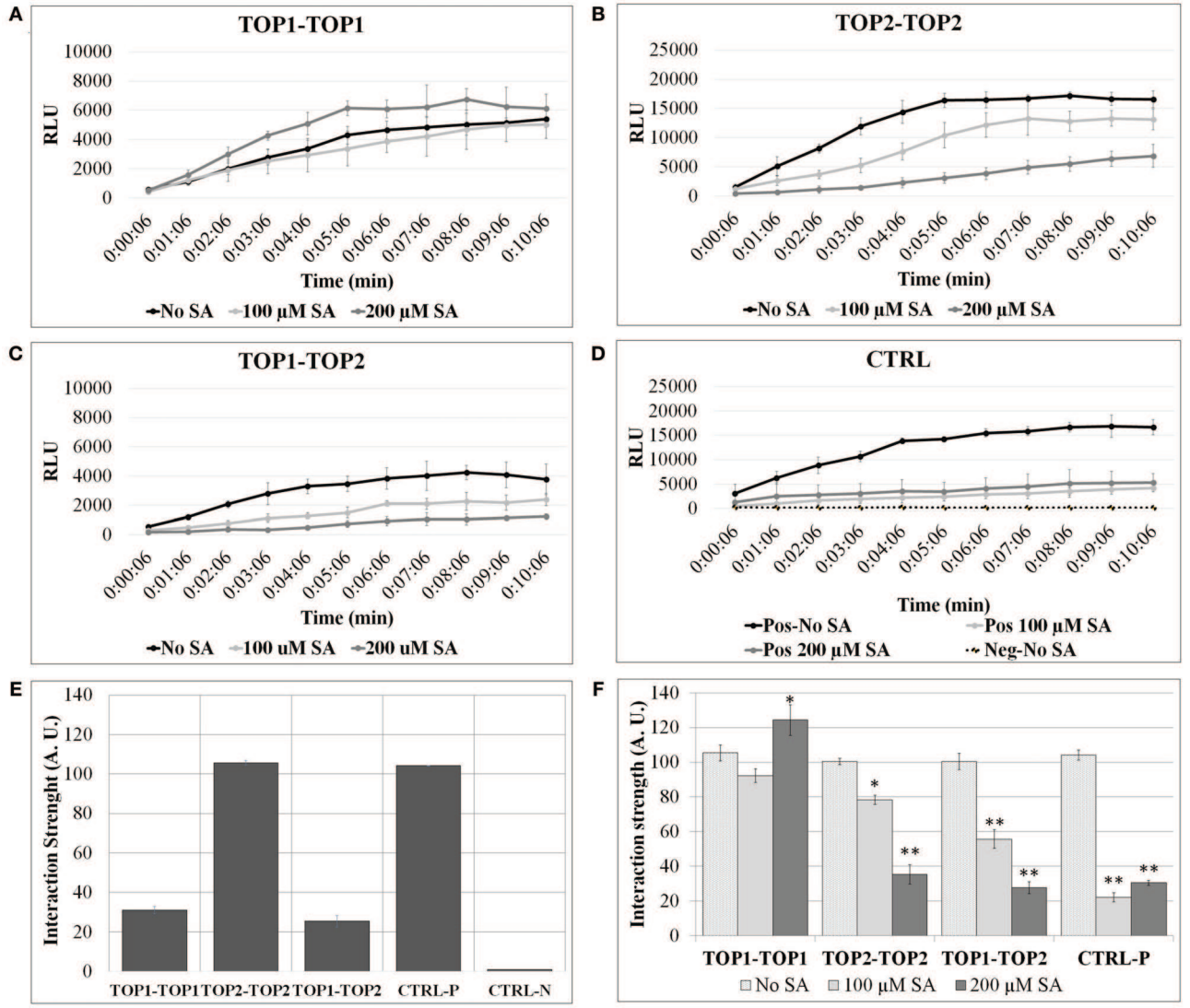


FIGURE 1 | TOP1 and TOP2 dimerize in a SA concentration-dependent manner. Split-luciferase assays were performed in *Arabidopsis thaliana* protoplasts in the presence or absence of SA. Detection of relative luminesce units (RFU) emitted was used to assess potential binding of the interacting pairs. **(A)** The relative luminescence of TOP1-TOP1 interactions over time in the presence or absence of SA. **(B)** The relative luminescence of TOP2-TOP2 interactions over time in the presence or absence of SA. **(C)** The relative luminescence of TOP1-TOP2 interactions in the presence or absence of SA. **(D)** The relative luminescence of control interacting partners (MKK5 and HopF2) in the presence or absence of SA. **(E)** TOP1 and TOP2 form homo- and hetero-dimers *in vivo*. Bars represent the normalized interaction strength, measured as *Renilla* luciferase intensity values normalized to a positive interaction set of proteins (Ctrl-P). **(F)** The interactions between TOP1 and TOP2 are modulated by salicylic acid (SA). Bars show interaction strength in SA and non-SA conditions. For both (**A,B**), the luciferase intensity values measured in the 07:06 and 10:06 min time interval after adding the luciferase substrate, were used to calculate the relative interaction strength of the TOP interactions (A.U. are arbitrary units); all intensity values were normalized to the values at 07:06 min time point for the CTRL-P protein pair and averaged for plotting; the error bars are standard deviations calculated relative to the negative control (CTRL-N) in **(A)**, and relative to the "no-SA" condition for each protein pair tested in **(B)**. Asterisks represent statistical significance (Student's *T*-test) (*$p < 0.05$ and **$p < 0.01$) calculated from 3 to 6 replicates per protein pair tested.

respect to the transit peptide in TOP1, the overall TOP1-TOP2 similarity is approximately 93%. Despite distinct patterns of subcellular localization, it is possible that TOP1 in transit to the organelles binds to TOP2 and the interaction may have a functional significance. Overall, we conclude that both TOP1 and TOP2 have the capacity to dimerize, albeit with distinct affinities.

TOP1 and TOP2 Form Transient Monomers and Dimers

To further investigate the dimerization potential of TOPs, we used gel filtration chromatography that separates proteins on the basis of mass. We first developed a uniform purification procedure for TOPs using His-tagging; full length *TOP1-His,* and *TOP2-His* were expressed in in the *E. coli* BL21 strain and total

lysates were run through a His-tag cobalt column to separate TOP1-His and TOP2-His protein preparation which were then chromatographed through a Superdex 200 gel filtration column.

Each of the proteins was eluted with two distinct peaks from the gel filtration columns. Protein fractions spanning the elution profiles from both preparations were analyzed by SDS/PAGE and Coomassie staining; TOP1-His and TOP2-His were detected only in the fractions corresponding to the major peaks in the elution profiles (**Figures 2A,B**). The apparent MW of the elution peaks was estimated by interpolation using the elution profile of conalbumin and aldolase. With the shorter-retention time elution peak, both TOP1-His and TOP2-His eluted at a volumes with approximate MWs 2-fold higher that their actual MWs; by contrast, with the longer retention time peak TOP1-His and TOP2-His eluted at their actual MW. We concluded that the two peaks correspond to the dimeric and monomeric forms, respectively, of TOP1 and TOP2. Based on the total absorption units of each peak, we calculated that the dimer:monomer ratio was approximately 1:3 in the case of each protein, indicating that under the experimental conditions used, the monomeric forms were favored.

Our results indicate that both TOP1 and TOP2 exist as both monomers and dimers; in the case of both TOPs, the monomers are more abundant than dimers in solution.

SA and Dithiothreitol Influence the Dimer-Monomer Balance of TOP1 and TOP2

Next, we were interested to investigate a possible functional relevance of the observed TOP dimerization. First, we tested the effect of SA on TOPs homodimerization. SA concentrations and incubation times used in our assays were optimized to maintain protoplast viability. Concentrations of SA beyond 200 μM resulted in significant protoplast lysis during incubation and the lethal effects of SA amounts beyond 500 μM were readily apparent even when the incubation time was shorter. Extending incubation time to over 3 h, in the presence of 100, 200 μM SA or higher SA concentrations, caused significant cell death (data not presented).

To test SA's effects on TOPs interactions, protoplasts expressing various pairs of TOP-Luc fusions were incubated with 100 or 200 μM SA for 3 h and restoration of luciferase activity was measured over time in increments of 1 min. SA treatment significantly lowered the intensity of the reconstituted luciferase in the case of TOP2-TOP2 and TOP1-TOP2 interactions compared to the no-SA condition (**Figure 1F**). The SA-dependent decrease in the luciferase activity occurred in a concentration dependent manner; 100 μM SA reduced luciferase intensity of TOP2-Luc dimers by approximately 30% and of TOP1-TOP2 dimers by 50%, while 200 μM SA reduced it by 75 and 80%, respectively. On the other hand, SA had a much reduced effect on TOP1-Luc dimerization compared to TOP2-TOP2 and TOP1-TOP2. While 100 μM SA did not significantly impair TOP1 interactions, 200 μM SA slightly increased the luciferase intensity of TOP1-Luc over the no-SA condition within the time interval with the maximum interaction strength. We can't preclude the possibility that the null/low SA sensitivity of the TOP1 dimer in this system is a result of its localization in chloroplasts and mitochondria or that the exogenous SA influences the cytosolic TOP1 in transit to the chloroplasts. The amount of the exogenously applied SA that may be transported into the organelles within our experimental timeframe is unknown; it may be that SA does not accumulate to

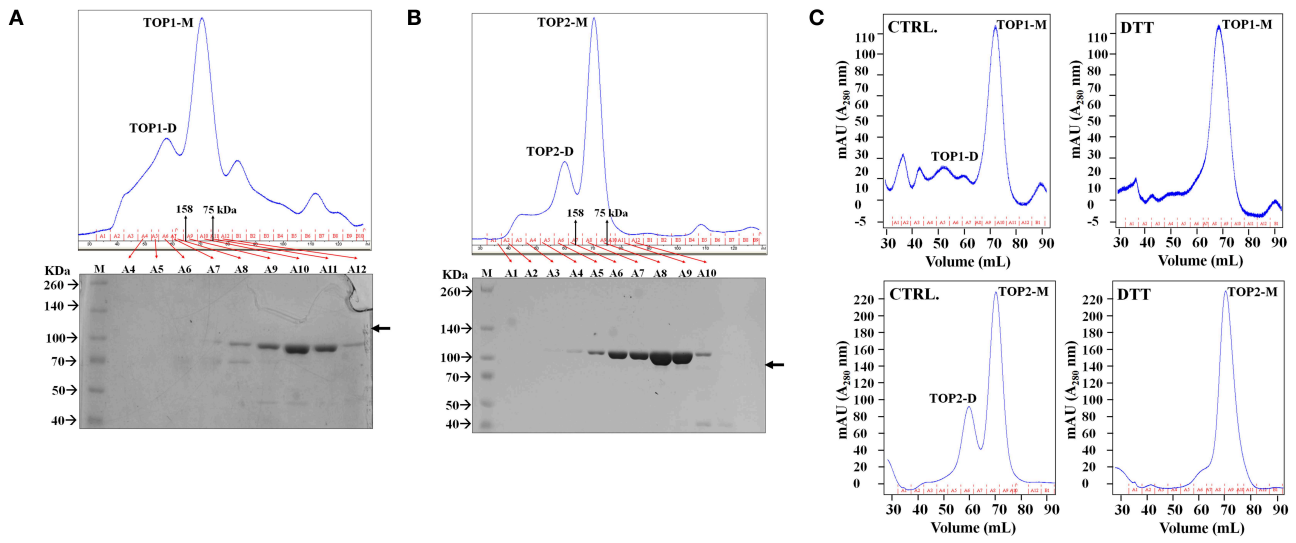


FIGURE 2 | TOPs Gel Filtration Elution Profile. Purified bacterially expressed TOP1 and TOP2 are subjected to size exclusion chromatography. **(A)** TOP1 obtained from cobalt His-tagged purification was subjected to size-exclusion chromatography. The elution profile in tandem with SDS PAGE of the fractions indicate that TOP1 elutes primarily at two different sizes corresponding to the sizes of the monomer and dimer. **(B)** Size-exclusion elution profile of TOP2 with corresponding fractions subjected to SDS-PAGE indicate that TOP2 elutes at two peaks corresponding with the dimer and monomer size. **(C)** The elution profile of TOP2 in the absence or after incubation with of dithiothreitol.

a threshold high enough to elicit an effect on TOP1 dimerization. The intensity of protoplasts expressing the positive interaction pair was decreased to similar levels (75%) in presence of 100 or 200 μM SA compared to the no-SA control. We conclude that TOP1 *in vivo* dimerization is more resistant to exogenous SA than TOP2 dimerization and both were more resistant than the control interaction pair.

The above results suggest that SA-induced shifts in the reducing-oxidative (*redox*) environment of the protoplasts might interfere with TOP monomer↔dimer shifts. To test the possibility of potential *redox* modulation of TOPs, we investigated the effects of dithiothreitol (DTT) which is a strong thiol-based reductant capable of modulating the activity of many *redox*-sensitive proteins (Cleland, 1964). Purified TOP1- and TOP2-His were incubated with 500 μM dithiothreitol (DTT) and passed through the Superdex 200 gel filtration column. TOPs elution profiles showed a dramatic shift toward the monomeric fractions—the approximate dimer:monomer ratio shifted to 1:15, with the second peak corresponding to the dimer being eliminated almost completely after incubation with the thiol-based reductant (**Figure 2C**). By comparing the total absorbance (A280) intensities of the monomer and dimer peaks before and after DTT treatment, we found that the total amount of TOP1 or TOP2 did not change after incubation with DTT, suggesting that the reduced amount of the dimer was a result of the reduction of *redox*-sensitive disulfide bonding. We conclude that thiol-based reducing conditions have the ability to directly modulate TOP dimerization, reductive conditions facilitating an increase in the monomer/dimer ratio.

Altogether, our results suggest that variations in *redox* conditions alter TOPs monomer/dimer ratio through, possibly, disruption of disulfide bonds.

Dithiothreitol Inhibits the Enzymatic Activity of TOP1 and TOP2

Our results so far indicate that TOP1 and TOP2 monomers and dimers co-exist in an approximately 3:1 equilibrium under physiological conditions and that the monomer/dimer ratio is modulated by the thiol-based reductant DTT. We were interested to investigate the peptidase activity of TOP1 and TOP2 monomeric and dimeric fractions and the potential effect of thiols on their activity.

The activity of freshly eluted TOP1-His and TOP2-His monomers and dimers was tested on a fluorogenic peptide substrate (Moreau et al., 2013) in the absence (control) or in the presence of increasing concentrations of DTT (reductive environment). Testing both the monomeric and dimeric fraction allowed us to assess the monomer↔dimer dynamics under a range of reductive conditions. TOP1 dimeric and monomeric fractions under control conditions reached the same level of activity after 10 min; however, their specific activities differed significantly over the 25 min recorded, with the dimers showing lower activity values than the monomers. Addition of 50 or 250 μM DTT inhibited the activity of TOP1 fractions up to 50% and notably, TOP1 dimers retain the lower average activity than the monomers at both DTT concentrations (**Figures 3A,B,G**). It may be that the inhibitory effect of the initially dimer TOP1 fraction in tandem with the inhibitory effects of DTT results in less substrate cleaved over the incubation time.

TOP2 monomers and dimers exhibited similar levels of activity under both control and reductive conditions, suggesting that unlike TOP1, TOP2 more readily achieves the monomer:dimer 3:1 equilibrium in non-reducing or reducing conditions and that the retained difference is a product of the initial inhibitory effect of the dimer on the enzyme activity in tandem with the inhibition by DTT. As with TOP1, both TOP2 monomeric and dimeric fractions were inhibited by the thiol-based reductant; TOP2 fractions maintained a higher level of activity at both 50 and 250 μM DTT—93 and 66%, respectively (**Figures 3D,E,G**). Further, to test TOP1 and TOP2 activity in a highly reductive environment, monomeric preparations were tested with 5 and 10 mM DTT. TOP1 maintained 10 and 7% activity, respectively, and TOP2 retained 33 and 18% activity respectively, compared to controls (**Figures 3C,F,G**). The difference in activity between the DTT treated monomer and untreated monomer indicates that potential intramolecular bonds may be affected by the reducing agent.

We conclude that in a thiol-driven reductive environment the activities of both TOPs are inhibited, both of the dimeric and monomeric fractions. Our data suggests that potential thiol-sensitive intra- and inter-molecular disulfide bonds in TOPs are critical for both the activity and the monomer-dimer oscillations of the proteins.

TOP1 and TOP2 Maintain their Enzymatic Activity in a Wide Range of pH

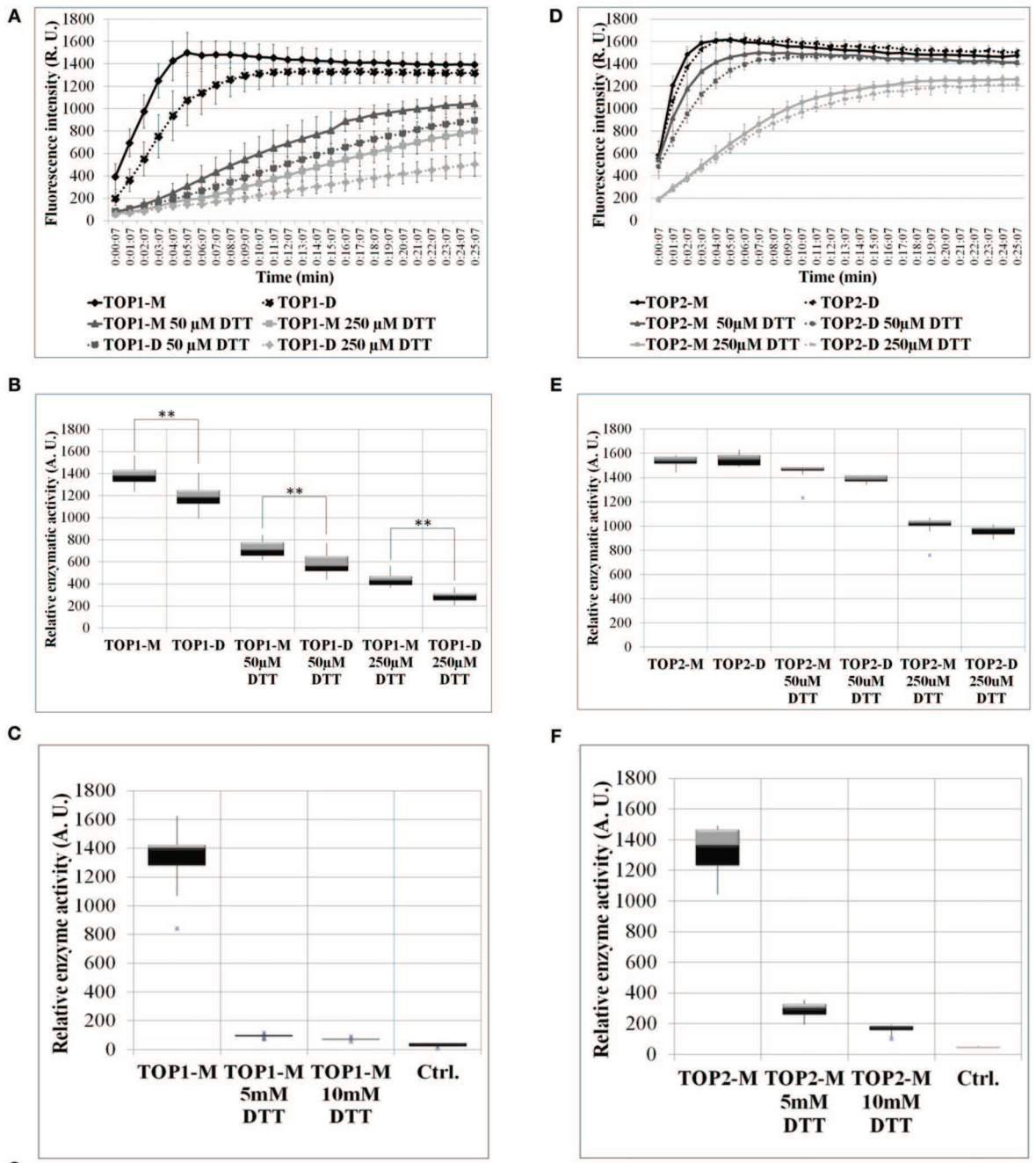
The transit peptide of TOP1 facilitates the dual transport of the protein to the chloroplast and mitochondria (Kmiec et al., 2013; Moreau et al., 2013). The function and activity of organellar enzymes is strongly influenced by pH changes in their environment caused by fluctuations in the light quality and quantity (Buchanan, 1980; Scheibe, 1991). The stroma and mitochondrial matrix typically represent an alkaline environment. The pH of the stroma fluctuates from 6.2 to 4.6 in light versus darkness (Smith and Raven, 1979). In contrast, the pH of the cytosol is more stable and centers around 7.1 (Gout et al., 1992).

To determine whether pH changes represent a potential regulatory mechanism of TOP activity, we examined the activity of recombinant TOP1- and TOP2-His under a range of pH conditions. We found that TOPs activity on the fluorogenic substrate is impervious to changes of pH both toward more acidic or basic values. TOP1 or TOP2 activity at pH 7.5 does not significantly differ from their activity at pH 6.5 or 8.5 (**Figures 4A,B**).

Thus, TOPs are robust enzymes with the capability of being functional in a range of pH values including values outside of the physiological pH span of the cytosol or organelles.

TOP1 and *TOP2* Participate in the Plant Response to Oxidative Stress Induced by Methyl Viologen

Prior evidence suggests that *TOP1* and *TOP2* peptidases contribute to plant defense against oxidative stress triggered by



G

	Activity Units (average)	STDV	% Activity
TOP1-M	1392.39	98.29	100.00
TOP1-M 50µM DTT	721.81	75.86	51.84
TOP1-M 250µM DTT	442.86	69.31	31.81
TOP1-M 5mM DTT	133.51	21.39	9.83
TOP1-M 10mM DTT	93.32	16.16	6.87
TOP2-M	1534.0	51.06	100.0
TOP2-M 50uM DTT	1439.9	80.41	93.9
TOP2-M 250uM DTT	1018.7	45.77	66.4
TOP2-M 5mM DTT	411.39	129.68	32.66
TOP2-M 10mM DTT	229.71	65.65	18.24

FIGURE 3 | The activity of TOP1 and TOP2 monomer or dimer in the presence or absence of dithiothreitol. 0.1 µg of purified recombinant TOP1 or TOP2 was incubated in the reaction buffer solution containing 20 µM of the MCA-peptide. MCA-peptide emit detectable fluorescence upon cleavage. The activity was assessed at λ excitation of 328 nm and λ.newline *(Continued)*

FIGURE 3 | Continued
emission of 393 nm over the course of 25 min. Quantification of fluorescence intensity was expressed in the levels of fluorescence emission per minute and shown as relative enzymatic activity. **(A)** Fluorescence intensity of TOP1 monomer and dimer activity over time in reaction buffer containing no DTT or 50/250 μM DTT. **(B)** The relative enzymatic activity of TOP1 monomer and dimer after incubation in reaction buffer or reaction buffer containing 50/250 μM DTT. **(C)** The enzymatic activity of TOP1 under 5 and 10 mM DTT. Control constitutes reaction buffer containing no TOP enzyme. **(D)** The fluorescence emitted by the cleavage by TOP2 of the substrate over time. **(E)** The quantification of TOP2 monomer and dimer activity in terms of relative enzymatic activity in the presence or absence of 50/250 μM DTT. **(F)** The relative activity of TOP2 upon incubation with 5 and 10 mM DTT. Reaction buffer without TOP2 enzyme is utilized as the control. **(G)** The average relative activity and percent activity of TOP1 and TOP2 monomer under increasing concentrations of DTT.

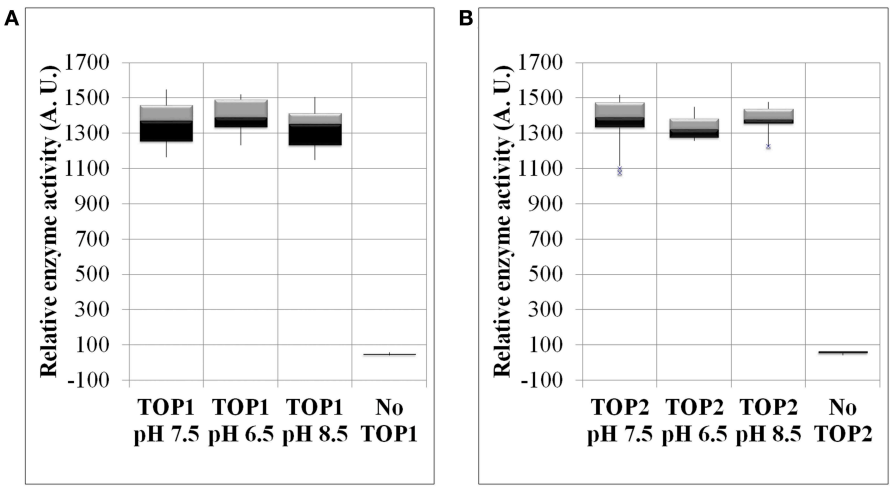


FIGURE 4 | The enzymatic activity of TOP1 and TOP2 under changing pH conditions. Recombinant bacterial expressed TOP1 or TOP2 was incubated with 20 μM MCA-peptide under varying pH conditions. **(A)** TOP1 activity in pH values of either: 6.5, 7.5, or 8.5. **(B)** TOP2 activity in pH values 6.5, 7.5, and 8.5. In all pH experiments, the buffer solution containing the MCA-peptide in the absence of TOP was used as a control. The activity was assessed at λ excitation of 328 nm and λ emission of 393 nm over the course of 30 min. The relative enzymatic activity was calculated by the sum of the fluorescence emission per minute. The error bars are the standard deviation of 12 replicates.

pathogens or abiotic factors (Polge et al., 2009; Moreau et al., 2013). We further explored the functions of *TOPs* to gain insight into the potential role of *TOP1/TOP2*-mediated pathways in ROS-triggered PCD.

Various treatments or abiotic stress conditions induce higher rates of ROS synthesis and drive their accumulation. We tested the phenotypes of *top* mutant seedlings to a panel of ROS-inducing factors including methyl viologen (MV), selenite, cadmium, antimycin A, exogenous hydrogen peroxide treatment, and salinity stress. With the exception of MV treatments, the other treatments produced no distinguishable phenotypes in *top* mutants.

MV impairs photosynthesis by interfering with electron transport of the photosystems and by generating toxic superoxide anions (Farrington et al., 1973; Härtel et al., 1992; Krieger-Liszkay et al., 2011), induces lipid peroxidation and interferes with electron transport in mitochondria (Dodge, 1971; Palmeira et al., 1995). The generation of ROS further damages the photosystems which inhibits growth, chloroplast homeostasis, and leads to PCD (Farrington et al., 1973).

To test the effect of MV on *top* mutants, seeds were sown on medium containing MV, stratified for 2 days at 4°C in darkness and grown in long-day (16 h) light conditions; the radicle and cotyledon emergence was assessed in both mutants and Col-0 control. First, we optimized our assay by testing multiple concentrations of MV (0.65, 7.5, 0.9, 0.95, 1, 1.5, and 2 μM). While radicle emergence could be seen at MV concentrations above 0.9 μM, green cotyledon emergence was strongly impeded at concentrations greater than 1 μM. The 0.9 μM MV condition became our established standard, as the differential effects of MV on radicle emergence was most apparent. In our assays, the percentages of radicle emergence after 2 days of growth on 0.9 μM MV-containing medium were assessed in mutants and Col-0 (**Figure 5A**). In the presence of MV, the emergence of radicles in *top2* mutant, but not *top1* or *top1 top2* mutants, was significantly inhibited compared to Col-0. The germination rates of *top* mutants on control medium (no-MV condition) showed no significant differences from that of Col-0 seedlings. MV applications on fully mature rosettes produced no significant differences between *top* mutants and Col-0. We conclude that *TOP2* positively modulates tolerance to MV exposure during seed germination; *TOP1* may potentially act in an opposite manner since combining *top1* and *top2* mutations partially rescued *top2* radicle hypersensitivity to MV.

MV induces ROS production during seed dormancy—short term MV treatment on dormant seeds within 6 h resulted in improved germination rates by breaking dormancy (Farrington

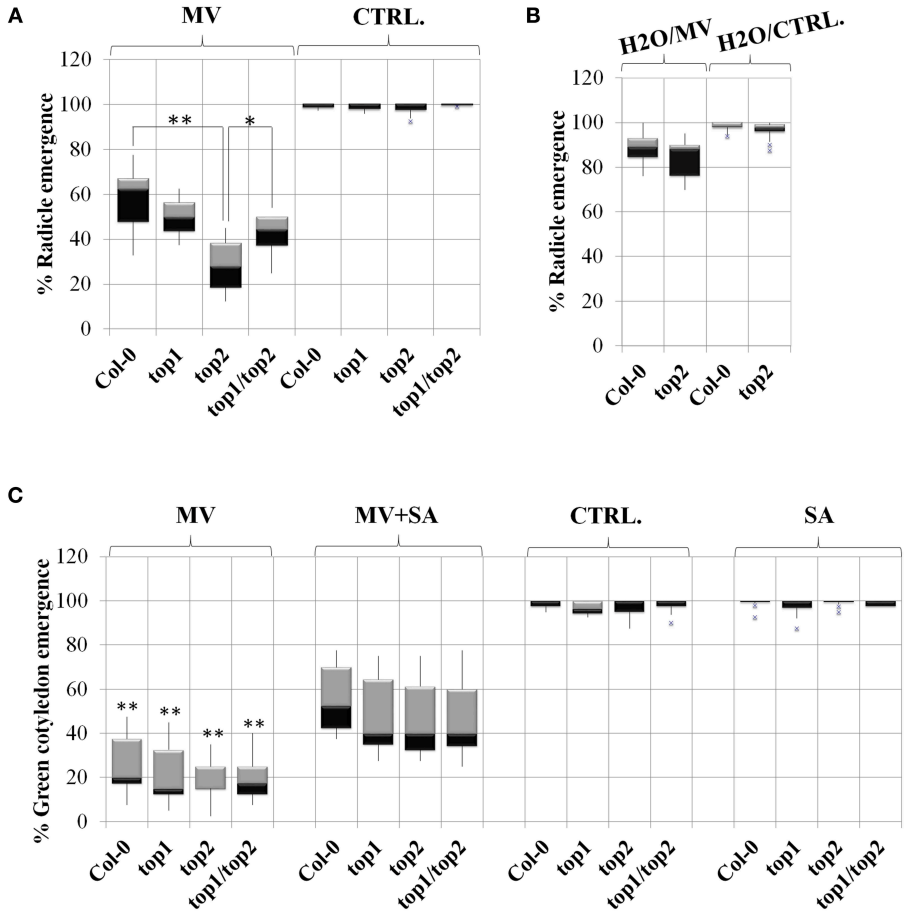


FIGURE 5 | TOPs involvement in methyl viologen stress is SA-independent. Radicle and cotyledon emergence in the presence or absence of salicylic acid (SA), Methyl Viologen (MV) or SA and MV, in the *top* mutants described in Moreau et al. (2013). Box plots represent percentage of radicle or cotyledon emergence under various treatments as noted above each plot. **(A)** MV inhibits radicle emergence in the *top2* mutant. Seeds were stratified directly on MV-containing medium or no-MV (CTRL) media; *p*-values (p) (Student's *T*-test) were calculated as shown in the figure. **(B)** Stratification in water rescues the MV toxicity of *top2*. The seeds were stratified in water and then plated on MV (H_2O/MV) or no-MV plates (H_2O/ CTRL) plates. **(C)** Exogenous SA alleviates the defects in green cotyledon emergence of both the *top* mutants and wild type Col-0. In **(A,B)**, asterisks represent statistical significance (*T*-test) relative to wild type Col-0 calculated from over 6 replicates per treatment. In **(C)**, asterisks represent a statistical significance (*T*-test) of the difference between line performance in MV+SA and in MV ($*p < 0.05$ and $**p < 0.01$).

et al., 1973). To determine whether the oxidative stress resulting from ROS production upon prolonged exposure (48 h) to MV during seed dormancy may be the cause of the hypersusceptibile phenotype of the *top* mutants, *top2* and Col-0 were no longer stratified in the presence of MV. Instead, Col-0 and *top2* were stratified on water for 2 days and then seeded on MV-containing plates. We found that under these conditions the germination rate of *top2* increased drastically so that the difference between *top2* and Col-0 germination was no longer significant (**Figure 5B**). Thus, the MV-mediated inhibition of the germination rate of *top2* only occurs when *top2* is exposed to the oxidative stress inducer during seed dormancy.

Altogether, our data indicates that *TOP1* and *TOP2* specifically mediate plant responses to MV during early development via, at least partially, distinct pathways; also, it suggest that *TOPs* do not have broad, unspecific, roles in mediating plant's oxidative stress response.

Exogenous SA Alleviates the MV Toxicity in a *TOP*-independent Manner

Low levels of exogenous SA lessened the damage caused by oxidative stress through the modulation of antioxidant-related activities (Lee et al., 2010) and alleviated the effects of MV on photosynthesis (Ananieva et al., 2002). In addition, our previous work indicated a connection between *TOP1*, *TOP2,* and SA-mediated signaling (Moreau et al., 2013). Thus, the possibility emerged that *TOPs* may contribute to the plant response to MV through an SA-mediated pathway.

To test this hypothesis, we sowed mutants and Col-0 seedlings on plates in the presence or absence of 10 μM SA and/or

0.95 μM MV and quantified their effects on the germination rate (**Figure 5C**). The quantification of cotyledon emergence instead of radicle emergence was done to assess the recovery effect of SA on photosynthesis. Seedlings were grown only in the presence of SA to determine its independent effect on germination. We found that all lines exhibited normal germination rates in the presence of 10 μM SA, as determined by measuring the emergence of green cotyledons indicating that, at this low concentration, exogenous SA does not impede germination. Next, we examined whether SA-induced signaling is functional in a *top* background by measuring the percentages of green cotyledons of seedlings grown in the presence of both 0.95 μM MV and 10 μM SA. We found that SA alleviated the negative effect of MV on photosynthesis on all lines to a similar extent; in average, the green cotyledon emergence rate of all lines increased by 45%. Thus, the SA-mediated signaling triggered by low amounts of exogenous SA is unaffected in *top* mutant background.

Taken together, our results suggest that the SA-mediated pathways activated by MV exposure function independently or are genetically downstream of the *TOP* pathways.

A Model of TOP-mediated Cellular Functions in Oxidative Stress

To understand how *TOP1*- and *TO2*-mediated pathways operate and influence each other in the context of SA signaling and stress response, we undertook an analytical approach to study their system-level dynamics. We developed a systems biology model that characterizes TOP1 and TOP2 functions in the context of the SA- and *redox*-triggered PCD (**Figure 6A**). The model was built by integrating experimental observations from the analysis of *top* mutants, biochemical analysis of TOPs and current knowledge on *SA* and the oxidative stress response pathways; the measurable cellular phenotype integrated in the model is the PCD. The architecture of the model relies on the relation between five main components: SA, ROS (H_2O_2), antioxidants (AOX), and TOP1/TOP2. Following is a description of the model development that includes the rationale for selecting molecular species, reactions, rate equations, and parameters.

SA in the Context of Redox Homeostasis and PCD Signaling

The core of the model is constituted by SA, ROS (H_2O_2), and AOX and their relationships. We included in the model the functions of SA in rapport to TOPs from Moreau et al. (2013) and the present study, pathways that represent the biochemical reactions related to production, degradation, and signaling functions of H_2O_2, and the cellular activities of antioxidants as symbolized by a generic AOX molecule. A large number of reactions related to antioxidant activities are known, however we omitted biochemical activities of specific AOX on TOPs as they are not yet understood. The main pathways that drive the SA, ROS, and AOX activities are described by the following reactions:

(i) The central regulators of the cellular redox homeostasis are antioxidant enzymes and small MW species (such as glutathione, ascorbate, and tocopherol), which participate in cellular detoxification through scavenging of ROS, reducing oxidized thiols, and functioning as *redox* buffers (*re33* and *re41*). Catalases constitute an important part of the plant's antioxidant system; SA inhibits the activity of catalases (Conrath et al., 1995; Rüffer et al., 1995) (*re43*, *re33*). ROS signaling mediates the activation of the antioxidant system (*re29, re42*).

(ii) SA synthesized in the chloroplasts (cSA) is transported into the cytosol (SA) (*re1*) (Fragniere et al., 2011; Serrano et al., 2013); H_2O_2 the most abundant ROS species produced from superoxide during photosynthesis diffuses and/or is transported across chloroplastic membranes (*re8)* (Bienert et al., 2006, 2007; Mubarakshina et al., 2010). The reduced form of glutathione (GSH) maintains a reductive environment in the cell (Han et al., 2013). GSH-dependent Glutathione Peroxidase catalyzes hydrogen peroxide detoxification and forms GSSG, the oxidized form of glutathione. Glutathione reductase (*GR*) catalyzes the reduction of GSSG to GSH and helps maintain a reducing cellular milieu (Meyer et al., 2012; Deponte, 2013). Accumulation of H_2O_2 regulates increases GSH/GSSG which in turn activates the Isochorismate syntase1 (ICS1)-dependent SA synthesis (Han et al., 2013) (*re31*).

(iii) *Redox* signals that drive the development of PCD (*re29, re37,* and *re5*) may be transduced via thiol-driven post-translational modifications in sensor proteins with higher chemical reactivity (Mou et al., 2003; Apel and Hirt, 2004; Buchanan and Balmer, 2005; Temple et al., 2005; D'Autreaux and Toledano, 2007). Such sensors contain residues whose location and ionic state render them sensitive to oxidation (Ghezzi et al., 2005; Nagy, 2013). *Re29* models the positive effect of ROS accumulation on the reversible oxidation of protein sensors (*ProtOx*), while antioxidant enzymes, such as the GSH-dependent glutaredoxins, catalyze the reverse reaction (Rouhier, 2010) (*re41)*. Under high oxidative stress or in mutants with a defective proteasome pathway, the accumulation of a partially degraded peptide (*re37*) triggers PCD through (*re5*). An independent SA-driven PCD pathway (*re46*), involves a transcriptional response via *NPRs* (Hoeberichts and Woltering, 2003; Jayakannan et al., 2015).

(iv) Antioxidant production is enhanced by SA (*re42*) though a transcriptional pathway (*re44*). This simulates the SA modulatory effect on the GSH/GSSG ratio—SA increases GSH cellular content by enhancing the transcription of enzymes in the glutathione cycle (Li et al., 2013).

TOP1/TOP2 Pathways

(i) *TOPs* are nuclear-encoded genes; in the model, the respective mRNAs (*mTOP1* and *mTOP2*) are translated into proteins *(re20* and *re19*).

(ii) *TOP* proteins have distinct subcellular localizations; *re20, re22*, and *re50* summarize the maturation, import from cytosol into the chloroplast and degradation of *TOP1*, while *re19* and *re49* describes the synthesis and degradation of cytosol-localized *TOP2* (Kmiec et al., 2013; Moreau et al., 2013).

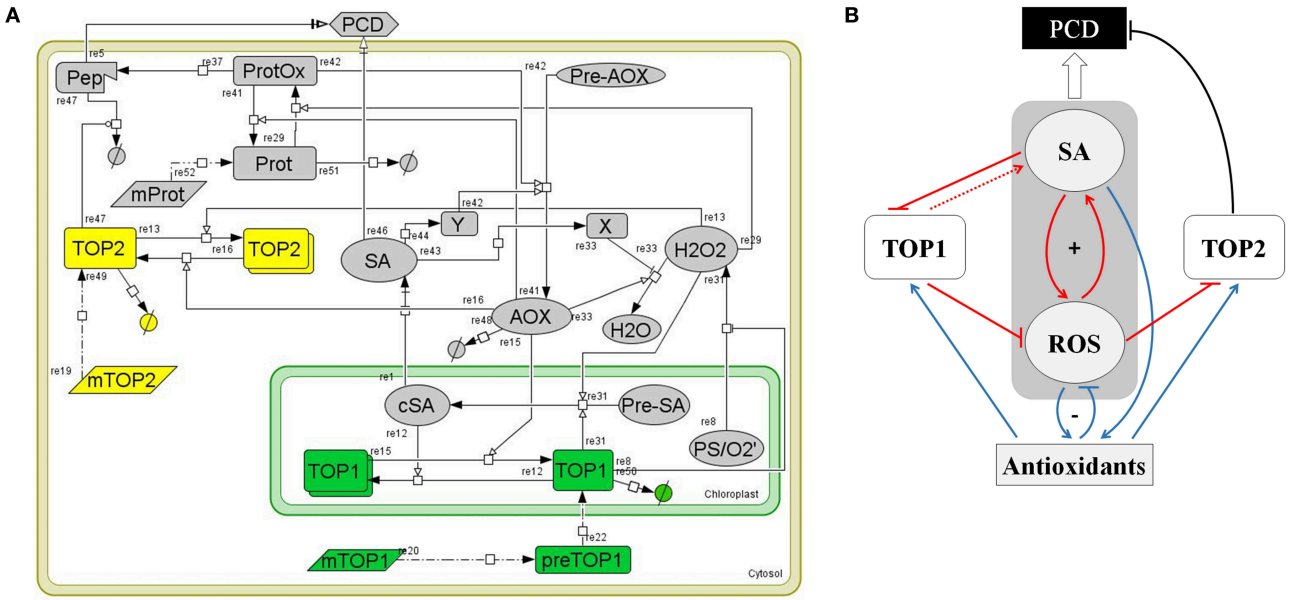


FIGURE 6 | A computational model of *TOP1* and *TOP2* functions in the oxidative stress response. (A) Graphical representation of the TOP model. The model represents a plant cell containing various cellular elements (transcripts, proteins, small molecules, peptides) linked by biochemical reactions (transcription, translation, transport, association/disassociation, inhibition, and catalysis) and the kinetic laws associated with the biochemical reactions. The model was build using *CellDesigner 4.4* software. **(B)** Logical diagram visualizing the relationships among TOP1, TOP2, SA, ROS, and Antioxidants in the context of the oxidative stress response. Pathways active during the oxidative burst phase are in red, pathways active during the reductive burst phase are in blue, and PCD-triggering pathways are in black. The symbol "+" indicates the SA-ROS positive feed-back loop; "−" shows the negative feed-back between ROS-Antioxidants. The punctuated line arrow depicts the hypothesized function of TOP1 in SA synthesis.

(iii) We hypothesized that SA and AOX modulate the enzymatic activities of TOP1 and TOP2 by adjusting the monomer/dimer ratios of TOP1 and TOP2. Specifically, the chloroplastic SA binds TOP1 and inhibits its activity by decreasing the monomer/dimer ratio (**Figure 1**; Moreau et al., 2013) (*re12*) while the AOXs' reducing activity increases TOP1's monomer/dimer ratio (*re15*) by promoting monomerization. Likewise, TOP2 activity is modulated by shifts in the AOX *redox* status whereby a reductive environment favors monomerization (*re16*) and increases TOP2 activity, while an oxidative environment favors TOP2 dimerization (*re13*) and inhibits its proteolytic activity (*re47*) (**Figures 2**, **3**).

(iv) TOP1 and TOP2 sustain organelle- and cytosol-specific proteolytic pathways respectively. We postulate that TOP1 activity sustains normal levels of ROS accumulation in chloroplasts (*re8*), possibly by participating in chloroplasts import of antioxidant enzymes (e.g., GPX) or degradation of oxidized proteins (Kmiec et al., 2013). We postulate that TOP1 activity faciliates SA accumulation by participating in the import of enzymes that catalyze SA synthesis (*re31*). On the other hand, TOP2 may modulate the execution of PCD as part of a cytosolic proteolytic pathway activated by MV or other factors causing oxidative stress (Polge et al., 2009). We hypothesize that TOP2 controls the accumulation of a signaling peptide—a positive regulator of PCD (*re5*); thus, the irreversible oxidation of proteins leads to their degradation via the proteosomal machinery (*re37*) and TOP2 (*re47*).

Species, Reactions, and Selection of Parameters

The model contains 22 molecular species (3 mRNAs, 8 small molecules, and 11 proteins), 25 reactions and 44 reaction parameters, described in **Supplemental Tables S1–S3**. We selected the species' initial values based on the published literature: SA (cSA) basal levels were selected in the interval (0.05–1 μM) (Enyedi et al., 1992; Abreu and Munné-Bosch, 2009); H_2O_2 levels within (1–100 μM) (Veljovic-Jovanovic et al., 2002) about 40-Jovanmol g^{-g} FW; and AOX levels within (1–100 μM). TOP1 and TOP2 expression was normalized to maintain a ratio of 1/2, with TOP1 expressed at 1/10 level in comparison to the large PS-I complex as a baseline, based on Genevestigator data (Hruz et al., 2008). The initial monomer:dimer ratio of TOPs was chosen to be 3:1 as observed in our assays. Molecules that are consumed during the simulation of the cellular stress phenotype (*mTOP1*, *mTOP2*, *mProt*, *Pre-SA*, *Pre-AOX*, *Prot*) were selected to the normalized value of 1 to limit their impact on the dynamics of TOPs pathways. The concentration of superoxide ($O_2^{\bullet -}$) was the input variable for controlling the oxidative stress. The remaining species (*Pep*, *X*, *Y*) were initialized to zero or low concentrations.

Several reactions (*re12*, *re13*, *re15*, *re16*, *re19*, *re20*, *re29*, *re41*, *re47*, *re48*, *re31*, *re42*, and partially *re33*) follow a Michelis-Menten rate law; the inhibitor has the rate equation:

$k_1^*E^*S/(k_2+S)$ (*E*-enzyme, *S*-substrate); the PCD-trigger reactions (*re5* and *re46*) were modeled with a Hill dynamics ($k_1^*S^n/(k_2^n+S^n)$) and the Hill coefficient ($n = 2$). Simple reactions (protein production, degradation, and transport) follow a mass action rate law (*re1*, *re22*, *re37*, *re43*, *re44*, *re49*, *re50*, *re51*, *re52*). The rate law for TOP1 inhibition of H_2O_2 production (*re8*) and catalysis of H_2O_2 reduction to H_2O (*re33*) is: $k_1*S/(1+E/k_2)$. The production of AOX (*re42*) was modeled by the addition of two independent reactions, one driven by SA and the other by the level of cellular oxidative stress (*ProtOx*). The reduction of H_2O_2 to H_2O (*re33*) was also obtained through two independent pathways—one driven by AOX and another inhibited by SA through a transcriptional pathway involving an unknown protein species (X) (Rao et al., 1997). The synthesis of cSA from precursors was modeled as a single pathway regulated by both H_2O_2 through the GSH/GSSG system and TOP1, probably through its proteolytic activity necessary to import and mature enzymes involved in SA synthesis. A background rate of SA synthesis independent of TOP1 activity was also necessary to explain the experimentally observed mild MV phenotype in *top1*.

Reactions parameters were selected such that enzymatic reactions occur at high rates (normalized to $k = 1.0\ s^{-1}$ or $k_{cat} = 1.0\ s^{-1}$) while reactions involving transcriptional control have one order of magnitude slower rates; the Michaelis constant was selected by default ($k_M = 1\,\mu M$) and was varied between 1 and 10 μM subsequently. The rate constants for the mass action kinetics describing protein production/degradation were chosen two orders of magnitude lower than the enzymatic reactions ($k = 0.01\ s^{-1}$). Since quantitative time series data for the reactions were not available, the parameters were initially selected as described above. Subsequently, a parameter scan was performed to observe the dynamics of species and reaction fluxes; the parameters were adjusted to fit the observed dynamics of the stress phenotypes of *top* mutants, of the ROS-AOX inhibition and SA-ROS amplification loops, and the hallmark aspects of the oxidative stress response—an initial oxidative wave followed by a strong reductive phase. We adjusted the reaction rates to establish the observed monomer/dimer ratio and to equilibrate the production and degradation of species. **Supplemental Table S3** lists the parameters for which PCD, ROS, AOX, and SA dynamics is robust and correlates with *top* phenotypes.

TOP Model Dynamics and Analysis of Oxidative Stress Phenotypes

The computational model in **Figure 6A** was summarized in a logical diagram (**Figure 6B**). The diagram contains the SA-ROS amplification loop driving the oxidation wave, the AOX wave stimulated by the SA accumulation, and the ROS-AOX inhibitory loop driving the *redox* reductive wave and the connected TOP1/TOP2 pathways. The expected activity and dynamics associated with the TOPs is as follows. We hypothesize that in the oxidative burst phase TOP1 has a positive control over the SA-ROS amplification loop, driving a higher SA accumulation while limiting the ROS accumulation in the chloroplast; in the reductive phase, TOP1 activity is enhanced via monomerization under the enzymatic activity of antioxidants—SA drives antioxidants that increase TOP1 activity which reduce ROS accumulation. On the other hand, in the oxidative burst phase the amount of TOP2 monomers decreases and, in consequence, TOP2 activity is inhibited which may trigger PCD via accumulation of oxidized products; the amplitude of the PCD correlates with the amplitude of the oxidative burst; in the reductive phase TOP2 increases in activity (via monomerization) suppressing the SA-independent pathway leading to PCD.

Using the model shown in **Figure 6A**, we simulated the dynamics of SA, H_2O_2, AOX, and the resultant PCD in Col-0, *top2*, and *top1 top2* backgrounds in conditions of MV-induced oxidative stress or the stress-free control (**Figure 7A**). Increased superoxide levels resulted in a rapid oxidative wave, which increased SA amount via the SA-ROS amplification loop and subsequently triggered an increase in the AOX accumulation and a longer reductive wave. The intensity of PCD caused by oxidative stress is determined by the level of SA accumulation—which is lower in *top1* (**Figure 7B**), and the accumulation of un-degraded peptide *Pep*—which reaches a higher level in *top2*. **Figure 7C** details the dynamics of ROS-AOX inhibition loop under oxidative stress (MV challenge): the initial fast increase in ROS accumulation triggers a gradual increase in the AOX level which negatively regulates the ROS and suppresses the oxidative burst. Furthermore, simulation outputs reproduce the observed *top2* MV hypersensitivity; accumulation of partially degraded peptides in *top2* results in a stronger PCD phenotype (**Figure 7D**). Thus, the model explains the milder *top1 top2* MV phenotype compared to *top2* by predicting lower levels of *SA-ROS* amplification in *top1 top2* where the TOP1-amplified oxidative burst does not function at full capacity and thus, partially alleviates the higher PCD levels of *top2*.

Taken together, we demonstrated that the expected dynamics and stress phenotypes of *top* mutants were correctly predicted by this qualitative model of TOP1 and TOP2 pathways.

Discussion

In this study, we investigate the characteristics, enzymatic properties, and cellular roles of *TOP1* and *TOP2* as they relate to the plant oxidative stress response. We generated a theoretical model of the functions of *TOP1* and *TOP2* in the context of an essential set of cellular elements and processes, deduced by integrating experimental data from the analysis of *top* mutants and the biochemical characterization of TOP proteins.

We determined that TOP1 and TOP2 are capable of self- and hetero-dimerization using two distinct systems: the *in vivo* protoplast system, which accounts for factors that may modulate TOPs interactions such as the cellular *redox* environment and potential post-translational modifications, and *in vitro* gel chromatography, a method using purified proteins which allows for fine adjustments of the assay conditions (**Figures 1**, **2**). Interestingly, TOP2 dimers showed a higher interactions affinity than TOP1 dimers in this assays. It is notable that TOP2 has one additional Cys residue in the peptidase domain compared to the chloroplastic TOP1; it is possible, that similar to the case of the poplar thioredoxins (Chibani et al., 2012), this Cys residue may be essential for TOP2's ability to form tighter dimers, and

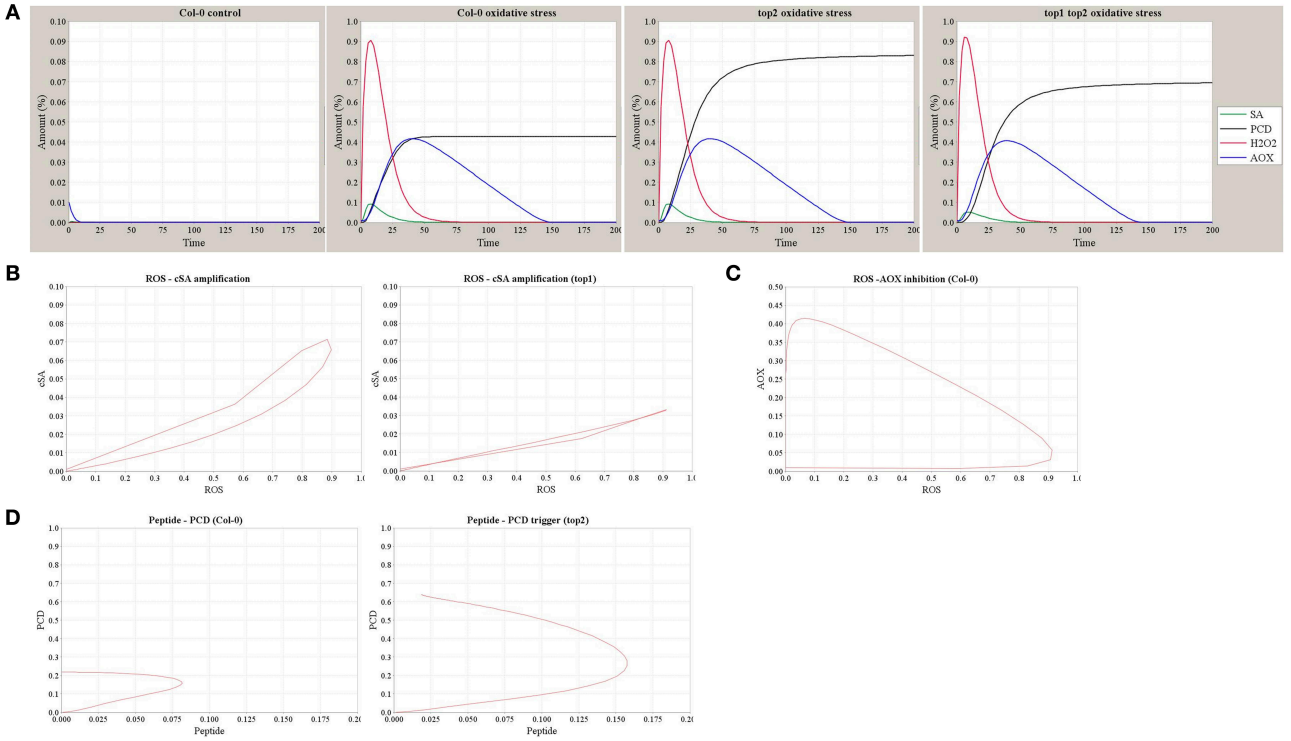


FIGURE 7 | Simulation outputs of the computational model of *TOP1* and *TOP2* functions in the oxidative stress response. (A) The dynamics of SA, H_2O_2, AOX, and PCD simulated in the Col-0 control (no MV stress) and in Col-0, *top2,* and *top1 top2* mutants in conditions of MV-induced oxidative stress. **(B)** The dynamics of ROS-SA amplification loop in Col-0 and the impaired amplification in *top1* mutant. **(C)** The dynamics of ROS-AOX inhibition loop under oxidative stress in Col-0. **(D)** The dynamics of PCD triggering and peptide accumulation in Col-0 and *top2* mutant.

may represent an evolutionary advantage in the highly reducing conditions of the cytosol.

TOP2 dimerization was influenced by treatments with SA and the thiol-based reductant DTT. We demonstrated that incubation of protoplasts with exogenous SA destabilized the TOP2 dimer. This result could be the effects of direct binding of TOP2 to SA, however this hypothesis is unlikely as we previously showed that TOP2-SA association has a low affinity and thus, is probably functionally irrelevant (Moreau et al., 2013). Alternatively, SA's effects on TOP2 self-association may be explained by the indirect consequences of SA treatment on the *redox* homeostasis of the protoplasts, akin to NPR1 monomerization and thioredoxins activation (Mou et al., 2003; Tada et al., 2008). Several observations strengthen the latter hypothesis: (1) we observed a concentration-dependent effect of SA on TOP2 dimerization suggesting that the inhibition of TOP2 dimerization may occur via disruption of multiple intra- or intermolecular disulfide bonds by the SA-mediated increase in the reductive potential of the protoplasts; (2) DTT treatment of purified TOP2 shifted the monomer/dimer ratio from 3:1 to 15:1 (**Figure 2**), indicating that a reductive environment destabilizes disulfide bridges and favors accumulation of TOP monomers; and (3) The effects of SA on TOPs dimerization is in contrast to its effect on the MKK5-HOPF2, an interaction not known to be mediated by disulfide bridges and which was drastically reduced by the lowest SA amount tested.

Given our results, it is plausible that *redox* changes induced by SA can potentially influence the TOPs monomer-dimer equilibrium. We postulate that SA-mediated *redox* shifts may lead either to dimerization during the oxidizing burst or monomerization during the reductive bursts. It is possible that direct or indirect effects of SA on TOPs' monomerization/dimerization state are further regulated by additional factors such as the SA local concentrations, TOPs sub-cellular localization and the temporal and compartment-specific redox environments following stress driven by the major glutathione-ascorbate antioxidant systems—all aspects that require further investigation. Altogether, our results support the hypothesis that TOPs are regulated on the basis of cellular *redox* state.

We examined the activity of TOP1 and TOP2 monomers and dimers to understand if TOPs self-associations and thiol-sensitivity have regulatory roles. Notably, TOP1 and TOP2 show a similar trend of sensitivity to the metazoan TOP toward high concentrations of DTT (5 and 10 mM DTT) indicating that akin to the proposed effects for the metazoan TOP (Lew et al., 1995; Shrimpton et al., 1997), high levels of DTT inhibit TOP1 and TOP2 by interfering with the zinc co-factor binding and/or disrupting intramolecular bonds. Remarkably, plant TOPs differ from the metazoan counterpart in one important aspect; unlike metazoan TOP, TOP1, and TOP2 activity is inhibited by low DTT concentrations (below 1 mM). While metazoan TOPs are

Cys-rich proteins, the comparatively lower number of Cys in TOP1 and TOP2 may potentially result in fewer intra- and intermolecular disulfide bonds, or, alternatively, form bonds that may be more accessible to reduction; in either case, the reduction of the intramolecular bonds may be deleterious to the structure of the monomeric TOPs and DTT able to limit their activity even at low concentrations. Altogether, our results on the activity levels displayed by various TOP1/TOP2 fractions in the absence or presence of DTT, alongside published studies on metazoan TOPs, lead us to hypothesize that TOPs contain disulfide bonds and that monomers are the active forms, while the dimers and potential multimers are inactive enzymatically.

TOPs functions in modulating pathogen-induced PCD (Moreau et al., 2013) and the newly-uncovered biochemical characteristics of TOPs motivated us to investigate their particular contributions to the plant oxidative stress response. The oxidative triggers modifications in the proteome composition and activity and *TOPs* unique attributes and functions render them likely participants in the *redox*-mediated signaling. A prior study on plant *TOPs* suggested their role in limiting oxidative damage following heavy metal stress (Polge et al., 2009). Although we have not detected any unusual responses of *top* mutants to heavy metal stress, we provide evidence that functional *TOPs* are required to mediate the damage caused by MV, a potent inducer of photo-oxidative stress. MV readily interferes with the photosystems resulting in severe chloroplastic-derived oxidative stress (Babbs et al., 1989). ROS accumulation is known to promote seed germination (Marino et al., 2012) and specifically, short term exposure to MV increased ROS levels and interrupted dormancy in *Arabidopsis* and *Helianthus* seeds (Oracz et al., 2007; Leymarie et al., 2012). Interestingly, *top2* responded with increased sensitivity to MV when scoring for radicle emergence indicating that the cytosolic TOP2, rather than the chloroplastic TOP1, is the critical *TOP* for MV tolerance in this organ. The importance of the cytosolic TOP2, alongside the chloroplastic TOP1, for the plants' MV tolerance is in line with studies indicating that chloroplastic stress induces expression of both cytosolic and chloroplastic antioxidant enzymes and supporting the role of cytosol as a major site for detoxification systems associated with photosynthesis (Mullineaux et al., 2000; Yabuta et al., 2004).

SA alleviated the germination of *top* mutants and Col-0 seeds grown in the presence of MV. The protective properties of treatments with physiological concentrations of SA are considered a general consequence of SA's antioxidant activities as evidenced by lowered cellular ROS or nitric oxide levels in *Arabidopsis* and crops, monocots and dicots, subjected to abiotic or biotic stress upon exogenous SA application (Lee et al., 2010; Gémes et al., 2011; Zhang et al., 2011; Wang and Liu, 2012). It is possible that a similar mechanism is responsible for relieving MV toxicity in *top* seeds. While the precise function of *TOPs* in modulating MV tolerance is unclear, it is likely that *TOPs* have ROS-protective roles by cleaving oxidized peptides and preventing their accumulation in the cytosol or chloroplast; in parallel, TOPs may indirectly participate in the ROS-mediated signaling by cleaving/degrading specific peptides with signaling properties that modulate PCD.

A qualitative model was constructed describing TOP1 and TOP2 functions in the development of PCD associated with oxidative stress, in the context of known SA- and *redox*-mediated signaling pathways (**Figures 6**, **7**). TOP1/TOP2 functions are described within the SA-ROS-Antioxidant framework. Perception of both abiotic and biotic stress triggers shifts in the *redox* potential of the cellular milieu, primarily driven by increases in the endogenous concentration of SA and ROS, which are critical for the downstream signal transduction and induction of defense transcriptome and metabolome. Two distinct *redox* phases have been described: (1) the oxidative burst, driven by fast reactions initiated by ROS, is controlled by the interplay between SA and ROS synthesis (Leon et al., 1995; Rao et al., 1997; Shirasu et al., 1997); (2) the reductive burst, which is a consequence of the oxidative burst and is regulated by SA and synthesis of antioxidants (Mou et al., 2003; Tada et al., 2008). We postulate that TOP1 activity of attenuating ROS increase is repressed by high SA accumulation during the oxidative phase and enhanced by the antioxidant actions during the reductive phase; on the other hand, TOP2—less active during the oxidative phase and more active during the reductive phase—is regulated by, but not a contributor to, the SA-ROS-Antioxidant driven *redox* oscillations.

Altogether, our study that combines analytical and experimental approaches supports the hypothesis that the interplay between TOP1-controlled chloroplastic events and the cytosolic TOP2 modulates the development of PCD through the ROS-SA-AOX axis.

Further studies of TOP1 and TOP2 effects on the plant oxidome alongside identification of their respective peptide substrates are required to validate or reject the hypotheses presented in the qualitative model. Undeniably, the SA signaling network during the biotic/abiotic stress comprises numerous elements with complex interactions; it would be a challenging task to assemble a quantitative model comprising all components and biochemical reactions and to demonstrate their precise spatial and temporal control.

Materials and Methods

Germination Treatments

The germination experiments were done on petri plates containing 35 mL of MS agar containing MV and/or SA. Germination on MS agar plates containing no MV or SA was used as controls. Seeds were either stratified directly on plates unless indicated otherwise. After stratification of 2 days in darkness, the seed were germinated under 16 h light/8 h dark conditions. Germination was counted by either assessing radicle emergence after 2 days or green cotyledon emergence after 5 days.

Split-luciferase Complementation Assays

Coding sequences of *TOP1* and *TOP2* were cloned into the pENTR vector. Fusion to N-terminal or C-terminal luciferase protein was done by their subsequent sub-cloning into pDuEx-DC6 and pDuEx-AC6 (Fujikawa and Kato, 2007). Vectors were transformed into DH5 alpha *E. coli*. *MKK5* and *HOPF2* were

cloned similarly and utilized as a positive control. Plasmid was extracted using Zymo's Zyppy Maxi Plasmid Prep kits (Zymo Research). Protoplast co-transformation with plasmids was done in 96 well plates using a modified protoplast extraction and PEG transformation protocol derived from Yoo et al. (2007), Wu et al. (2009) and Singh et al. (2014). At 12–16 h post-transformation, the protoplasts were incubated with the luciferase substrate in the presence or absence of SA. Luminescence was detected in a Synergy Microwell plate reader.

Protein Purification

His-tagged TOP1 was expressed in pET-28(a) vector in BL21. His-Tagged TOP2 was expressed in pET-32 vector in BL21. After the induction of His-tagged protein production, a crude extract of proteins were subjected to HisPur Cobalt Column purification (Thermo Scientific). The proteins were further purified using size-exclusion chromatography. Additional purification and concentrations were done using the Amicon Ultra 50 k Centrifuge Filter (EMD Millipore) in the case of the monomer. Pierce Concentrators 150 k MWCO (Thermo Scientific) was used to further purify and concentration the dimer. Small aliquots were stored or utilized in the same day.

Enzymatic Activity

Measurements of TOP activity was done by utilizing Synergy 4 micro well reader with the capacity of λ excitation of 328 nm and λ emission of 393 nm. 0.1 μg purified peptidase was incubated in a 99 μL reaction solution contacting TRIS-HCl buffer and 20 μM of the fluorogenic peptide substrate. The fluorogenic substrate, Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys (Dnp)-OH (Enzo Life Sciences), emits detectable fluorescence upon cleavage. Unless stated otherwise, the pH of the reaction mix was 7.5.

Gel Filtration Chromatography

Proteins eluted from cobalt column was subjected to size exclusion chromatography. Maximum volume of 4 mL of the eluted extract were injected to the gel filtration apparatus into the Superdex 200 column. The filtration apparatus facilitated the elution of cobalt purified protein based on size. UV at absorbance 280 nm while elution the respective fractions allowed the quantification of protein concentration within the sample. In the case of the DTT treated TOP samples, the cobalt TOP extractions were incubated with 500 μM DTT overnight and then subjected to size-exclusion chromatography.

Systems Biology Modeling and Simulation

The model was described in System Biology Mark-up language (SBML) format and analyzed using Cell Designer 4.4 (Funahashi et al., 2006) simulation tools. Model stability was assessed by observing stability over a range of parameters for species concentrations and reaction constants. The model was stable in the parameter range studied. The SBML model is available upon request.

Acknowledgments

We gratefully acknowledge Amanda Gray, Gary Horvath, Dr. Hyong Woo Choi, and Dr. Murli Manohar, the high school students Anna Knapp and Julia Wang for their assistance with various aspects of this study, and Dr. Nabil Elrouby for valuable comments on the manuscript. This work was supported by the National Science Foundation (project IOS-1025642 to SCP) and by CNCSIS-UEFISCDI and MEN-CDI (project number PN-II-PT-PCCA-2011-3.1-1350 and NUCLEU-PN0939/2009 to GVP).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00327/abstract

Table S1 | Model variables.

Table S2 | Model reactions.

Table S3 | Model parameters.

References

Abreu, M. E., and Munné-Bosch, S. (2009). Salicylic acid deficiency in NahG transgenic lines and sid2 mutants increases seed yield in the annual plant *Arabidopsis thaliana*. *J. Exp. Bot.* 60, 1261–1271. doi: 10.1093/jxb/ern363

An, C., and Mou, Z. (2011). Salicylic acid and its function in plant immunityF. *J. Integr. Plant Biol.* 53, 412–428. doi: 10.1111/j.1744-7909.2011.01043.x

Ananieva, E. A., Alexieva, V. S., and Popova, L. P. (2002). Treatment with salicylic acid decreases the effects of paraquat on photosynthesis. *J. Plant Physiol.* 159, 685–693. doi: 10.1078/0176-1617-0706

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399. doi: 10.1146/annurev.arplant.55.031903.141701

Babbs, C. F., Pham, J. A., and Coolbaugh, R. C. (1989). Lethal hydroxyl radical production in paraquat-treated plants. *Plant Physiol.* 90, 1267–1270. doi: 10.1104/pp.90.4.1267

Bienert, G. P., Møller, A. L., Kristiansen, K. A., Schulz, A., Møller, I. M., Schjoerring, J. K., et al. (2007). Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J. Biol. Chem.* 282, 1183–1192. doi: 10.1074/jbc.M603761200

Bienert, G. P., Schjoerring, J. K., and Jahn, T. P. (2006). Membrane transport of hydrogen peroxide. *Biochim. Biophys. Acta* 1758, 994–1003. doi: 10.1016/j.bbamem.2006.02.015

Buchanan, B. B. (1980). Role of light in the regulation of chloroplast enzymes. *Annu. Rev. Plant Physiol.* 31, 341–374. doi: 10.1146/annurev.pp.31.060180.002013

Buchanan, B. B., and Balmer, Y. (2005). Redox regulation: a broadening horizon. *Annu. Rev. Plant Biol.* 56, 187–220. doi: 10.1146/annurev.arplant.56.032604.144246

Chibani, K., Tarrago, L., Gualberto, J. M., Wingsle, G., Rey, J.-P., Jacquot, P., et al. (2012). Atypical thioredoxins in poplar: the glutathione-dependent thioredoxin-like 2.1 supports the activity of target enzymes possessing a single redox active cysteine. *Plant Physiol.* 159, 592–605. doi: 10.1104/pp.112.197723

Chu, T. G., and Orlowski, M. (1985). Soluble metalloendopeptidase from rat brain: action on enkephalin-containing peptides and other bioactive peptides*. *Endocrinology* 116, 1418–1425. doi: 10.1210/endo-116-4-1418

Cleland, W. (1964). Dithiothreitol, a new protective reagent for SH groups*. *Biochemistry* 3, 480–482. doi: 10.1021/bi00892a002

Conrath, U., Chen, Z., Ricigliano, J. R., and Klessig, D., F. (1995). Two inducers of plant defense responses, 2, 6-dichloroisonicotinec acid and salicylic acid, inhibit

catalase activity in tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7143–7147. doi: 10.1073/pnas.92.16.7143

D'Autreaux, B., and Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol.* 8, 813–824. doi: 10.1038/nrm2256

Denancé, N., Sánchez-Vallet, A., Goffner, D., and Molina, A. (2013). Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. *Front. Plant Sci.* 4:155. doi: 10.3389/fpls.2013.00155

Deponte, M. (2013). Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. *Biochim. Biophys. Acta* 1830, 3217–3266. doi: 10.1016/j.bbagen.2012.09.018

Dodge, A. (1971). The mode of action of the bipyridylium herbicides, paraquat and diquat. *Endeavour* 30, 130–135. doi: 10.1016/0160-9327(71)90039-1

Enyedi, A. J., Yalpani, N., Silverman, P., and Raskin, I. (1992). Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. U.S.A.* 89, 2480–2484. doi: 10.1073/pnas.89.6.2480

Farrington, J., Ebert, M., Land, E. J., and Fletcher, K. (1973). Bipyridylium quaternary salts and related compounds. V. Pulse radiolysis studies of the reaction of paraquat radical with oxygen. Implications for the mode of action of bipyridyl herbicides. *Biochim. Biophys. Acta* 314, 372–381. doi: 10.1016/0005-2728(73)90121-7

Ferreira, J. C., Icimoto, M. Y., Marcondes, M. F., Oliveira, V., Nascimento, O. R., and Nantes, I. L. (2013). Recycling of the high valence states of heme proteins by cysteine residues of Thimet-Oligopeptidase. *PLoS ONE* 8:e79102. doi: 10.1371/journal.pone.0079102

Foyer, C. H., and Noctor, G. (2013). Redox signaling in plants. *Antioxid. Redox Signal.* 18, 2087–2090. doi: 10.1089/ars.2013.5278

Fragniere, C., Serrano, M., Abou-Mansour, E., Metraux, J.-P., and L'Haridon, F. (2011). Salicylic acid and its location in response to biotic and abiotic stress. *FEBS Lett.* 585, 1847–1852. doi: 10.1016/j.febslet.2011.04.039

Fujikawa, Y., and Kato, N. (2007). Split luciferase complementation assay to study protein-protein interactions in Arabidopsis protoplasts. *Plant J.* 52, 185–195. doi: 10.1111/j.1365-313X.2007.03214.x

Funahashi, A., Matsuoka, Y., Jouraku, A., Kitano, H., and Kikuchi, N. (2006). CellDesigner 3.5: a modeling tool for biochemical networks. *Proc. IEEE* 96, 1254–1265. doi: 10.1109/jproc.2008.925458

Gémes, K., Poór, P., Horváth, E., Kolbert, Z., Szopkó, D., Szepesi, Á., et al. (2011). Cross-talk between salicylic acid and NaCl-generated reactive oxygen species and nitric oxide in tomato during acclimation to high salinity. *Physiol. Plant.* 142, 179–192. doi: 10.1111/j.1399-3054.2011.01461.x

Ghezzi, P., Bonetto, V., and Fratelli, M. (2005). Thiol–disulfide balance: from the concept of oxidative stress to that of redox regulation. *Antioxid. Redox Signal.* 7, 964–972. doi: 10.1089/ars.2005.7.964

Gout, E., Bligny, R., and Douce, R. (1992). Regulation of intracellular pH values in higher plant cells. Carbon-13 and phosphorus-31 nuclear magnetic resonance studies. *J. Biol. Chem.* 267, 13903–13909.

Han, Y., Chaouch, S., Mhamdi, A., Queval, G., Zechmann, B., and Noctor, G. (2013). Functional analysis of Arabidopsis mutants points to novel roles for glutathione in coupling H2O2 to activation of salicylic acid accumulation and signaling. *Antioxid. Redox Signal.* 18, 2106–2121. doi: 10.1089/ars.2012.5052

Härtel, H., Haseloff, R., Ebert, B., and Rank, B. (1992). Free radical formation in chloroplasts. Methyl viologen action. *J. Photochem. Photobiol. B Biol.* 12, 375–387. doi: 10.1016/1011-1344(92)85042-S

Hoeberichts, F. A., and Woltering, E. J. (2003). Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. *Bioessays* 25, 47–57. doi: 10.1002/bies.10175

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., et al. (2008). Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics* 2008:420747. doi: 10.1155/2008/420747

Jayakannan, M., Bose, J., Babourina, O., Shabala, S., Massart, A., Poschenrieder, C., et al. (2015). The NPR1-dependent salicylic acid signalling pathway is pivotal for enhanced salt and oxidative stress tolerance in Arabidopsis. *J. Exp. Bot.* 66, 1865–1875. doi: 10.1093/jxb/eru528

Kmiec, B., Teixeira, P. F., Berntsson, R. P.-A., Murcha, M. W., Branca, R. M., Radomiljac, J. D., et al. (2013). Organellar oligopeptidase (OOP) provides a complementary pathway for targeting peptide degradation in mitochondria and chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 110, E3761–E3769. doi: 10.1073/pnas.1307637110

Krieger-Liszkay, A., Kós, P. B., and Hideg, É. (2011). Superoxide anion radicals generated by methylviologen in photosystem I damage photosystem II. *Physiol. Plant.* 142, 17–25. doi: 10.1111/j.1399-3054.2010.01416.x

Lee, S., Kim, S. G., and Park, C. M. (2010). Salicylic acid promotes seed germination under high salinity by modulating antioxidant activity in Arabidopsis. *New Phytol.* 188, 626–637. doi: 10.1111/j.1469-8137.2010.03378.x

Leon, J., Lawton, M. A., and Raskin, I. (1995). Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. *Plant Physiol.* 108, 1673–1678.

Lew, R. A., Hey, N. J., Tetaz, T. J., Glucksman, M. J., Roberts, J. L., and Smith, A. I. (1995). Substrate specificity differences between recombinant rat testes endopeptidase EC 3.4. 24.15 and the native brain enzyme. *Biochem. Biophys. Res. Commun.* 209, 788–795. doi: 10.1006/bbrc.1995.1569

Leymarie, J., Vitkauskaité, G., Hoang, H. H., Gendreau, E., Chazoule, V., Meimoun, P., et al. (2012). Role of reactive oxygen species in the regulation of Arabidopsis seed dormancy. *Plant Cell Physiol.* 53, 96–106. doi: 10.1093/pcp/pcr129

Li, G., Peng, X., Wei, L., and Kang, G. (2013). Salicylic acid increases the contents of glutathione and ascorbate and temporally regulates the related gene expression in salt-stressed wheat seedlings. *Gene* 529, 321–325. doi: 10.1016/j.gene.2013.07.093

Marino, D., Dunand, C., Puppo, A., and Pauly, N. (2012). A burst of plant NADPH oxidases. *Trends Plant Sci.* 17, 9–15. doi: 10.1016/j.tplants.2011.10.001

Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, J.-P., and Riondet, C. (2012). Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. *Antioxid. Redox Signal.* 17, 1124–1160. doi: 10.1089/ars.2011.4327

Moreau, M., Westlake, T., Zampogna, G., Popescu, G., Tian, M., Noutsos, C., et al. (2013). The Arabidopsis oligopeptidases TOP1 and TOP2 are salicylic acid targets that modulate SA−mediated signaling and the immune response. *Plant J.* 76, 603–614. doi: 10.1111/tpj.12320

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Mubarakshina, M. M., Ivanov, B. N., Naydov, I. A., Hillier, W., Badger, M. R., and Krieger-Liszkay, A. (2010). Production and diffusion of chloroplastic H2O2 and its implication to signalling. *J. Exp. Bot.* 61, 3577–3587. doi: 10.1093/jxb/erq171

Mullineaux, P., Ball, L., Escobar, C., Karpinska, B., Creissen, G., and Karpinski, S. (2000). Are diverse signalling pathways integrated in the regulation of Arabidopsis antioxidant defence gene expression in response to excess excitation energy? *Philos. Trans. R. Soc. B Biol. Sci.* 355, 1531–1540. doi: 10.1098/rstb.2000.0713

Nagy, P. (2013). Kinetics and mechanisms of thiol-disulfide exchange covering direct substitution and thiol oxidation-mediated pathways. *Antioxid. Redox Signal.* 18, 1623–1641. doi: 10.1089/ars.2012.4973

Oracz, K., Bouteau, H. E. M., Farrant, J. M., Cooper, K., Belghazi, M., Job, C., et al. (2007). ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant J.* 50, 452–465. doi: 10.1111/j.1365-313X.2007.03063.x

Palmeira, C. M., Moreno, A. J., and Madeira, V. (1995). Mitochondrial bioenergetics is affected by the herbicide paraquat. *Biochim. Biophys. Acta* 1229, 187–192. doi: 10.1016/0005-2728(94)00202-G

Polge, C., Jaquinod, M., Holzer, F., Bourguignon, J., Walling, L., and Brouquisse, R. (2009). Evidence for the existence in *Arabidopsis thaliana* of the proteasome proteolytic pathway activation in response to cadmium. *J. Biol. Chem.* 284, 35412–35424. doi: 10.1074/jbc.M109.035394

Rao, M. V., Paliyath, G., Ormrod, D. P., Murr, D. P., and Watkins, C. B. (1997). Influence of salicylic acid on H2O2 production, oxidative stress, and H2O2-metabolizing enzymes (salicylic acid-mediated oxidative damage requires H2O2). *Plant Physiol.* 115, 137–149. doi: 10.1104/pp.115.1.137

Rivas-San Vicente, M., and Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *J. Exp. Bot.* 62, 3321–3338. doi: 10.1093/jxb/err031

Rouhier, N. (2010). Plant glutaredoxins: pivotal players in redox biology and iron–sulphur centre assembly. *New Phytol.* 186, 365–372. doi: 10.1111/j.1469-8137.2009.03146.x

Rüffer, M., Steipe, B., and Zenk, M. H. (1995). Evidence against specific binding of salicylic acid to plant catalase. *FEBS Lett.* 377, 175–180. doi: 10.1016/0014-5793(95)01334-2

Scheibe, R. (1991). Redox-modulation of chloroplast enzymes A common principle for individual control. *Plant Physiol.* 96, 1–3. doi: 10.1104/pp.96.1.1

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., et al. (2013). Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiol.* 162, 1815–1821. doi: 10.1104/pp.113.218156

Shirasu, K., Nakajima, H., Rajasekhar, V. K., Dixon, R. A., and Lamb, C. (1997). Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell* 9, 261–270. doi: 10.1105/tpc.9.2.261

Shivakumar, B. R., Wang, Z., Hammond, T. G., and Harris, R. C. (2005). EP24. 15 interacts with the angiotensin II type I receptor and bradykinin B2 receptor. *Cell Biochem. Funct.* 23, 195–204. doi: 10.1002/cbf.1176

Shrimpton, C. N., Glucksman, M. J., Lew, R. A., Tullai, J. W., Margulies, E. H., Roberts, J. L., et al. (1997). Thiol activation of endopeptidase EC 3.4. 24.15 A novel mechanism for the regulation of catalytic activity. *J. Biol. Chem.* 272, 17395–17399. doi: 10.1074/jbc.272.28.17395

Singh, D. K. M., Calviño, E. K., Brauer, N., Fernandez-Pozo, S., Strickler, R., Yalamanchili, H., et al. (2014). The tomato kinome and the tomato kinase library ORFeome: novel resources for the study of kinases and signal transduction in tomato and solanaceae species. *Mol. Plant Microbe Interact.* 27, 7–17. doi: 10.1094/MPMI-08-13-0218-TA

Smith, F. A., and Raven, J. A. (1979). Intracellular pH and its regulation. *Annu. Rev. Plant Physiol.* 30, 289–311. doi: 10.1146/annurev.pp.30.060179.001445

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Temple, M. D., Perrone, G. G., and Dawes, I. W. (2005). Complex cellular responses to reactive oxygen species. *Trends Cell Biol.* 15, 319–326. doi: 10.1016/j.tcb.2005.04.003

Tisljar, U., and Barrett, A. J. (1990). Thiol-dependent metallo-endopeptidase characteristics of Pz-peptidase in rat and rabbit. *Biochem. J.* 267, 531–533.

Veljovic-Jovanovic, S., Noctor, G., and Foyer, C. H. (2002). Are leaf hydrogen peroxide concentrations commonly overestimated? The potential influence of artefactual interference by tissue phenolics and ascorbate. *Plant Physiol. Biochem.* 40, 501–507. doi: 10.1016/S0981-9428(02)01417-1

Wang, Y., Li, J., Hou, S., Wang, X., Li, Y., Ren, D., et al. (2010). A Pseudomonas syringae ADP-ribosyltransferase inhibits Arabidopsis mitogen-activated protein kinase kinases. *Plant Cell* 22, 2033–2044. doi: 10.1105/tpc.110.075697

Wang, Y., and Liu, J.-H. (2012). Exogenous treatment with salicylic acid attenuates occurrence of citrus canker in susceptible navel orange (*Citrus sinensis* Osbeck). *J. Plant Physiol.* 169, 1143–1149. doi: 10.1016/j.jplph.2012.03.018

Wrzaczek, M., Brosché, M., and Kangasjärvi, J. (2013). ROS signaling loops—production, perception, regulation. *Curr. Opin. Plant Biol.* 16, 575–582. doi: 10.1016/j.pbi.2013.07.002

Wu, F.-H., Shen, S.-C., Lee, L.-Y., Lee, S.-H., Chan, M.-T., and Lin, C.-S. (2009). Tape-Arabidopsis Sandwich-a simpler Arabidopsis protoplast isolation method. *Plant Methods* 5, 1–10. doi: 10.1186/1746-4811-5-16

Yabuta, Y., Maruta, T., Yoshimura, K., Ishikawa, T., and Shigeoka, S. (2004). Two distinct redox signaling pathways for cytosolic APX induction under photooxidative stress. *Plant Cell Physiol.* 45, 1586–1594. doi: 10.1093/pcp/pch181

Yoo, S.-D., Cho, Y.-H., and Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature protoc.* 2, 1565–1572. doi: 10.1038/nprot.2007.199

York, I. A., Mo, A. X., Lemerise, K., Zeng, W., Shen, Y., Abraham, C. R., et al. (2003). The cytosolic endopeptidase, thimet oligopeptidase, destroys antigenic peptides and limits the extent of MHC class I antigen presentation. *Immunity* 18, 429–440. doi: 10.1016/S1074-7613(03)00058-X

Zhang, F., Zhang, H., Xia, Y., Wang, G., Xu, L., and Shen, Z. (2011). Exogenous application of salicylic acid alleviates cadmium toxicity and reduces hydrogen peroxide accumulation in root apoplasts of *Phaseolus aureus* and *Vicia sativa*. *Plant Cell Rep.* 30, 1475–1483. doi: 10.1007/s00299-011-1056-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 13 April 2015
doi: 10.3389/fpls.2015.00228

Signal regulators of systemic acquired resistance

Qing-Ming Gao[1†], Shifeng Zhu[1,2†], Pradeep Kachroo[1] and Aardra Kachroo[1]*

[1] Department of Plant Pathology, University of Kentucky, Lexington, KY, USA, [2] Department of Plant Biology and Ecology, College of Life Sciences, Nankai University, Tianjin, China

Salicylic acid (SA) is an important phytohormone that plays a vital role in a number of physiological responses, including plant defense. The last two decades have witnessed a number of breakthroughs related to biosynthesis, transport, perception and signaling mediated by SA. These findings demonstrate that SA plays a crictical role in both local and systemic defense responses. Systemic acquired resistance (SAR) is one such SA-dependent response. SAR is a long distance signaling mechanism that provides broad spectrum and long-lasting resistance to secondary infections throughout the plant. This unique feature makes SAR a highly desirable trait in crop production. This review summarizes the recent advances in the role of SA in SAR and discusses its relationship to other SAR inducers.

Keywords: systemic resistance, plant defense, glycerol-3-phosphate, lipids, reactive oxygen species

OPEN ACCESS

Edited by:
Hua Lu,
University of Maryland, Baltimore County, USA

Reviewed by:
Kemal Kazan,
Commonwealth Scientific and Industrial Research Organization, Australia
Steven H. Spoel,
University of Edinburgh, UK

***Correspondence:**
Aardra Kachroo,
Department of Plant Pathology, University of Kentucky, 201F Plant Science Building, 1405 Veterans drive, Lexington, KY 40546, USA
apkach2@uky.edu

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *23 January 2015*
Accepted: *23 March 2015*
Published: *13 April 2015*

Citation:
Gao Q-M, Zhu S, Kachroo P and Kachroo A (2015) Signal regulators of systemic acquired resistance. Front. Plant Sci. 6:228. doi: 10.3389/fpls.2015.00228

Introduction

Plants being sessile are constantly exposed to a number of pathogenic microbes, which based on their infectious lifestyles can be broadly divided into biotrophs and necrotrophs (Glazebrook, 2005; Mengiste, 2012; Lai and Mengiste, 2013). Biotrophic pathogens rely on nutrients from living host cells, whereas necrotrophic pathogens feed on dead cells. Plants employ distinct immune responses to counter these pathogens and this aspect has been covered in detail in several recent reviews (Spoel and Dong, 2012; Dangl et al., 2013). This first layer of host defense involves the recognition of pathogen (or microbe) associated-molecular patterns (PAMPs/MAMPs), such as bacterial flagellin, lipopolysaccharides, and peptidoglycans. PAMPs are recognized by specialized transmembrane proteins in the plant, termed pattern recognition receptors (PPRs). PRR-mediated recognition of PAMPs triggers downstream signaling leading to the activation of basal resistance termed PAMP-triggered immunity (PTI; Schwessinger and Ronald, 2012). PTI can be suppressed by pathogen encoded effector proteins commonly known as avirulence (*avr*) factors (Göhre and Robatzek, 2008; Cunnac et al., 2009; Bozkurt et al., 2011; Caillaud et al., 2012; Marrtin and Kamoun, 2012; Cheong et al., 2013; Cui et al., 2013; Dangl et al., 2013; Giraldo and Valent, 2013). The avr factors are in turn recognized by the host encoded resistance (R) proteins, which confer more durable and robust resistance termed *R* gene- or effector-triggered immunity (ETI; Bogdanove and Martin, 2000; Mackey et al., 2002, 2003; Gu et al., 2005; Jones and Dangl, 2006; Narusaka et al., 2009; Cesari et al., 2013). ETI is generally associated with programmed cell death (PCD) at the site of infection and this phenomenon is called hypersensitive response (HR; Holliday et al., 1981; Dangl et al., 1996; Morel and Dangl, 1997).

Induction of local responses is associated with the transport of defense signals throughout the plant resulting in broad-spectrum disease resistance against secondary infections. This phenomenon, known as systemic acquired resistance (SAR), is conserved among diverse

plants and confers long-lasting resistance to unrelated pathogens (Chaturvedi et al., 2008; Dempsey and Klessig, 2012; Fu and Dong, 2013; Kachroo and Robin, 2013; Lucas et al., 2013; Shah and Zeier, 2013; Wendehenne et al., 2014). Several studies have shown that the establishment of SAR involves the generation and transport of signals via phloem to the uninfected distal tissues (Guedes et al., 1980; Tuzun and Kuc, 1985). Among the signals contributing to SAR are salicylic acid (SA) and several components of the SA pathway including the methylated derivative of SA (methyl SA,MeSA, Park et al., 2007). Additionally, the diterpenoid dehydroabietinal (DA, Chaturvedi et al., 2012), the nine carbon (C9) dicarboxylic acid azelaic acid (AzA, Jung et al., 2009), an amino acid derivative pipecolic acid (Pip; Návarová et al., 2012), auxin (Truman et al., 2010), the phosphorylated sugar glycerol-3-phosphate (G3P, Chanda et al., 2011; Mandal et al., 2012; Yu et al., 2013), the free radicals nitric oxide (NO) and reactive oxygen species (ROS; Wang et al., 2014a; El-Shetehy et al., 2015), galactolipids (Gao et al., 2014), factors contributing to cuticle formation (Xia et al., 2009, 2010, 2012) and the lipid transfer proteins (LTPs) DIR1 (Defective in Induced Resistance, Maldonado et al., 2002) and AZI1 (AzA insensitive, Jung et al., 2009), have all been proposed to serve as SAR signals. Here, we review the role of SA in SAR and discuss its relationship to these various SAR signals.

SA Biosysnthesis and SAR

Salicylic acid biosynthesis occurs via the shikimic acid pathway, which forms two distinct sub-branches both of which synthesize SA. These branched pathways, designated as isochorismate synthase (ICS)- and the phenylalanine ammonia-lyase (PAL)-derived pathways, utilize chorismate as the common precursor (Shah, 2003; Chen et al., 2009; Kachroo and Kachroo, 2009; Yu et al., 2010; An and Mou, 2011; Dempsey et al., 2011; Vlot et al., 2009; Singh et al., 2013; **Figure 1**). The first step of the PAL pathway involves conversion of phenylalanine (Phe) to *trans*-cinnamic acid and this reaction is catalyzed by PAL, a key enzyme of this pathway that is induced by pathogen infection. The *Arabidopsis* genome encodes four *PAL* isoforms and *PAL* quadruple mutants or wild-type plants treated with the PAL inhibitor, 2-aminoindan-2-phosphonic acid contain reduced SA, show increased susceptibility to pathogens and are unable to induce SAR (Yalpani et al., 1993; Mauch-Mani and Slusarenko, 1996; Pallas et al., 1996; Huang et al., 2010). Although relative contributions of PAL versus ICS branches toward SA biosynthesis vary between different plant species, at least in *Arabidopsis* majority of the pathogen-induced SA appears to be derived from the ICS branch. The ICS branch involves conversion of chorismate to isochorismate by ICS followed by coversion of isochorismate to SA by isochorismate pyruvate lyase (IPL). The *Arabidopsis* genome encodes two isoforms of ICS, of which ICS1 (SID2) accounts for ~95% of basal- or pathogen-induced SA (Strawn et al., 2007; Garcion et al., 2008). A mutation in *ICS1* also impairs SAR (Wildermuth et al., 2001; Jung et al., 2009; Chanda et al., 2011; Wang et al., 2014a), suggesting that SA contributed by both PAL- and ICS-pathways is critical for the induction and/or establishment of SAR. This together with the compromised SAR phenotype of transgenic plants expressing bacterial salicylate hydroxylase (NahG; Vernooij et al., 1994), an enzyme that catalyzes the conversion of SA to catechol, reemphasize the importance

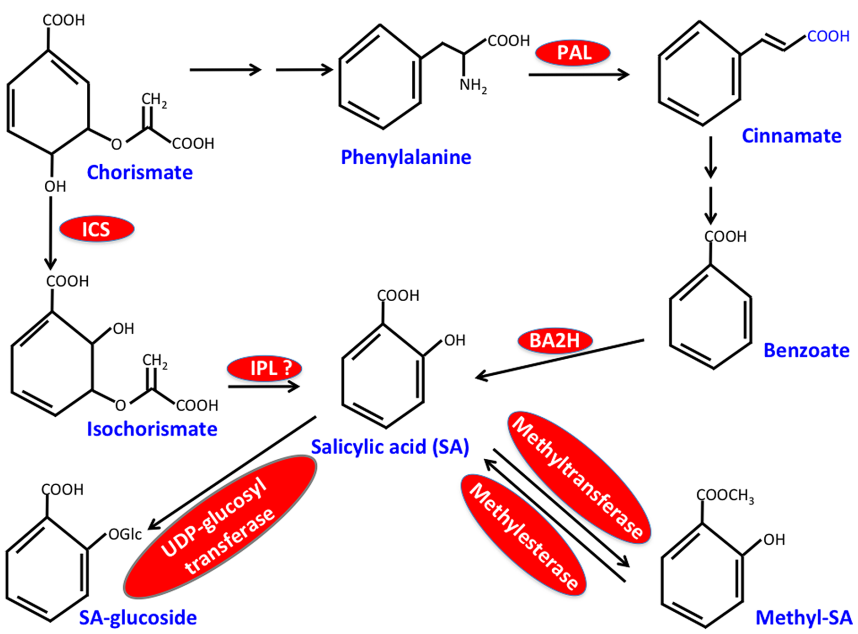


FIGURE 1 | Simplified scheme for salicylic acid (SA) biosynthesis in plants. Critical enzymes are shown in red. ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; PAL, phenylalanine ammonia-lyase; BA2H, benzoic acid 2-hydroxylase.

of SA in SAR. It is unclear what factors govern the specific recruitment of the PAL or ICS pathways for SA biosynthesis.

Salicylic acid synthesized in the chloroplasts is exported out to the cytosol via EDS5, a member of the MATE (Multidrug and Toxin Extrusion) transporter family, located in the chloroplast envelope (Nawrath et al., 2002; Serrano et al., 2013). Notably, a mutation in *EDS5* results in complete shut down of SA biosynthesis rather than SA accumulation within the chloroplasts. Thus, mutations in *ICS1* and *EDS5* similarly affect SA levels and the corresponding mutants thereby exhibit overlapping defense defects. This is likely due to negative feed-back regulation of ICS1 by SA (Fragnière et al., 2011; Serrano et al., 2013). The triphosphate tunnel metalloenzyme 2 is a negative regulator of the SA feed-back loop and functions in defense signal amplification (Ung et al., 2014). Pathogen induced expression of *ICS1* requires the binding of calmodulin binding protein CBP60g and its homolog, non-calmodulin binding SARD1 (SAR Deficient 1) to the *ICS1* promoter. CBP60g and SARD1 specifically bind the GAAATTTTGG sequence in the ICS1 promoter (Truman and Glazebrook, 2012). The induction of *ICS1* and thereby SA biosynthesis is inhibited in *cbp60g sard1* double mutant, resulting in compromised SAR (Zhang et al., 2010).

Although a number of studies have demonstrated the critical requirement of SA in SAR, a specific requirement for SA accumulation beyond basal levels during SAR has not been established. For instance, plants lacking a functional R protein RPS2 accumulate normal levels of SA in their distal tissues in response to infection by *Pseudomonas syringae pv. tomato* expressing *avrRpt2,* yet these plants are compromised for SAR (Cameron et al., 1999). Additionally, exogenous application of either G3P or AzA, which induce SAR in wild-type plants, do not induce SA accumulation. However, neither G3P nor AzA can confer SAR in *ics1* (*sid2*) mutant plants, which contain significantly reduced basal- and pathogen-induced SA. Thus, although SA is clearly critical for SAR, accumulation of SA alone is insufficient to establish SAR. Furthermore, although SA has been shown to accumulate to varying levels in the distal tissues of SAR induced plants (**Table 1**), there is no evidence suggesting that this accumulation is essential for SAR.

In comparison to local tissues, the distal tissues of SAR-induced plants have been shown to accumulate a broad range of SA ranging from as low as 10 ng/ g FW to ∼2.6 μg/g FW (**Table 1**; Rasmussen et al., 1991; Yalpani et al., 1991; Meuwly and Métraux, 1993; Molders et al., 1994; Vernooij et al., 1994; Lawton et al., 1995; Shulaev et al., 1995; Cameron et al., 1999; Kiefer and Slusarenko, 2003; Mishina and Zeier, 2006; Attaran et al., 2009; Liu et al., 2010, 2011; Xia et al., 2010; Chanda et al., 2011; Gao et al., 2014). The inability to accumulate SA in distal tissues has also been suggested to contribute to impaired SAR in *ald1* (*agd2*-Like Defense response protein 1) and *fmo1* (Flavin Monooxygenase 1) mutants, both of which accumulate normal SA in the local tissue (Song et al., 2004a,b; Mishina and Zeier, 2006). ALD1 encodes an aminotransferase that catalyzes the biosynthesis of the SAR inducer Pip, (Song et al., 2004b; Návarová et al., 2012) and FMO1 has been suggested to function downstream of Pip (Návarová et al., 2012). Thus, other factors besides SA might contribute to the SAR defect of *ald1* and *fmo1* mutants. One possibility is that SAR can be induced via SA-independent factors so long as a minimum basal level of SA can be maintained. Alternatively, SA accumulation in distal tissues might contribute to the priming process resulting in the activation of stronger defense responses upon secondary infections (Návarová et al., 2012; Gruner et al., 2013).

SA-Derivatives and SAR

A majority of the synthesized SA is converted and stored as biologically inactive derivatives via glucosylation, methylation and amino acid conjugation since accumulation of the acidic SA has adverse physiological consequences (Heil and Baldwin, 2002; Heidel et al., 2004). These include SA 2-*O*-β-D-glucose (SAG), SA glucose ester (SGE), methyl SA (MeSA), and SA-amino acid conjugates (Pierpoint, 1994; Vlot et al., 2009; Dempsey et al., 2011). Most recently, SA was shown to be derivatized to 2,3-dihydroxybenzoic acid (2,3-DHBA) and this reaction is catalyzed by SA 3-hydroxylase (S3H; Zhang et al., 2013). As predicted *s3h* knockout plants contain very high levels of SA, while plants expressing *S3H* gain-of-function mutations accumulate high amounts of 2,3-DHBA (Zhang et al., 2013). SA derivatives serve as storage forms that can be converted back to free SA (Hennig et al., 1993; Kawano et al., 2004; Kachroo and Kachroo, 2012). With the exception of MeSA however, the exact role of SA derivatives in SAR remains unclear.

Methyl SA is a volatile and phloem mobile SA derivative, which accumulates in infected and distal tissues in response to pathogen infection. Like MeSA, SA also accumulates in the phloem of tobacco leaves infected with tobacco mosaic virus or *Colletotrichum lagenarium* and in cucumber leaves infected with tobacco necrosis virus (Malamy et al., 1990; Métraux et al., 1990; Park et al., 2007). For SAR, MeSA must be converted to SA in the distal tissues between the 48–72 h period post primary infection. This time-frame correlates with that of pathogen-inducible MeSA accumulation in infected and systemic tissues. The biosynthesis of MeSA is catalyzed by SA methyltransferases (SAMT/BSMT), and the conversion of MeSA back to SA is mediated by methyl esterase (MES; Chen et al., 2003; Effmert et al., 2005; Koo et al., 2007). The tobacco MES was first identified based on its ability to bind SA, and therefore designated as SA-binding protein 2 (SABP2; Kumar and Klessig, 2003). Grafting studies in tobacco plants silenced for SABP2 have shown that SABP2 activity in scions, but not root-stocks is required for normal SAR (Park et al., 2007). Furthermore, the synthetic SA analog, 2,2,2,2′-tetra-fluoroacetophenone, which inhibits the esterase activity of SABP2, also inhibits SAR (Park et al., 2009). As in tobacco, homologs of SABP2 (AtMES9) and SAMT AtBSMT1 are required for SAR in *Arabidopsis* (Liu et al., 2011). Thus, the ability to derivatize SA to MeSA and reconvert MeSA back to SA are critical for SAR. Intriguingly, the requirement for *AtBSMT1* in SAR can be bypassed by prolonged exposure to light after pathogen inoculation (Attaran et al., 2009; Liu et al., 2011). However, the role of light signaling in SAR and how it might compensate for MeSA

TABLE 1 | Free and bound salicylic acid (SA) levels reported in distal tissues of mock-and pathogen-inoculated plants.

Free SA (ng/gFW)		Bound SA (SAG; ng/gFW)		Pathogen used, and concentration	Host	HPI[a]	Analytical procedure used	Reference
Mock	SAR- induced tissues	Mock	SAR- induced tissues					
~80	~1250	NA[b]	NA	*Psm*[d] *ES4326* expressing *AvrRpm1*, $OD_{600} = 0.01$	*Arabidopsis*	48	GC-MS[g]	Attaran et al. (2009)
~200	~2600	NA	NA	*Pst*[e] *DC3000 expressing AvrRpt2*, 1×10^7 CFU[f]/ml	*Arabidopsis*	48	HPLC[h]	Singh et al. (2013)
~ 50–150	~370–1200	~100–250	~300–1000	Tobacco mosaic virus	Tobacco	144	HPLC	Shulaev et al. (1995)
~30–70	~30–300	NA	NA	*Psm DC3000 expressing AvrRpml*, 5×10^7 CFU/ml	*Arabidopsis*	48	HPLC	Kiefer and Slusarenko (2003)
~100	~450	NA	NA	*Psm ES4326* expressing *AvrRpml*, $OD_{600} = 0.02$	*Arabidopsis*	48	GC-MS	Mishina and Zeier (2006)
~5.1	~21.3	ND[c]	~287.3	*Pseudomonas lachrymans*, 4×10^8 CFU/ml	Cucumber	120	HPLC	Meuwly and Métraux (1993)
~70	~200	~130	~400	*Pst DC3000 expressing AvrRpt2*, 10^7 CFU/ml	*Arabidopsis*	48	HPLC	Lawton et al. (1995)
~160	~400	~1300	~1800	*Pst DC3000 expressing AvrRpt2*,	*Arabidopsis*	48	HPLC	Gao et al. (2014)
~420	~500	~500	~1600	*Coronatine-deficient Psm expressing AvrRpt2*,1×10^6 CFU/ml	*Arabidopsis*	48	HPLC	Liu et al. (2011)
~80	~100	~470	~700	*Pst DC3000 expressing AvrRpt2*,	*Arabidopsis*	48	HPLC	Cameron et al. (1999)
~35	~60	~700	~1600	*Pst DC3000 expressing AvrRpt2*, 1×10^6 CFU/ml	*Arabidopsis*	48	HPLC	Chanda et al. (2011)
~220	~300	~210	~1200	*Coronatine-deficient Psm expressing*	*Arabidopsis*	60	HPLC	Liu et al. (2010)
~40	~60	~450	~800	*Pst DC3000 expressing AvrRpt2*, 1×10^6 CFU/ml	*Arabidopsis*	48	HPLC	Wang et al. (2014a)
~40	~65	~600	~1600	*Pst DC3000 expressing AvrRpt2*, 1×10^6 CFU/ml	*Arabidopsis*	48	HPLC	Xia et al. (2009)
~32–52	~51–83	~70–164	~82–196	Tobacco mosaic virus	Tobacco	168	HPLC	Vernooij et al. (1994)
~40	~90	~20	~85	Tobacco necrosis virus	Cucumber	53	HPLC	Molders et al. (1994)

[a]*HPI, hour post infection;* [b]*NA, not available;* [c]*ND, not detected;* [d]*Psm, P. syringae pv maculicola;* [e]*Pst, P. syringae pv tomota;*[f]*CFU, colony-forming unit;* [g]*GC-MS, gas chromatography-mass spectrometry;* [h]*HPLC, high performance liquid hromatography.*

is unclear. It is also not known whether MeSA merely functions to deliver SA to the distal tissues or has other function(s) in SAR. Notably, a certain percentage of SA is always transported from the inoculated to distal tissues (Meuwly et al., 1995; Kiefer and Slusarenko, 2003). The biological significance of this transport is unclear, particularly in view of the fact that SA is not considered to be the mobile SAR signal since wild-type tobacco scions grafted onto NahG root-stocks exhibit normal SAR (Vernooij et al., 1994; Meuwly et al., 1995; Kiefer and Slusarenko, 2003).

Regulation of SA Accumulation and SAR

Besides proteins that directly contribute to SA biosynthesis (ICS and PAL) or transport (EDS5), a number of other proteins have been identified that participate in pathogen induced SA accumulation and thereby SAR. These include EDS1 (Enhanced Disease Susceptibility 1), PAD4 (Phytoalexin Deficient 4), and NDR1 (Non-race-specific Disease Resistance 1; Century et al., 1995, 1997; Falk et al., 1999; Jirage et al., 1999; McDowell et al., 2000; Feys et al., 2001; Coppinger et al., 2004; Ishihara et al., 2008; Bhattacharjee et al., 2011; Cacas et al., 2011; Heidrich et al., 2011; Knepper et al., 2011; Lu et al., 2013). Unlike *ICS1* and *EDS5*, mutations in *EDS1*, *PAD4*, or *NDR1* cause partial reduction in SA levels. EDS1 and PAD4 are lipase-like proteins, which together with another lipase-like protein SAG101 (Senescence Associate Gene 101) form binary and ternary complexes (Feys et al., 2005; Zhu et al., 2011). EDS1 interacts with PAD4 in both cytosol and nucleus, and with SAG101 only in the nucleus. EDS1, PAD4, and SAG101 function cooperatively as well as independently in pathogen defense (Feys et al., 2005; Venugopal et al., 2009; Rietz et al., 2011; Zhu et al., 2011). For instance, all three proteins are required for R-mediated resistance against Turnip crinkle virus (TCV) but only PAD4 is required for the SA-mediated induction of the *R* gene which confers HR against TCV (HRT; Chandra-Shekara et al., 2004, 2006, 2007). Interestingly, EDS1, but not PAD4 or SAG101, interacts with HRT and potentiates HRT-mediated HR to TCV (Zhu et al., 2011). Similarly, only PAD4 is required for resistance to the green peach aphid, whereas EDS1 and SAG101 are not (Pegadaraju et al., 2005, 2007; Louis et al., 2010, 2012). Both nuclear and extranuclear localization of EDS1 is important for its defense function (García et al., 2010). However, the role of binary or ternary complex formation between EDS1, PAD4, and SAG101 proteins remains unknown. EDS1 was recently shown to participate in both SAR signal generation in the local tissues as well as perception in the distal leaves (Breitenbach et al., 2014).

The *Arabidopsis* genome encodes two isoforms of EDS1 that function redundantly and can compensate for each other (Zhu et al., 2011). However, some *Arabidopsis* ecotypes, such as Wassilewskija, Landsberg, and Dujon, contain only one functional EDS1 isoform, and this is sufficient for normal resistance in these ecotypes. Like *Arabidopsis*, soybean also contains two EDS1 isoforms. Interestingly, *Arabidopsis eds1* mutant expressing the soybean *EDS1* orthologs is only partially restored in SA levels, but completely restored in bacterial resistance (Wang et al., 2014b). This further questions the requirement for increased SA accumulation during defense activation and raises the possibility that a certain threshold of SA may be sufficient to induce appropriate defense responses. The soybean *EDS1* orthologs are unable to potentiate TCV coat protein-derived activation of HRT even though they do interact with HRT (Wang et al., 2014b). This suggests that EDS1 orthologs in different plants may have evolved to perform overlapping as well as distinct functions.

SA Signaling Components

Salicylic acid-mediated signaling leading to SAR is dependent on the ankyrin repeat containing protein NPR1 [Non-expressor of *Pathogenesis-Related* (*PR*) genes] (Dong, 2004). Under basal or uninduced conditions, NPR1 exists as a cytosolic inactive oligomer formed by intermolecular disulfide bonding (Mou et al., 2003). Reducing conditions resulting from accumulation of SA cause dissociation of the NPR1 oligomer into active monomers and the monomeric form of NPR1 is translocated into the nucleus (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2008). Nuclear localization of NPR1 facilitates its interaction with members of the TGACG motif binding (TGA) transcription factors that belong to the basic leucine zipper (bZIP) protein family (Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000; Zhou et al., 2000; Chern et al., 2001; Fan and Dong, 2002; Kim and Delaney, 2002). This in turn enhances binding of the TGA factors to promoter elements of NPR1-dependent target genes (Wang et al., 2006, 2011). Like NPR1, TGA factors are also required for SAR; the *tga2 tga5 tga6* triple mutant is non-responsive to SA and is defective in SAR (Zhang et al., 2003). Recent studies have shown that NPR1 and TGA1 also undergo *S*-nitrosylation, which is necessary for the proper functioning of NPR1 in immunity and increases the DNA binding activity of TGA1 (Tada et al., 2008; Lindermayr et al., 2010). On the other hand, thiol *S*-nitrosylation has also been shown to promote NPR1 oligomerization and thereby its inactivation (Tada et al., 2008). The nuclear NPR1 is phosphorylated and degraded in a proteasome-dependent manner (Spoel et al., 2009), and the turnover of NPR1 is essential for SAR establishment. The *Arabidopsis* genome contains five paralogs of NPR1 (Liu et al., 2005). Like NPR1, NPR3, and NPR4 also interact with TGA proteins (Zhang et al., 2006). The *npr3 npr4* mutant plants accumulate elevated levels of NPR1 and are consequently defective in SAR. NPR3 and NPR4 bind SA and function as adaptors of the Cullin 3 ubiquitin E3 ligase to mediate NPR1 degradation in an SA-dependent manner (Fu et al., 2012). However, the two differ in that NPR3 has higher affinity for SA than NPR4, and SA promotes the NPR1–NPR3 interaction but inhibits the NPR1–NPR4 interaction. These contrasting effects might offer a possible explanation for the nuances underlying NPR1-dependent immunity under different levels of SA. For instance, high concentration of SA in infected tissues might favor binding of NPR3 with SA, which would mediate degradation of the cell-death suppressor NPR1, and initiate PCD and local immunity. On the other hand, lower SA levels in the distal uninfected tissue would minimize NPR3-SA binding, thereby inhibiting PCD. Interestingly, in yet another study, NPR1 was

also shown to bind SA via the transition metal copper (Wu et al., 2012; Manohar et al., 2015). The binding of SA to NPR was suggested to induce a conformational change in NPR1 (Wu et al., 2012), which in turn is important for NPR1-dependant *PR1* expression.

NPR1 is also required for transgenerational SAR, which in turn involves epigenetic changes (Jaskiewicz et al., 2011; Luna et al., 2012). NPR1 othologs have been characterized from a number of plants including rice, tobacco, soybean, and cacao (Chern et al., 2001, 2005, 2014; Ekengren et al., 2003; Zwicker et al., 2007; Sandhu et al., 2009; Shi et al., 2010; Chen et al., 2013). Transgenic expression of *Arabidopsis NPR1* confers enhanced resistance in heterologous plants (Lin et al., 2004; Shi et al., 2010; Chen et al., 2012). Conversely, transgenic expression of soybean orthologs can complement the *Arabidopsis npr1* mutation (Sandhu et al., 2009). Overexpression of NPR1 also enhances pathogen resistance in monocots (Chern et al., 2005; Yuan et al., 2007). However, studies in rice and barley suggest that NPR1 function may not be fully conserved in monocots and dicots and that SA signaling and SAR in monocots might involve NPR1-independent pathways (Shimono et al., 2007; Dey et al., 2014). Transcription analysis in distal tissues revealed that bacteria-triggered SAR in barley was likely associated with jasmonic acid, ethylene and ABA, rather than SA. In contrast, SAR in maize is associated with SA accumulation in local and distal leaves (Balmer et al., 2013). Additionally, petiole exudates from pathogen infected *Arabidopsis* plants induced SAR in wheat (Chaturvedi et al., 2008). This suggests that SAR signaling in barley may not be similar to that in other monocots like maize and wheat.

The stability of NPR1 is dependent on Mediator (MED) 16 [allelic to Sensitive to Freezing (SFR) 6] (McKown et al., 1996; Warren et al., 1996), a subunit of the MED complex which functions as a bridge between transcription factors and the general RNA polymerase II transcriptional machinery (Zhang et al., 2012). A mutation in *MED16* compromises SAR and SA-induced defense responses but does not affect SA levels or nuclear localization of NPR1. Thus, MED16 likely functions downstream of SA in the SAR pathway. Interestingly, MED16 is also required for jasmonic acid/ethylene-responsive gene expression and resistance to necrotrophic pathogens (Zhang et al., 2012). Thus, MED16 might function by relaying signals from transcription factors that are specific to the SA and JA/ethylene pathways. A mutation in another MED subunit, MED 15 (isolated in a screen for non-recognition-of-the SA analog, BTH, *nrb4*), also attenuates SAR and SA responsiveness (Canet et al., 2012). However, MED15 is not required for NPR1 stability or localization and likely functions downstream of NPR1.

SA versus Other SAR Inducers

Systemic acquired resistance is a complex phenomenon that involves the interplay of a diverse group of chemicals and associated proteins, besides SA. Most of these molecules can now be placed in one of two main branches that comprise the SAR pathway. One branch involves SA and its signaling component NPR1, and the other branch involves the free radicals NO and ROS, which function directly upstream of AzA, which in turn is upstream of G3P (Wang et al., 2014a; Wendehenne et al., 2014; El-Shetehy et al., 2015). Unlike G3P and AzA, exogenous application of Pip or DA induces SA accumulation in the absence of pathogen infection (Chaturvedi et al., 2012; Návarová et al., 2012). Therefore, Pip and DA likely function in the SA branch of SAR. The presence of two SAR branches is supported by the fact that SA cannot restore SAR in mutants defective in NO, ROS, or G3P biosynthesis, while NO/ROS cannot confer SAR on mutants defective in SA synthesis or signaling. Furthermore, pharmacological inhibitors of NO synthesis or NO scavengers attenuate SA-induced SAR in tobacco (Song and Goodman, 2001). Interestingly, unlike SA, both NO and ROS function in a concentration dependent manner because they can confer SAR only when present at an optimal concentration (Wang et al., 2014a). Free radicals are well known to operate similarly in animal systems where too little or too much can produce opposing physiological effects (Delledonne et al., 1998; Besson-Bard et al., 2008; Wink et al., 2011). Free radicals are thought to participate in SAR by mediating the oxidation of carbon (C) 18 unsaturated fatty acids (FAs) containing a double bond on C 9. This results in the formation of 9-oxo nonanoic acid (ONA), which is converted to the di-carboxylic acid AzA by the addition of a carboxylic group. AzA is unable to confer SAR on mutants unable to synthesize G3P, indicating it functions upstream of G3P. Exogenous AzA increases the expression of the G3P synthesizing *GLY1* and *GLI1* genes, which encode G3P dehydrogenase and glycerol kinase, respectively. G3P operates in a feedback loop with the LTPs DIR1 and AZI1 such that lack of DIR1 or AZI1 impairs pathogen-induced G3P accumulation while lack of G3P results in reduced *DIR1* and *AZI1* transcripts (Yu et al., 2013). DIR1 and AZI1 form homo- and hetromers suggesting that a complex comprising these proteins might function in SAR. Perhaps such a complex or the individual LTPs serve in transporting SAR essential signal(s) to the distal tissues. G3P appears to be the logical choice for such a transported signal since it is a precursor for lipid biogenesis. However, no direct interaction could be detected between G3P and DIR1 raising the possibility that G3P may be derivatized and this derivative may then be transported from infected to distal tissues. Radiolabel feeding experiments showed that G3P is indeed converted to an as yet unidentified derivative which can translocate from infected to distal tissues in a DIR1-dependent manner (Chanda et al., 2011).

Recent studies have shown that the C 18 FAs which serve as precusors for AzA are derived from the major plastidal lipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which comprise ∼80% of the total lipids in plants (Zoeller et al., 2012; Gao et al., 2014). Thus, besides SA, NO, ROS and G3P, chloroplasts also serve as an important site for AzA biosynthesis. Notably, both galactose sugars in DGDG appear to be important for SAR since *dgd1* plants producing α-glucose-β-galactose diacylglycerol via transgenic expression of a bacterial glucosyltransferase, are not restored in SAR even though they are partially restored in chloroplast function. Thus it appears that the position of the hydroxyl group on C 4 of

galactose may be important for SAR since glucose and galactose are sterioisomeric sugars which differ only in the position of their axial hydroxyl group at C 4.

Cross Talk between SA and NO Pathways in SAR

Monogalactosyldiacylglycerol and DGDG galactolipids also serve additional functions in SAR. For instance, DGDG is required for SA and NO biosynthesis (Gao et al., 2014) and for AzA responsiveness. Interestingly, in spite of their impaired SA and NO synthesis, petiole exudates from pathogen-infected *dgd1* plants were able to confer SAR in wild-type plants. This suggests that *dgd1* plants can make signals that are capable of inducing SA- and NO-synthesis in plants with normal DGDG levels. These results show that SAR involves DGDG-dependent retrograde signaling between the chloroplast and nucleus and emphasizes the fact that the two branches of SAR are intricately linked (Gao et al., 2014).

In fact it is well known that there is cross talk between SA- and NO-mediated signaling. For example, NO mediated *S*-nitrosylation of NPR1 can result in the oligomerization and nuclear localization of NPR1 (Tada et al., 2008; Lindermayr et al., 2010). Moreover, SA has been suggested to regulate chloroplast structure since exogenous SA can cause swelling of grana thylakoids, coagulation of the stroma and increased chloroplast volume (Uzunova and Popova, 2000; Rivas-San Vicente and Plasencia, 2011). Regulation of SA and AzA levels by EDS1 is another case in point (Wittek et al., 2014). Together, these results suggest that the parallel operation of the interlinked SA- and NO-pathways might allow multiple points of regulation in fine tuning the optimal onset of SAR. This may be particularly relevant for signals like NO and ROS, which are functional within specific concentration ranges (Wang et al., 2014a).

Conclusion and Perspectives

Recent work on SAR has identified a number of chemical and protein signals and placed them in a common pathway that comprises at least two parallel branches (**Figure 2**). However, these studies also indicate the involvement of additional unknown signal(s) that function upstream of the branchpoint separating SA-NPR1- and NO-ROS-AzA-G3P-derived pathways. In addition, several chemical signals, including G3P and AzA, undergo derivatization into unknown compounds and at least one of the G3P-derivative is SAR bioactive (unpublished data). Identification of these

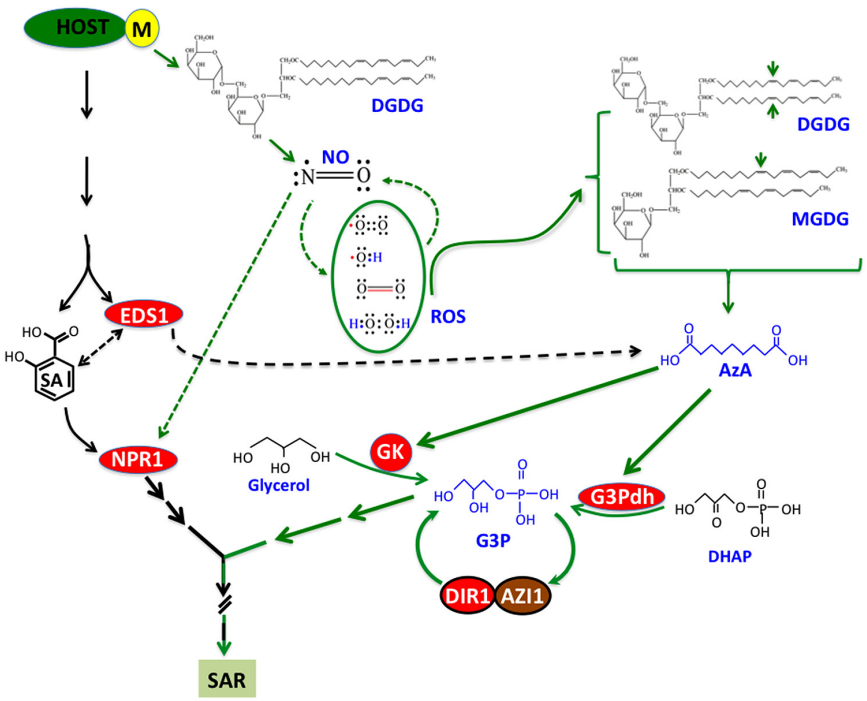


FIGURE 2 | A simplified model showing pathways, chemicals, and proteins involved in SAR. Infection with avirulent (avr) pathogen induces accumulation of SA and nitric oxide (NO) in a digalactosyldiacylglycerol (DGDG)-dependent manner. NO operates in a feedback loop with reactive oxygen species (ROS), which catalyze oxidation of C18 unsaturated fatty acids (FA) present on monogalactosyldiacylglycerol (MGDG) and DGDG lipids. Oxidation of C18 FAs at C9 carbon (indicated by the arrows) generates azelaic acid (AzA), which triggers biosynthesis of glycerol-3-phosphate (G3P) via upregulation of genes encoding G3P biosynthetic enzymes (glycerol kinase, GK and G3P dehydrogenase, G3Pdh). G3P-mediated signaling is dependent on DIR1 and AZI1, which interact with each other and require G3P for the stability of their respective transcripts. Conversely, DIR1 and AZI1 are also required for G3P biosynthesis, suggesting that G3P and DIR1/AZI1 regulate SAR via a feedback loop. In the SA branch, EDS1 regulates both SA and AzA levels. NPR1 is a key downstream component in the SA branch which is nitrosylated by NO.

signals should provide useful insights into signaling events leading to the induction and establishment of SAR. Another area of SAR research that has not received much attention is the transport and perception of signals in the distal tissues. Although cuticle was implicated in the perception of SAR signals (Xia et al., 2009), later studies on cuticle mutants have suggested that perception might relate to the severity of cuticular damage or perhaps other unknown factors (Xia et al., 2012). These aspects of SAR should provide exciting avenues for studying how SAR overlaps with basic physiological processes and the distinct events that decide the onset of SAR versus normal growth and development.

Acknowledgments

We thank all members (past and present) of the Kachroo (AK and PK) laboratories who contributed to SA and SAR related work. Work in our laboratories is funded from the National Science Foundation (IOS# 0749731, #051909), Kentucky Soybean Board and the Kentucky Science and Engineering Foundation (KSF-2923-RDE-016). The information reported in this article (No. 15-12-039) is part of a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

References

An, C., and Mou, Z. (2011). Salicylic acid and its function in plant immunity. *J. Integr. Plant Biol.* 53, 412–428. doi: 10.1111/j.1744-7909.2011.01043.x

Attaran, E., Zeier, T. E., Griebel, T., and Zeier, J. (2009). Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis*. *Plant Cell* 21, 954–971. doi: 10.1105/tpc.108.063164

Balmer, D., de Papajewski, D. V., Planchamp, C., Glauser, G., and Mauch-Mani, B. (2013). Induced resistance in maize is based on organ-specific defence responses. *Plant J.* 74, 213–225. doi: 10.1111/tpj.12114

Besson-Bard, A., Pugin, A., and Wendehenne, D. (2008). New insights into nitric oxide signaling in plants. *Annu. Rev. Plant Biol.* 59, 21–39. doi: 10.1146/annurev.arplant.59.032607.092830

Bhattacharjee, S., Halane, M. K., Kim, S. H., and Gassmann, W. (2011). Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators. *Science* 334, 1405–1408. doi: 10.1126/science.1211592

Bogdanove, A. J., and Martin, G. B. (2000). AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8836–8840. doi: 10.1073/pnas.97.16.8836

Bozkurt, T. O., Schornack, S., Win, J., Shindo, T., Ilyas, M., Oliva, R., et al. (2011). Phytophthora infestans effector AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20832–20837. doi: 10.1073/pnas.1112708109

Breitenbach, H. H., Wenig, M., Wittek, F., Jordá, L., Maldonado-Alconada, A. M., Sarioglu, H., et al. (2014). Contrasting roles of the apoplastic aspartyl protease apoplastic, enhanced disease susceptibility1-dependent1 and legume lectin-like protein1 in *arabidopsis* systemic acquired resistance. *Plant Physiol.* 165, 791–809. doi: 10.1104/pp.114.239665

Cacas, J. L., Petitot, A. S., Bernier, L., Estevan, J., Conejero, G., Mongrand, S., et al. (2011). Identification and characterization of the non-race specific disease resistance 1 (NDR1) orthologous protein in coffee. *BMC Plant Biol.* 11:144. doi: 10.1186/1471-2229-11-144

Caillaud, M. C., Piquerez, S. J., Fabro, G., Steinbrenner, J., Ishaque, N., Beynon, J., et al. (2012). Subcellular localization of the Hpa RxLR effector repertoire identifies a tonoplast-associated protein HaRxL17 that confers enhanced plant susceptibility. *Plant J.* 69, 252–265. doi: 10.1111/j.1365-313X.2011.04787.x

Cameron, R., Paiva, N. L., Lamb, C. J., and Dixon, R. A. (1999). Accumulation of salicylic acid and PR-1 gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. tomato in *Arabidopsis*. *Physiol. Mol. Plant Pathol.* 55, 121–130. doi: 10.1006/pmpp.1999.0214

Canet, J. V., Dobón, A., and Tornero, P. (2012). Non-recognition-of-BTH4, an *Arabidopsis* mediator subunit homolog, is necessary for development and response to salicylic acid. *Plant Cell* 24, 4220–4235. doi: 10.1105/tpc.112.103028

Century, K. S., Holub, E. B., and Staskawicz, B. J. (1995). NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6597–6601. doi: 10.1073/pnas.92.14.6597

Century, K. S., Shapiro, A. D., Repetti, P. P., Dahlbeck, D., Holub E., and Staskawicz, B. J. (1997). NDR1, a pathogen-induced component required for *Arabidopsis* disease resistance. *Science* 278, 1963–1965. doi: 10.1126/science.278.5345.1963

Cesari, S., Thillieza, G., Ribota, C., Chalvona, V., Michela, C., Jauneauc, A., et al. (2013). The rice resistance protein pair RGA4/RGA5 recognizes the magnaporthe oryzae effectors AVR-Pia and AVR1-CO39 by direct binding. *Plant Cell* 25, 1463–1481. doi: 10.1105/tpc.112.107201

Chanda, B., Xia, Y., Mandal, M. K., Sekine, K. T., Gao, Q. M., Selote, D., et al. (2011). Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nat. Genet.* 43, 421–427. doi: 10.1038/ng.798

Chandra-Shekara, A. C., Gupte, M, Navarre, D., Raina, S., Raina, R., Klessig, D. F., et al. (2006). Light-dependent hypersensitive response and resistance signaling to *turnip crinkle virus* in *Arabidopsis*. *Plant J.* 45, 320–334. doi: 10.1111/j.1365-313X.2005.02618.x

Chandra-Shekara, A. C., Navarre, D., Kachroo, A., Kang, H. G., Klessig, D. F., and Kachroo, P. (2004). Signaling requirements and role of salicylic acid in HRT- and rrt-mediated resistance to *turnip crinkle virus* in *Arabidopsis*. *Plant J.* 40, 647–659. doi: 10.1111/j.1365-313X.2004.02241.x

Chandra-Shekara, A. C., Venugopal, S. C., Barman, S. R., Kachroo, A., and Kachroo, P. (2007). Plastidial fatty acid levels regulate resistance gene-dependent defense signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7277–7282. doi: 10.1073/pnas.0609259104

Chaturvedi, R., Krothapalli, K., Makandar, R., Nandi, A., Sparks, A., Roth, M., et al. (2008). Plastid omega3-fatty acid desaturase-dependent accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. *Plant J.* 54, 106–117. doi: 10.1111/j.1365-313X.2007.03400.x

Chaturvedi, R., Venables, B., Petros, R. A., Nalam, V., Li, M., Wang, X., et al. (2012). An abietane diterpenoid is a potent activator of systemic acquired resistance. *Plant J.* 71, 161–172. doi: 10.1111/j.1365-313X.2012.04981.x

Chen, F., D'Auria, J. C., Tholl, D., Ross, J. R., Gershenzon, J., Noel, J. P., et al. (2003). An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J.* 36, 577–588. doi: 10.1046/j.1365-313X.2003.01902.x

Chen, J. C., Lu, H. C., Chen, C. E., Hsu, H. F., Chen, H. H., and Yeh, H. H. (2013). The NPR1 ortholog PhaNPR1 is required for the induction of PhaPR1 in *Phalaenopsis aphrodite*. *Bot. Stud.* 54, 31. doi: 10.1186/1999-3110-54-31

Chen, X. K., Zhang, J. Y., Zhang, Z., Du, X. L., Du, B. B., and Qu, S. C. (2012). Overexpressing MhNPR1 in transgenic Fuji apples enhances resistance to apple powdery mildew. *Mol. Biol. Rep.* 39, 8083–8089. doi: 10.1007/s11033-012-1655-3

Chen, Z., Zheng, Z., Huang, J., Lai, Z., and Fan, B. (2009). Biosynthesis of salicylic acid in plants. *Plant Signal. Behav.* 4, 493–496. doi: 10.4161/psb.4.6.8392

Cheong, H., Kim, C. Y., Jeon, J. S., Lee, B. M., Moon S. J., and Hwang, I. (2013). *Xanthomonas oryzae* pv. oryzae type III effector XopN targets OsVOZ2 and a putative thiamine synthase as a virulence factor in rice. *PLoS ONE* 8:e73346. doi: 10.1371/journal.pone.0073346

Chern, M., Bai, W., Ruan, D., Oh, T., Chen, X., and Ronald, P. C. (2014). Interaction specificity and coexpression of rice NPR1 homologs 1 and 3 (NH1

and NH3), TGA transcription factors and Negative Regulator of Resistance (NRR) proteins. *BMC Genomics* 15:461. doi: 10.1186/1471-2164-15-461

Chern, M., Fitzgerald, H. A., Canlas, P. E., Navarre, D. A., and Ronald, P. C. (2005). Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. *Mol. Plant Microbe Interact.* 6, 511–520. doi: 10.1094/MPMI-18-0511

Chern, M. S., Fitzgerald, H. A., Yadav, R. C., Canlas, P. E., Dong, X., and Ronald, P. C. (2001). Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in *Arabidopsis*. *Plant J.* 27, 101–113. doi: 10.1046/j.1365-313x.2001.01070.x

Coppinger, P., Repetti, P. P., Day, B., Dahlbeck, D., Mehlert, A., and Staskawicz, B. J. (2004). Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. *Plant J.* 40, 225–237. doi: 10.1111/j.1365-313X.2004.02203.x

Cui, F., Wu, S., Sun, W., Coaket, G., Kunkel, B., He, P., et al. (2013). The *Pseudomonas syringae* type III effector AvrRpt2 promotes pathogen virulence via stimulating *Arabidopsis* auxin/indole acetic acid protein turnover. *Plant Physiol.* 162, 1018–1029. doi: 10.1104/pp.113.219659

Cunnac, S., Lindeberg, M., and Collmer, A. (2009). *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. *Curr. Opin. Microbiol.* 12, 53–60. doi: 10.1016/j.mib.2008.12.003

Dangl, J. L., Dietrich, R. A., and Richberg, M. H. (1996). Death don't have nomercy: cell death programs in plant-microbe interactions. *Plant Cell* 8, 1793–1807. doi: 10.1105/tpc.8.10.1793

Dangl, J. L., Horvath, D. M., and Staskawicz, B. J. (2013). Pivoting the plant immune system from dissection to deployment. *Science* 341, 746–751. doi: 10.1126/science.1236011

Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* 394, 585–588. doi: 10.1038/29087

Dempsey, D. A., and Klessig, D. F. (2012). SOS-too many signals for systemic acquired resistance? *Trends Plant Sci.* 17, 538–545. doi: 10.1016/j.tplants.2012.05.011

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arabidopsis Book* 9, e0156. doi: 10.1199/tab.0156

Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P. R. (2000). The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12, 279–290. doi: 10.1105/tpc.12.2.279

Dey, S., Wenig, M., Langen, G., Sharma, S., Kugler, K. G., Knappe, C., et al. (2014). Bacteria-triggered systemic immunity in barley is associated with WRKY and ETHYLENE RESPONSIVE FACTORs but not with salicylic acid. *Plant Physiol.* 166, 2133–2151. doi: 10.1104/pp.114.249276

Dong, X. (2004). NPR1, all things considered. *Curr. Opin. Plant Biol.* 7, 547–552. doi: 10.1016/j.pbi.2004.07.005

Effmert, U., Saschenbrecker, S., Ross, J., Negre, F., Fraser, C. M., Noel, J. P., et al. (2005). Floral benzenoid carboxyl methyltransferases: from in vitro to in planta function. *Phytochemistry* 66, 1211–1230. doi: 10.1016/j.phytochem.2005.03.031

Ekengren, S. K., Liu, Y., Schiff, M., Dinesh-Kumar, S. P., and Martin, G. B. (2003). Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. *Plant J.* 36, 905–917. doi: 10.1046/j.1365-313X.2003.01944.x

El-Shetehy, M., Wang, C., Shine, M. B., Yu, K., Navarre, D., Wendehenne, D., et al. (2015). Nitric oxide and reactive oxygen species are required for systemic acquired resistance in plants. *Plant Signal. Behav.* In Press.

Falk, A., Feys, B. J., Frost, L. N., Jonathan, D. G., Jones, D. G., Michael, J., et al. (1999). EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3292–3297. doi: 10.1073/pnas.96.6.3292

Fan, W., and Dong, X. (2002). In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in *Arabidopsis*. *Plant Cell* 14, 1377–1389. doi: 10.1105/tpc.001628

Feys, B. J., Moisan, L. J., Newman, M. A., and Parker, J. E. (2001). Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. *EMBO J.* 20, 5400–5411. doi: 10.1093/emboj/20.19.5400

Feys, B. J., Wiermer, M., Bhat, R. A., Moisan, L. J., Medina-Escobar, N., Neu, C., et al. (2005). *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell* 17, 2601–2613. doi: 10.1105/tpc.105.033910

Fragnière, C., Serrano, M., Abou-Mansour, E., Métraux, J. P., and L'Haridon, F. (2011). Salicylic acid and its location in response to biotic and abiotic stress. *FEBS Lett.* 585, 1847–1852. doi: 10.1016/j.febslet.2011.04.039

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gao, Q. M., Xia, Y., Yu, K., Navarre, D., Kachroo, A., and Kachroo, P. (2014). Galactolipids are required for nitric oxide biosynthesis and systemic acquired resistance. *Cell Rep.* 9, 1681–1691. doi: 10.1016/j.celrep.2014.10.069

García, A. V., Blanvillain-Baufumé, S., Huibers, R. P., Wiermer, M., Li, G., Gobbato, E., et al. (2010). Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS Pathog.* 6:e1000970. doi: 10.1371/journal.ppat.1000970

Garcion, C., Lohmann, A., Lamodière, E., Catinot, J., Buchala, A., Doermann, P., et al. (2008). Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of *Arabidopsis*. *Plant Physiol.* 147, 1279–1287. doi: 10.1104/pp.108.119420

Giraldo, M., and Valent, B. (2013). Filamentous plant pathogen effectors in action. *Nat. Rev. Microbiol.* 11, 800–814. doi: 10.1038/nrmicro3119

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Göhre, V., and Robatzek, S. (2008). Breaking the barriers: microbial effector molecules subvert plant immunity. *Annu. Rev. Phytopathol.* 46, 189–215. doi: 10.1146/annurev.phyto.46.120407.110050

Gruner, K., Griebel, T., Návarová, H., Attaran, E., and Zeier, J. (2013). Reprogramming of plants during systemic acquired resistance. *Front. Plant Sci.* 4:252. doi: 10.3389/fpls.2013.00252

Gu, K., Yang, B., Tian, D., Wu, L., Wang, D., Sreekala, C., et al. (2005). R gene expression induced by a type-III effector triggers disease resistance in rice. *Nature* 435, 1122–1125. doi: 10.1038/nature03630

Guedes, M. E. M., Richmond, S., and Kuc, J. (1980). Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with *Colletotrichum* lagenarium and the onset of flowering and fruiting. *Physiol. Plant Pathol.* 17, 229–233. doi: 10.1016/0048-4059(80)90056-9

Heidel, A. J., Clarke, J. D., Antonovics, J., and Dong, X. (2004). Fitness costs of mutations affecting the systemic acquired resistance pathway in *Arabidopsis thaliana*. *Genetics* 168, 2197–2206. doi: 10.1534/genetics.104.032193

Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L., and Parker, J. E. (2011). *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* 334, 1401–1404. doi: 10.1126/science.1211641

Heil, M., and Baldwin, I. T. (2002). Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends Plant Sci.* 7, 61–66. doi: 10.1016/S1360-1385(01)02186-0

Hennig, J., Malamy, J., Grynkiewicz, G., Indulski, J., and Klessig, D. (1993). Interconversion of the salicylic acid signal and its glucoside in tobacco. *Plant J.* 4, 593–600. doi: 10.1046/j.1365-313X.1993.04040593.x

Holliday, M. J., Keen, N. T., and Long, M. (1981). Cell death patterns and accumulation of fluorescent material in the hypersensitive response of soybean leaves to *Pseudomonas syringae* pv. glycinea. *Physiol. Plant Pathol.* 18, 279–287. doi: 10.1016/S0048-4059(81)80079-3

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y. H., et al. (2010). Functional analysis of the *Arabidopsis* PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiol.* 153, 1526–1538. doi: 10.1104/pp.110.157370

Ishihara, T., Sekine, K. T., Hase, S., Kanayama, Y., Seo, S., Ohashi, Y., et al. (2008). Overexpression of the *Arabidopsis thaliana* EDS5 gene enhances resistance to viruses. *Plant Biol.* 10, 451–461. doi: 10.1111/j.1438-8677.2008.00050.x

Jaskiewicz, M., Conrath, U., and Peterhänsel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* 12, 50–55. doi: 10.1038/embor.2010.186

Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., et al. (1999). *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13583–13588. doi: 10.1073/pnas.96.23.13583

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jung, H. W., Tschaplinkski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91. doi: 10.1126/science.1170025

Kachroo, A., and Kachroo, P. (2009). Fatty acid-derived signals in plant defense. *Annu. Rev. Phytopathol.* 47, 153–176. doi: 10.1146/annurev-phyto-080508-081820

Kachroo, A., and Robin, G. P. (2013). Systemic signaling during plant defense. *Curr. Opin. Plant Biol.* 16, 527–533. doi: 10.1016/j.pbi.2013.06.019

Kachroo, P., and Kachroo, A. (2012). "The roles of salicylic acid and jasmonic acid in plant immunity," in *Molecular Plant Immunity,* ed. G. Sessa (Oxford: Wiley-Blackwell). doi: 10.1002/9781118481431.ch4

Kawano, T., Furuichi, T., and Muto, S. (2004). Controlled free salicylic acid levels and corresponding signaling mechanisms in plants. *Plant Biotechnol.* 21, 319–335. doi: 10.5511/plantbiotechnology.21.319

Kiefer, I. W., and Slusarenko, A. J. (2003). The pattern of systemic acquired resistance induction within the *Arabidopsis* rosette in relation to the pattern of translocation. *Plant Physiol.* 132, 840–847. doi: 10.1104/pp.103.021709

Kim, H. S., and Delaney, T. P. (2002). Over-expression of TGA5, which encodes a bZIP transcription factor that interacts with NIM1/NPR1, confers SAR-independent resistance in *Arabidopsis thaliana* to *Peronospora parasitica*. *Plant J.* 32, 151–163. doi: 10.1046/j.1365-313X.2001.01411.x

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.1105/tpc.12.12.2339

Knepper, C., Savory, E. A., and Day, B. (2011). *Arabidopsis* NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. *Plant Physiol.* 156, 286–300. doi: 10.1104/pp.110.169656

Koo, Y. J., Kim, M. A., Kim, E. H., Song, J. T., Jung, C., Moon, J. K., et al. (2007). Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic acid-mediated pathogen resistance in *Arabidopsis thaliana*. *Plant Mol. Biol.* 64, 1–15. doi: 10.1007/s11103-006-9123-x

Kumar, D., and Klessig, D. F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid stimulated lipase activity. *Proc. Natl. Acad. Sci. U.S.A.* 100, 16101–16106. doi: 10.1073/pnas.0307162100

Lai, Z., and Mengiste, T. (2013). Genetic and cellular mechanisms regulating plant responses to necrotrophic pathogens. *Curr. Opin. Plant Biol.* 16, 505–512. doi: 10.1016/j.pbi.2013.06.014

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol. Plant Microbe Interact.* 8, 863–870. doi: 10.1094/MPMI-8-0863

Lin, W. C., Lu, C. F., Wu, J. W., Cheng, M. L., Lin, Y. M., Yang, N. S., et al. (2004). Transgenic tomato plants expressing the *Arabidopsis* NPR1 gene display enhanced resistance to a spectrum of fungal and bacterial diseases. *Transgenic Res.* 13, 567–581. doi: 10.1007/s11248-004-2375-9

Lindermayr, C., Sell, S., Müller, B., Leister, D., and Durner, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *Plant Cell* 22, 2894–2907. doi: 10.1105/tpc.109.066464

Liu, G., Holub, E. B., Alonso, J. M., Ecker, J. R., and Fobert, P. R. (2005). An *Arabidopsis* NPR1-like gene, NPR4, is required for disease resistance. *Plant J.* 41, 304–318. doi: 10.1111/j.1365-313X.2004.02296.x

Liu, P. P., von Dahl, C. C., and Klessig, D. F. (2011). The extent to which methyl salicylate is required for signaling systemic acquired resistance is dependent on exposure to light after infection. *Plant Physiol.* 157, 2216–2226. doi: 10.1104/pp.111.187773

Liu, P. P., Yang, Y., Pichersky, E., and Klessig, D. F. (2010). Altering expression of benzoic acid/salicylic acid carboxyl methyltransferase 1 compromises systemic acquired resistance and PAMP-triggered immunity in *Arabidopsis*. *Mol. Plant Microbe Interact.* 23, 82–90. doi: 10.1094/MPMI-23-1-0082

Louis, J., Gobbato, E., Mondal, H. A., Feys, B. J., Parker, J. E., and Shah, J. (2012). Discrimination of *Arabidopsis* PAD4 activities in defense against green peach aphid and pathogens. *Plant Physiol.* 158, 1860–1872. doi: 10.1104/pp.112.193417

Louis, J., Leung, Q., Pegadaraju, V., Reese, J., and Shah, J. (2010). PAD4-dependent antibiosis contributes to the ssi2-conferred hyper-resistance to the green peach aphid. *Mol. Plant Microbe Interact.* 23, 618–627. doi: 10.1094/MPMI-23-5-0618

Lu, H., Zhang, C., Albrecht, U., Shimizu, R., Wang, G., and Bowman, K. D. (2013). Overexpression of a citrus NDR1 ortholog increases disease resistance in *Arabidopsis*. *Front. Plant Sci.* 4:157. doi: 10.3389/fpls.2013.00157

Lucas, W. J., Groover, A., Lichtenberger, R., Furuta, K., Yadav, S. R., Helariutta, Y., et al. (2013). The plant vascular system: evolution, development and functions. *J. Integr. Plant Biol.* 55, 294–388. doi: 10.1111/jipb.12041

Luna, E., Bruce, T. J., Roberts, M. R., Flors, V., and Ton, J. (2012). Next-generation systemic acquired resistance. *Plant Physiol.* 158, 844–853. doi: 10.1104/pp.111.187468

Mackey, D., Belkhadir, Y., Alonso, J. M., Ecker, J. R., and Dangl, J. L. (2003). *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112, 379–389. doi: 10.1016/S0092-8674(03)00040-0

Mackey, D., Holt, B. F., Wiig, A., and Dangl, J. L. (2002). RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell* 108, 743–754. doi: 10.1016/S0092-8674(02)00661-X

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250, 1002–1004. doi: 10.1126/science.250.4983.1002

Maldonado, A. M., Doerner, P., Dixon, R. A., Lamb, C. J., and Cameron, R. K. (2002). A putative lipid transfer protein involved in systemic resistance signaling in *Arabidopsis*. *Nature* 419, 399–403. doi: 10.1038/nature00962

Mandal, M. K., Chandra-Shekara, A. C., Jeong, R. D., Yu, K., Zhu, S., Chanda, B., et al. (2012). Oleic acid-dependent modulation of NITRIC OXIDE ASSOCIATED1 protein levels regulates nitric oxide-mediated defense signaling in *Arabidopsis*. *Plant Cell* 24, 1654–1674. doi: 10.1105/tpc.112.096768

Manohar, M., Tian, M., Moreau, M., Park, S. W., Choi, H. W., Fei, Z., et al. (2015). Identification of multiple salicylic acid-binding proteins using two high throughput screens. *Front. Plant Sci.* 5:777. doi: 10.3389/fpls.2014.00777

Marrtin, F., and Kamoun, S. (2012). *Effectors in Plant-microbe Interactions*. Chichester; Ames, IA: Wiley-Blackwell.

Mauch-Mani, B., and Slusarenko, A. J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell* 8, 203–212. doi: 10.1105/tpc.8.2.203

McDowell, J. M., Cuzick, A., Can, C., Beynon, J., Dangl, J. L., and Holub, E. B. (2000). Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. *Plant J.* 22, 523–529. doi: 10.1046/j.1365-313x.2000.00771.x

McKown, R., Kuroki, G., and Warren, G. (1996). Cold responses of *Arabidopsis* mutants impaired in freezing tolerance. *J. Exp. Bot.* 47, 1919–1925. doi: 10.1093/jxb/47.12.1919

Mengiste, T. (2012). Plant immunity to necrotrophs. *Annu. Rev. Phytopathol.* 50, 267–294. doi: 10.1146/annurev-phyto-081211-172955

Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006. doi: 10.1126/science.250.4983.1004

Meuwly, P., and Métraux, J. P. (1993). Ortho-anisic acid as internal standard for the simultaneous quantitation of salicylic acid and its putative biosynthetic precursors in cucumber leaves. *Anal. Biochem.* 214, 500–505. doi: 10.1006/abio.1993.1529

Meuwly, P., Molders, W., Buchala, A., and Metraux, J. P. (1995). Local and systemic biosynthesis of salicylic acid in infected cucumber plants. *Plant Physiol.* 109, 1107–1114.

Mishina, T. E., and Zeier, J. (2006). The *Arabidopsis* flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiol.* 141, 1666–1675. doi: 10.1104/pp.106.081257

Molders, W., Meuwly, P., Summermatter, K., and Métraux, J. P. (1994). Salicylic acid content in cucumber plants infected with *Pseudomonas* lachrymans and *tobacco necrosis virus*. *Acta Hortic.* 381, 375–378.

Morel, J. B., and Dangl, J. L. (1997). The hypersensitive response and the induction of cell death in plants. *Cell Death Differ.* 4, 671–683. doi: 10.1038/sj.cdd.4400309

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., et al. (2009). RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens. *Plant J.* 60, 218–226. doi: 10.1111/j.1365-313X.2009.03949.x

Návarová, H., Bernsdorff, F., Döring, A. C., and Zeier, J. (2012). Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *Plant Cell* 24, 5123–5141. doi: 10.1105/tpc.112.103564

Nawrath, C., Heck, S., Parinthawong, N., and Metraux, J. P. (2002). EDS5, an essential component of salicylic acid dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell* 14, 275–286. doi: 10.1105/tpc.010376

Niggeweg, R., Thurow, C., Kegler, C., and Gatz, C. (2000). Tobacco transcription factor TGA2.2 is the main component of as-1-binding factor ASF-1 and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. *J. Biol. Chem.* 275, 19897–19905. doi: 10.1074/jbc.M909267199

Pallas, J. A., Paiva, N. L., Lamb, C., and Dixon, R. A. (1996). Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by *tobacco mosaic virus*. *Plant J.* 10, 281–293. doi: 10.1046/j.1365-313X.1996.10020281.x

Park, S. W., Kaiyomo, E., Kumar, D., Mosher, S. L., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Park, S. W., Liu, P. P., Forouhar, F., Vlot, A. C., Tong, L., Tietjen, K., et al. (2009). Use of a synthetic salicylic acid analog to investigate the roles of methyl salicylate and its esterases in plant disease resistance. *J. Biol. Chem.* 284, 7307–7317. doi: 10.1074/jbc.M807968200

Pegadaraju, V., Knepper, C., Reese, J., and Shah, J. (2005). Premature leaf senescence modulated by the *Arabidopsis* PHYTOALEXIN DEFICIENT 4 gene is associated with defense against the phloem-feeding green peach aphid1. *Plant Physiol.* 139, 1927–1934. doi: 10.1104/pp.105.070433

Pegadaraju, V., Louis, J., Singh, V., Reese, J. C., Bautor, J., Feys, B. J., et al. (2007). Premature leaf senescence modulated by the *Arabidopsis* PHYTOALEXIN DEFICIENT4 gene is associated with defense against the phloem-feeding green peach aphid1. *Plant J.* 52, 332–341. doi: 10.1111/j.1365-313X.2007.03241.x

Pierpoint, W. S. (1994). Salicylic acid and its derivatives in plants: medicines, metabolites and messenger molecules. *Bot. Res.* 20, 163–235. doi: 10.1016/S0065-2296(08)60217-7

Rasmussen, J. B., Hammerschmidt, R., and Zook, M. N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. *Plant Physiol.* 97, 1342–1347. doi: 10.1104/pp.97.4.1342

Rietz, S., Stamm, A., Malonek, S., Wagner, S., Becker, D., Medina-Escobar, N., et al. (2011). Different roles of enhanced disease susceptibility1 (EDS1) bound to and dissociated from phytoalexin deficient4 (PAD4) in *Arabidopsis* immunity. *New Phytol.* 191, 107–119. doi: 10.1111/j.1469-8137.2011.03675.x

Rivas-San Vicente, M., and Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *J. Exp. Bot.* 62, 3321–3338. doi: 10.1093/jxb/err031

Sandhu, D., Tasma, I. M., Frasch, R., and Bhattacharyya, M. K. (2009). Systemic acquired resistance in soybean is regulated by two proteins, Orthologous to *Arabidopsis* NPR1. *BMC Plant Biol.* 9:105. doi: 10.1186/1471-2229-9-105

Schwessinger, B., and Ronald, P. C. (2012). Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* 63, 451–482. doi: 10.1146/annurev-arplant-042811-105518

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., et al. (2013). Export of salicylic acid from the chloroplast requires the MATE-like transporter EDS5. *Plant Physiol.* 162, 1815–1821. doi: 10.1104/pp.113.218156

Shah, J. (2003). The salicylic acid loop in plant defense. *Curr. Opin. Plant Biol.* 6, 365–371. doi: 10.1016/S1369-5266(03)00058-X

Shah, J., and Zeier, J. (2013). Long-distance communication and signal amplification in systemic acquired resistance. *Front. Plant Sci.* 4:30. doi: 10.3389/fpls.2013.00030

Shi, Z., Maximova, S. N., Liu, Y., Verica, J., and Guiltinan, M. J. (2010). Functional analysis of the *Theobroma cacao* NPR1 gene in *Arabidopsis*. *BMC Plant Biol.* 10:248. doi: 10.1186/1471-2229-10-248

Shimono, M., Sugano, S., Nakayama, A., Jiang, C. J., Ono, K., Toki, S., et al. (2007). Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance. *Plant Cell* 19, 2064–2076. doi: 10.1105/tpc.106.046250

Shulaev, V., Leon, J., and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? *Plant Cell* 7, 1691–1701. doi: 10.1105/tpc.7.10.1691

Singh, V., Roy, S., Giri, M. K., Chaturvedi, R., Chowdhury, Z., Shah, J., et al. (2013). Arabidopsis thaliana FLOWERING LOCUS D is required for systemic acquired resistance. *Mol. Plant Microbe Interact.* 26, 1079–1088. doi: 10.1094/MPMI-04-13-0096-R

Song, F., and Goodman, R. M. (2001). Activity of nitric oxide is dependent on, but is partially required for function of, salicylic acid in the signaling pathway in tobacco systemic acquired resistance. *Mol. Plant Microbe Interact.* 14, 1458–1462. doi: 10.1094/MPMI.2001.14.12.1458

Song, J. T., Lu, H., McDowell, J. M., and Greenberg, J. T. (2004a). A key role for ALD1 in activation of local and systemic defenses in *Arabidopsis*. *Plant J.* 40, 200–212. doi: 10.1111/j.1365-313X.2004.02200.x

Song, J., Lu, H., and Greenberg, J. (2004b). Divergent roles in *Arabidopsis thaliana* development and defense of two homologous genes, ABERRANT GROWTH AND DEATH2 and AGD2-LIKE DEFENSE RESPONSE PROTEIN1, encoding novel aminotransferases. *Plant Cell* 16, 353–366. doi: 10.1105/tpc.019372

Spoel, S., and Dong, X. (2012). How do plants achieve immunity? Defence withoug specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Spoel, S. H., Mou, Z., Tada, Y., Spivey, N. W., Genschik, P., and Dong, X. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual role in regulating plant immunity. *Cell* 137, 860–872. doi: 10.1016/j.cell.2009.03.038

Strawn, M. A., Marr, S. K., Inoue, K., Inada, N., Zubieta, C., and Wildermuth, M. C. (2007). *Arabidopsis* isochorismate synthase functional in pathogen-induced salicylate biosynthesis exhibits properties consistent with a role in diverse stress responses. *J. Biol. Chem.* 282, 5919–5933. doi: 10.1074/jbc.M605193200

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Truman, W. M., Bennett, M. H., Turnbull, C. G., and Grant, M. R. (2010). *Arabidopsis* auxin mutants are compromised in systemic acquired resistance and exhibit aberrant accumulation of various indolic compounds. *Plant Physiol.* 152, 1562–1573. doi: 10.1104/pp.109.152173

Truman, W., and Glazebrook, J. (2012). Co-expression analysis identifies putative targets for CBP60g and SARD1 regulation. *BMC Plant Biol.* 12:216. doi: 10.1186/1471-2229-12-216

Tuzun, S., and Kuc, J. (1985). Movement of a factor in tobacco infected with *Peranospora tabacina* Adam which systemically protects against blue mold. *Physiol. Plant Pathol.* 26, 321–330. doi: 10.1016/0048-4059(85)90007-4

Ung, H., Moeder, W., and Yoshioka, K. (2014). *Arabidopsis* triphosphate tunnel metalloenzyme2 is a negative regulator of the salicylic acid-mediated feedback amplification loop for defense responses. *Plant Physiol.* 166, 1009–1021. doi: 10.1104/pp.114.248757

Uzunova, A. N., and Popova, L. P. (2000). Effect of salicylic acid on leaf anatomy and chloroplast ultrastructure of barley plants. *Photosynthetica* 38, 243–250. doi: 10.1023/A:1007226116925

Venugopal, S. C., Jeong, R. D., Mandal, M., Zhu, S., Chandra-Shekara, A. C., Xia, Y., et al. (2009). ENHANCED DISEASE SUSCEPTIBILITY and salicylic acid act redundantly to regulate resistance gene-mediated signaling. *PLoS Genet.* 5:e1000545. doi: 10.1371/journal.pgen.1000545

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., et al. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* 6, 959–965. doi: 10.1105/tpc.6.7.959

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Wang, C., El-Shetehy, M., Shine, M. B., Yu, K., Navarre, D., Wendehenne, D., et al. (2014a). Free radicals mediate systemic acquired resistance. *Cell Rep.* 7, 348–355 doi: 10.1016/j.celrep.2014.03.032

Wang, J., Shine, M. B., Gao, Q. M., Navarre, D., Jiang, W., Liu, C., et al. (2014b). Enhanced Disease Susceptibility1 mediates pathogen resistance and virulence function of a bacterial effector in soybean. *Plant Physiol.* 165, 1269–1284. doi: 10.1104/pp.114.242495

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2:e123. doi: 10.1371/journal.ppat.0020123

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tör, M., Caldelari, D., et al. (2011). Timing of plant immune responses by a central circadian regulator. *Nature* 470, 110–114. doi: 10.1038/nature09766

Warren, G., McKown, R., Marin, A., and Teutonico, R. (1996). Isolation of mutations affecting the development of freezing tolerance in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol.* 111, 1011–1019. doi: 10.1104/pp.111.4.1011

Wendehenne, D., Gao, Q. M., Kachroo, A., and Kachroo, P. (2014). Free radical-mediated systemic immunity in plants. *Curr. Opin. Plant Biol.* 20, 127–134. doi: 10.1016/j.pbi.2014.05.012

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Wink, D. A., Hines, H. B., Cheng, R. Y. S., Switzer, C. H., Flores-Santana, W., Vitek, M. P., et al. (2011). Nitric oxide and redox mechanisms in the immune response. *J. Leukoc. Biol.* 89, 873–891. doi: 10.1189/jlb.1010550

Wittek, F., Hoffmann, T., Kanawati, B., Bichlmeier, M., Knappe, C., Wenig, M., et al. (2014). *Arabidopsis* ENHANCED DISEASE SUSCEPTIBILITY1 promotes systemic acquired resistance via azelaic acid and its precursor 9-oxo nonanoic acid. *J. Exp. Bot.* 65, 5919–5931. doi: 10.1093/jxb/eru331

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 6, 639–647. doi: 10.1016/j.celrep.2012.05.008

Xia, Y., Gao, Q. M., Yu, K., Navarre, D., Hildebrand, D., Kachroo, A., et al. (2009). An intact cuticle in distal tissues is essential for the induction of systemic acquired resistance in plants. *Cell Host Microbe.* 5, 151–165. doi: 10.1016/j.chom.2009.01.001

Xia, Y., Yu, K., Gao, Q. M., Wilson, E. V., Navarre, D., Kachroo, P., et al. (2012). Acyl CoA binding proteins are required for cuticle formation and plant responses to microbes. *Front. Plant Sci.* 3:224. doi: 10.3389/fpls.2012.00224

Xia, Y., Yu, K., Navarre, D., Seebold, K., Kachroo, A., and Kachroo, P. (2010). The glabra1 mutation affects cuticle formation and plant responses to microbes. *Plant Physiol.* 154, 833–846. doi: 10.1104/pp.110.161646

Yalpani, N., Leon, J., Lawton, M. A., and Raskin, I. (1993). Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. *Plant Physiol.* 103, 315–321.

Yalpani, N., Silverman, P., Wilson, T. M., Kleier, D. A., and Raskin, I. (1991). Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell* 3, 809–818. doi: 10.1105/tpc.3.8.809

Yu, J., Gao, J., Wang, X. Y., Wei, Q., Yang, L. F., Qiu, K., et al. (2010). The pathway and regulation of salicylic acid biosynthesis in probenazole-treated *Arabidopsis*. *J. Plant Biol.*53, 417–424. doi: 10.1007/s12374-010-9130-y

Yu, K., Soares, J. M., Mandal, M. K., Wang, C., Chanda, B., Gifford, A. N., et al. (2013). A feedback regulatory loop between G3P and lipid transfer proteins DIR1 and AZI1 mediates azelaic-acid-induced systemic immunity. *Cell Rep.* 3, 1266–1278. doi: 10.1016/j.celrep.2013.03.030

Yuan, Y., Zhong, S., Li, Q., Zhu, Z., Lou, Y., Wang, L., et al. (2007). Functional analysis of rice NPR1-like genes reveals that OsNPR1/NH1 is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. *Plant Biotechnol. J.* 5, 313–324. doi: 10.1111/j.1467-7652.2007.00243.x

Zhang, K., Halitschke, R., Yin, C., Liu, C. J., and Gan, S. S. (2013). Salicylic acid 3-hydroxylase regulates *Arabidopsis* leaf longevity by mediating salicylic acid catabolism. *Proc. Natl. Acad. Sci. U.S.A.* 110, 14807–14812. doi: 10.1073/pnas.1302702110

Zhang, X., Wang, C., Zhang, Y., Sun, Y., and Mou, Z. (2012). The *Arabidopsis* mediator complex subunit16 positively regulates salicylate-mediated systemic acquired resistance and jasmonate/ethylene-induced defense pathways. *Plant Cell* 24, 4294–4309. doi: 10.1105/tpc.112.103317

Zhang, Y., Cheng, Y. T., Qu, N., Zhao, Q., Bi, D., and Li, X. (2006). Negative regulation of defense responses in *Arabidopsis* by two NPR1 paralogs. *Plant J.* 48, 647–656. doi: 10.1111/j.1365-313X.2006.02903.x

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528. doi: 10.1073/pnas.96.11.6523

Zhang, Y., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653. doi: 10.1105/tpc.014894

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y. T., He, J., et al. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18220–18225. doi: 10.1073/pnas.1005225107

Zhou, J. M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., et al. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol. Plant Microbe Interact.* 13, 191–202. doi: 10.1094/MPMI.2000.13.2.191

Zhu, S., Jeong, R. D., Venugopal, S. C., Lapchyk, L., Navarre, D., Kachroo, A., et al. (2011). SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against *Turnip crinkle virus*. *PLoS Pathog.* 7:e1002318. doi: 10.1371/journal.ppat.1002318

Zoeller, M., Stingl, N., Krischke, M., Fekete, A., Waller, F., Berger, S., et al. (2012). Lipid profiling of the *Arabidopsis* hypersensitive response reveals specific lipid peroxidation and fragmentation processes: biogenesis of pimelic and azelaic acid. *Plant Physiol.* 160, 365–378. doi: 10.1104/pp.112.202846

Zwicker, S., Mast, S., Stos, V., Pfitzner, A. J., and Pfitzner, U. M. (2007). Tobacco NIMIN2 proteins control PR gene induction through transient repression early in systemic acquired resistance. *Mol. Plant Pathol.* 4, 385–400. doi: 10.1111/j.1364-3703.2007.00399.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 19 March 2015
doi: 10.3389/fpls.2015.00174

Interconnection between flowering time control and activation of systemic acquired resistance

*Zeeshan Z. Banday and Ashis K. Nandi **

School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

The ability to avoid or neutralize pathogens is inherent to all higher organisms including plants. Plants recognize pathogens through receptors, and mount resistance against the intruders, with the help of well-elaborated defense arsenal. In response to some local infections, plants develop systemic acquired resistance (SAR), which provides heightened resistance during subsequent infections. Infected tissues generate mobile signaling molecules that travel to the systemic tissues, where they epigenetically modify expression of a set of genes to initiate the manifestation of SAR in distant tissues. Immune responses are largely regulated at transcriptional level. Flowering is a developmental transition that occurs as a result of the coordinated action of large numbers of transcription factors that respond to intrinsic signals and environmental conditions. The plant hormone salicylic acid (SA) which is required for SAR activation positively regulates flowering. Certain components of chromatin remodeling complexes that are recruited for suppression of precocious flowering are also involved in suppression of SAR in healthy plants. FLOWERING LOCUS D, a putative histone demethylase positively regulates SAR manifestation and flowering transition in *Arabidopsis*. Similarly, incorporation of histone variant H2A.Z in nucleosomes mediated by PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1, an ortholog of yeast chromatin remodeling complex SWR1, concomitantly influences SAR and flowering time. SUMO conjugation and deconjugation mechanisms also similarly affect SAR and flowering in an SA-dependent manner. The evidences suggest a common underlying regulatory mechanism for activation of SAR and flowering in plants.

Keywords: SAR, flowering, SA, FLD, chromatin remodeling, epigenetic

OPEN ACCESS

Edited by:
Jean Toby Greenberg, The University of Chicago, USA

Reviewed by:
Robin Katrina Cameron, McMaster University, Canada
Yusuke Saijo, Max Planck Institute for Plant Breeding Research, Germany

***Correspondence:**
Ashis K. Nandi, School of Life Sciences, Jawaharlal Nehru University, Room 415, New Delhi-110067, Delhi, India
ashis_nandi@yahoo.com

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *11 November 2014*
Accepted: *04 March 2015*
Published: *19 March 2015*

Citation:
Banday ZZ and Nandi AK (2015) Interconnection between flowering time control and activation of systemic acquired resistance. Front. Plant Sci. 6:174. doi: 10.3389/fpls.2015.00174

Introduction

Immobility precludes plants from evading pathogens. However, the presence of strong immune system most often keeps them healthy. Higher animals like vertebrates, are capable of retaining an infection memory with the help of dedicated immune system and circulatory cells. The generated infection memory facilitates stronger immune response during subsequent interactions with the same pathogen (adaptive immunity). Despite not having dedicated immune cells, plants are equally capable of using infection-induced molecular memories to resist subsequent infections. This heightened resistance based on past experience is called systemic acquired resistance (SAR; Ross, 1961). Unlike adaptive immunity-based learning in animals, SAR-mediated protection in

plants is not limited to the same pathogen, but is effective against a wide range of microbial pathogens (Sticher et al., 1997; Durrant and Dong, 2004).

While pre-existing cell wall and structural components such as cuticular wax provide resistance against pathogens, most defense responses are induced upon pathogen infection. Resistance against pathogens in plants relies both on fortification of structural barriers and production of antimicrobial chemicals and proteins. Microbe/pathogen associated molecular patterns (MAMPs/PAMPs) are recognized by the plasma membrane (PM) resident pattern recognition receptors (PRRs; Nimchuk et al., 2003). Recognition of MAMP/PAMP by PRRs activates signaling cascades involving kinases, proteases, protein modifiers and transcription regulators, which eventually results in cell wall strengthening, production of antimicrobial proteins and phytoalexins (Schwessinger and Ronald, 2012). The defense hormones such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) function as second messengers of the signaling events (Verhage et al., 2010). PRR activation induces biosynthesis of these hormones, which in turn leads to transcriptional reprogramming in favor of defense. Pathogen infection induces transcription of large number of genes, a subset of these are *pathogenesis related* (*PR*) genes (van Loon et al., 2006). Several PR proteins are secreted out of the host cell and negatively affect the growth of pathogens due to their antimicrobial properties (van Loon et al., 2006). Immune responses as described above, triggered by activation of PRRs is known as pattern triggered immunity (PTI). Some pathogens, however, release effector molecules to suppress the plant PTI response (Jones and Dangl, 2006). Plants can overcome the effects of pathogen effectors using R gene-mediated resistance, in which R receptors interact directly or indirectly with pathogen effectors to initiate effector-triggered immunity (ETI). ETI is an exaggerated form of PTI (Jones and Dangl, 2006).

When a plant succeeds in restricting the growth of a pathogen, it develops SAR; a state of preparedness that provides elevated resistance during subsequent infections (Durrant and Dong, 2004; Iriti and Faoro, 2007; Vlot et al., 2008). Besides pathogens, certain chemicals such as SA and its chemical analogs are capable of inducing SAR in plants (Lawton et al., 1996). During the SAR inducing infection, mobile signals are synthesized in the infected tissue and get distributed throughout the plant, via phloem (**Figure 1**; Guedes et al., 1980; Tuzun and Kuc, 1985). It has been demonstrated that upon localized pathogen inoculation, the pathogen free distal tissues show immune responses like the infected tissues, but to a moderate level. For example, distal tissues show fortification of cell wall, accumulation defense hormones and expression of PR-proteins (Ward et al., 1991; Ryals et al., 1996; Fu and Dong, 2013). But more importantly, an experienced plant activates priming, a SAR induced mechanism that results in robust induction of defense responses compared to a naive plant, during subsequent pathogen infections (Jung et al., 2009; Slaughter et al., 2012; Singh et al., 2013). Genetic and biochemical experiments, mostly on model plants, identified several compounds such as SA, methyl salicylate, JA, dihydroabetinal, azelaic acid, glycerol-3-phosphate, pipecolic acid, and lipid transfer protein DIR1 as potential mobile signals of SAR (Dempsey and Klessig, 2012; Navarova et al., 2012; Champigny et al., 2013). Petiole exudates enriched for phloem sap collected from pathogen-inoculated leaves, carrying these mobile signals are capable of inducing SAR in naive plants (Chaturvedi et al., 2008; Chanda et al., 2011).

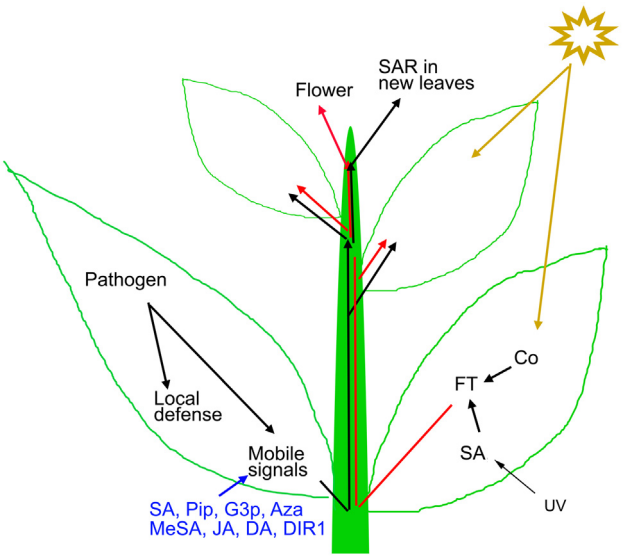


FIGURE 1 | Mechanism of SAR and flowering induction. Local pathogen inoculation induces local defense and mobile SAR signals (such as SA, Pip, G3P, Aza, MeSA, JA, DA, and DIR1) that travel to distal parts through phloem for SAR activation. The generated SAR signals are capable of SAR induction in leaves that develop after the primary induction (Caruso and Kuc, 1977). Exogenous application of these mobile signals also induces resistance throughout the plant. Light quality and quantity influences floral regulators like FT that also travels through the phloem to modify the shoot apex to produce flowers instead of leaves.

Mechanism of the development of infection memory, subsequent to receiving the mobile signal in distal tissues, is not elucidated well. The mobile signals by themselves are not antimicrobial (Jung et al., 2009; Chanda et al., 2011; Chaturvedi et al., 2012; Navarova et al., 2012). The metabolic signals do not directly provide SAR, as they are elevated only transiently, while SAR lasts for weeks to months as observed in cucumber, watermelon, muskmelon, and other plants (Caruso and Kuc, 1977; Kuc and Richmond, 1977). Thus, for the induction of SAR, the systemic tissues must perceive and decode the SAR signals. Recent studies, mostly with the model plant *Arabidopsis* provide evidence that upon infection, epigenetic modifications takes place in systemic tissues, which contribute to infection memory formation. Promoters of the plant specific WRKY transcription factors have been reported to accumulate elevated levels of modified histones that are normally associated with epigenetic control of gene expression (Jaskiewicz et al., 2011; Luna et al., 2012; Singh et al., 2014b). Modified histones on WRKY genes involved in SAR could be part of infection memory. It's not clear how this epigenetic mechanism relates to SAR memory.

Recent studies indicate a close interconnection between flowering time control and SAR activation mechanisms. The transition to flowering is an irreversible process for annual plants, when the shoot apical meristem becomes an inflorescence meristem that produces flowers instead of leaves. The timing of this transition is a major factor for the reproductive success of plants. Regulation of flowering time involves complex regulatory network consisting of multiple set of genes (Simpson and Dean, 2002). The flowering molecular switch ensures that plants flower at a time when internal resources are adequate and the ambient environmental conditions are optimum for pollination and seed development (Simpson et al., 1999). A large number of gene products affect both flowering and SAR (**Figure 2**). This review article discusses the possible mechanistic overlap in regulation of flowering time and SAR.

Flowering Control by Salicylic Acid and other SAR Inducers

Functions of SA and its derivatives are intricately associated with SAR. SA and its chemical analogs are potential SAR inducers when exogenously applied to plants (Yalpani et al., 1991; Gaffney et al., 1993). When a plant is infected by a pathogen, high level of SA accumulates in the pathogen-infected tissue and to a lesser extent in pathogen free systemic tissues (Metraux et al., 1990; Nandi et al., 2004). SA promotes nuclear localization and activation of NON-EXPRESSOR OF PR-1 (NPR1), a *trans*-activator protein, which is required for SAR (Kinkema et al., 2000; Wu et al., 2012). NPR1 interacts with TGA transcription factors, and together induce expression of *PR* genes (Dong, 2004). Expression of PR-*1* gene is typically associated with the activation of SA signaling and thus serves as its marker. The mutants such as *suppressor of fatty acid desaturase 1* (*sfd1*), *reduced systemic immunity 1* (*rsi1*), *azelaic acid induced 1* (*azi1*) of *Arabidopsis* that are impaired in SAR induction are defective in systemic SA accumulation, and priming induced expression of *PR-1* (Chaturvedi et al., 2008; Jung et al., 2009; Singh et al., 2013). SA has been implicated as an integral component of SAR signaling (Ryals et al., 1996; Sticher et al., 1997; Conrath, 2011; Fu and Dong, 2013).

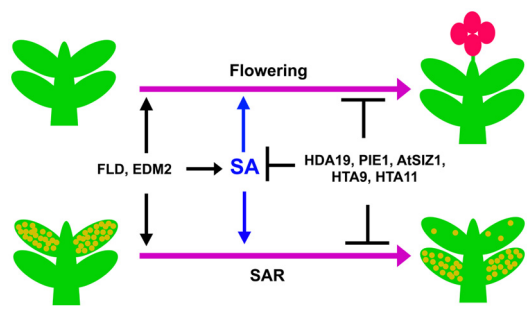


FIGURE 2 | Transition from vegetative stage to reproductive stage and development of SAR is controlled by SA. Upon attaining the right developmental stage, plants show transition from the vegetative to the reproductive phase of growth (upper two plants). A plant that has previously experienced a pathogen develops fewer disease symptoms after subsequent infections due to SAR (lower right plant) compared to an inexperienced plant (lower left plant). SA positively influences both of these processes. The genes mentioned in the figure similarly affect SA accumulation, flowering and SAR.

Interestingly, SA also influences flowering time to a great extent. Involvement of SA in common regulation of SAR/pathogen response and flowering is reflected in many reports (discussed in the following sections; **Figure 2**). *ENHANCED DOWNY MILDEW 2* (*EDM2*) gene of *Arabidopsis* is required for *RPP7*-mediated resistance against downy mildew pathogen *Hyaloperonospora parasitica* (Eulgem et al., 2007). The mutants of EDM2 fail to accumulate pathogen induced SA, and also cause flowering time delay (Tsuchiya and Eulgem, 2010).

Effect of Light on SA, SAR, and Flowering

Light plays very important role in biosynthesis of SA and immune responses (Zeier et al., 2004; Kangasjarvi et al., 2012). Pathogen induced SA biosynthesis takes place in chloroplast, in light (UV-C) dependent manner (Fragniere et al., 2011). A large number of genes that are induced upon flg22 (bacterial flagellin derived PAMP peptide) treatment require light (Sano et al., 2014). Light composition, intensity, and duration affect defense responses (Chandra-Shekara et al., 2006; Griebel and Zeier, 2008; Ballare et al., 2012; de Wit et al., 2013). Red light stimulates disease resistance against many pathogens (Islam et al., 2008; Wang et al., 2010). In contrast, addition of far-red light (leading to reduced red:far-red ratio) negatively influences defense responses (de Wit et al., 2013). Plants perceive red and far-red lights by two interconvertible forms of phytochrome photo-receptors, Pr and Pfr, which absorb red and far-red light, respectively. The Pr is the inactive form, which converts into the active form Pfr, upon absorbing red light, whereas, the Pfr form converts back into the Pr form by absorbing far-red light (Smith, 2000). The *phyAphyB* double mutant plants are susceptible against virulent pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (Psm; Griebel and Zeier, 2008). The results suggest that phytochrome signaling plays a very significant role in disease defense. Does phytochrome signaling have any specific role in SAR? The experimental evidence is insufficient at the present time to draw this conclusion. Griebel and Zeier, 2008, reported that the phytochrome signaling is more pertinent for SAR than local defense. However, this conclusion may be accepted with certain reservations. The *phyAphyB* mutant plants are highly susceptible to Psm, and support modestly higher growth of Psm carrying the avirulence gene *avrRpm1* (Psm-AvrRpm1) compared to wild-type plants. The *phyAphyB* plants, but not the mutants of other photoreceptors such as cryptochromes (*cryAcryB*) and phototropins (*phot1 phot2*) are defective activation of SAR. Surprisingly, authors used Psm as primary pathogen for SAR induction, against which *phyAphyB* plants were compromised for local defense, instead of Psm-AvRpm1 (Griebel and Zeier, 2008). Moreover, other studies, such as the effect of red light in promoting disease defense, and low red-far red ratio affecting general defense responses, also counter argues for phytochromes having specific roles in SAR.

The role of light in flowering is much well-established. Amongst the environmental factors that affect flowering, light

plays the most important role. According to the photoperiod dependence for flowering, angiosperms are grouped into long-day (LD), short-day (SD), and day-neutral plants. *Arabidopsis* is a facultative LD plant that flowers early in LD, and show delayed flowering under SD condition. The striking similarity between SAR and photo-period induced flowering is the requirement of long distance signal movement through phloem (**Figure 1**; Zeevaart, 2006). Grafting and girdling experiments suggested that the flowering inducers are phloem transmissible (Knott, 1934; Chailakhyan, 1936). By the perception of the day-length effect, leaves generate a mobile signal for flowering. The signal moves to the growing apex via phloem; the apex modifies to produce flower instead of leaves (Knott, 1934; Chailakhyan, 1936). In recent years, it has been shown that the phloem mobile flowering promoting factor is a protein; *flowering locus T* (FT) in *Arabidopsis* (Corbesier et al., 2007). FT orthologs have been identified in many plants suggesting that the vascular conductance is a universal feature for flowering in plants (Tamaki et al., 2007; Varkonyi-Gasic et al., 2013; Li et al., 2014).

Interestingly, SA is also reported as an inducer of photoperiod-mediated flowering. Abiotic stress such as UV-C induces expression of *FT* as well as flowering in SA dependent manner in *Arabidopsis* (Martinez et al., 2004). The transgenic plants expressing NahG fail to induce *FT* expression and early flowering by UV-C treatment (Martinez et al., 2004). The phloem sap, or the honeydew produced by aphid infestation, on *Xanthium strumarium* is capable of inducing flowering in the long-day plant *Lemna gibba* (Cleland and Ajami, 1974). Purification of flowering inducing component from aphid-honeydew by TLC, followed by GLC and mass-spectrometric analysis identified SA as the active ingredient of flowering inducer in phloem sap (Cleland and Ajami, 1974). Exogenous application of SA in the growing medium or in leaves, in several plants promote flowering (Cleland and Ajami, 1974; Khurana and Cleland, 1992; Wada et al., 2014). Thus, SA may be considered as a common inducer for both flowering and SAR (**Figure 1**). A similar dual role is also reported for pipecolic acid (Pip), another mobile signal for SAR induction (Navarova et al., 2012). Flowering inducing activity guided fractionation identified Pip and nicotinamide as flowering inducing substances in *L. gibba* leaf extracts (Fujioka et al., 1987).

FLD Regulates the Transition to Flowering and SAR

At a defined time in their life-cycle, annual plants undergo a developmental transition from the vegetative to the reproductive stage. This transition is controlled by environmental as well as endogenous developmental cues. The environmental factors include day length (photoperiod), quality and quantity of light (composition and photon density), prolonged cold exposure (vernalization), and nutrient and water availability, whereas, plant age and vegetative growth provide developmental cues for transition (see Amasino, 1996; Aukerman and Amasino, 1996). In *Arabidopsis*, mutational analysis has identified numerous genes that affect flowering time. The CONSTANS protein accumulates in long-days and positively regulates expression of *FT*, and SUPPRESOR OF CO 1 (SOC1), and thereby promotes flowering (Suarez-Lopez et al., 2001). In contrast, *FLOWERING LOCUS C* (*FLC*) negatively regulates *FT* and SOC1, and helps plants to avoid premature flowering (Michaels and Amasino, 1999; Helliwell et al., 2006). FLC codes for a MADS box protein that binds to promoters of FT and SOC1 repressing transcription of these genes (Helliwell et al., 2006). A large number of genes, whose expression is modulated by developmental cues and environmental factors, affect expression of *FLC* and control flowering time (Henderson and Dean, 2004). The FLC locus is epigenetically regulated through histone modifications. FLOWERING LOCUS D (FLD) negatively regulates expression of *FLC* and thereby promotes flowering (He et al., 2003; He and Amasino, 2005; Liu et al., 2007). Thus, flowering is delayed in *fld* loss-of-function mutants (He et al., 2003; Singh et al., 2013).

A genetic screen selecting for SAR-impaired mutants from EMS treated *Arabidopsis* plants, identified *reduced in systemic immunity 1* (*rsi1*), which is a loss-of-function allele of *FLD* (Singh et al., 2013). The *rsi1* mutant is defective in systemic accumulation of SA and priming of *PR-1*, *WRKY6,* and *WRKY29* genes (Singh et al., 2013, 2014b). Petiole exudates from inoculated *rsi1* leaves activate SAR on WT plants, whereas, SAR inducible petiole exudates from WT plants fail to induce SAR in *rsi1*. Moreover, SAR is not induced in *rsi1* plants by exogenous application of SAR inducers such as dihydroabetinal and azelaic acid. Thus, the *rsi1* and the allelic *fld* mutants are capable of generating SAR mobile signals after primary infection, but fail to decode the signal in the distal tissues. These data suggest that FLOWERING LOCUS D function is required for generating infection memory, subsequent to receiving the SAR signal. FLD expression is induced both in the primary SAR-induced and systemic tissues (Singh et al., 2013). As a consequence, FLC expression maybe suppressed in by SAR induction. Indeed, transcript analysis following SA treatment showed suppression of FLC expression (Martinez et al., 2004). Although, *FLC* expression is suppressed by SA, its function is probably not associated with SAR (Singh et al., 2013). The *flc* mutant has no defect in SAR activation, and the *flc* mutation does not rescue the SAR defect in the *rsi1*/*fld* mutant. Thus FLD may function as branch point between flowering time control and SAR activation in *Arabidopsis* (**Figure 3**).

The mechanism of FLD expression in response to SAR induction is not known. Brassinosteroid (BR) signaling has recently been associated with FLD expression (Zhang et al., 2013b). BR is perceived by the receptor kinase, BR INSENSITIVE1 (BRI1) along with BRI1 ASSOCIATED KINASE1 (BAK1; Yang et al., 2011). Binding of BR activates both BAK1 and BRI1 through auto- and *trans*-phosphorylation, which in turn release the receptor-like cytoplasmic kinases BRASSINOSTEROID SIGNALING KINASES (BSKs) and CONSTITUTIVE DIFFERENTIAL GROWTH1 (CDG1; Tang et al., 2008; Kim et al., 2011). BSKs and CDG1 phosphorylate and activate BRI1 SUPPRESSOR1 (BSU1), a phosphatase that dephosphorylates BIN2 (Clouse, 2011; Kim et al., 2011). BIN2 negatively regulates BR signaling by phosphorylating and thereby promoting cytoplasmic retention of transcription

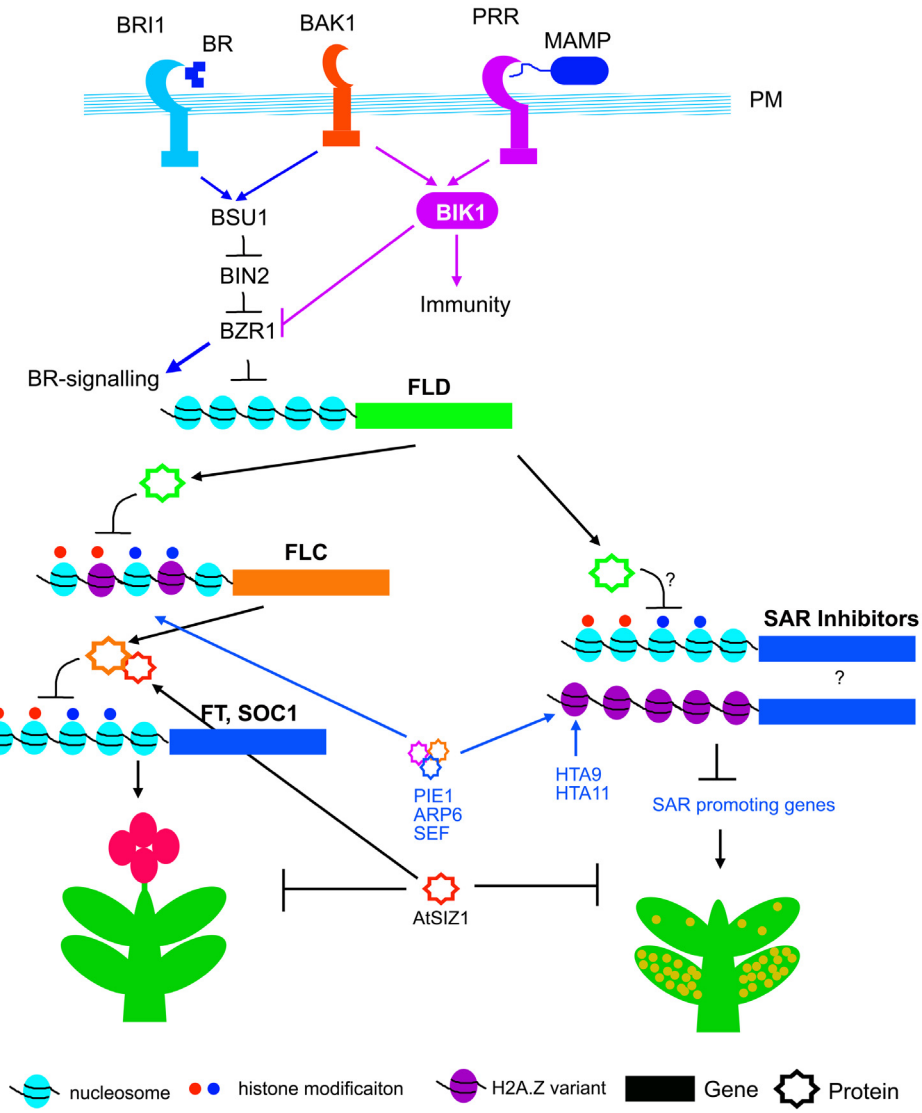


FIGURE 3 | Genetic and epigenetic control of flowering and SAR. Plasma membrane (PM) resident BAK1 associates with both BRI1 and PRRs (pattern recognition receptors) which are required for BR and PTI signaling respectively. BSU1 phosphatase is activated by BRI1 and BAK1. BSU1 dephosphorylates and inactivates BIN2, and thereby activates BZR1 and BR signaling. BZR1 negatively regulates FLD expression. Activation of PTI activates BIK1, which suppress BZR1 and thereby may promote expression of FLD. FLD transcriptionally suppress FLC, the floral repressor. FLC protein is stabilized through interaction with AtSIZ1. AtSIZ1 functions as negative regulator for both flowering and SAR. The PIE1, ARP6 and SEF complex, and HTA9, HTA11 promote histone variant incorporation and biosynthesis, and thus promote transcription of FLC and unknown SAR suppressors.

factors, such as BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1 EMS SUPPRESSOR1 (BES1), through interaction with 14-3-3 protein (Yang et al., 2011). Dephosphorylation of BZR1 and BES1 by protein phosphatase 2A, relieve cytoplasmic retention, allowing their nuclear translocation and binding to target promoters (Tang et al., 2011). Interestingly, the promoter of FLD contains one BR-responsive element (BRRE; Zhang et al., 2013b). Electrophoretic mobility shift assay (EMSA) shows that BRRE of FLOWERING LOCUS D promoter binds with the recombinant MBP-BZR1 protein but not the maltose binding protein (MBP; Zhang et al., 2013b). In addition, chromatin immuno-precipitation with GFP antibody shows enrichment of CFP-BZR1 in FLD promoter. The physical association and transcriptional analyses suggest that BZR1 binds to promoter of FLD and negatively regulates its expression (Zhang et al., 2013b). In immune signaling, FLAGELIN SENSING2 (FLS2) and ELONGATION FACTOR TU RECEPTOR (EFR), the pattern receptors for bacterial flagellin and elongation factor Tu respectively, heteromerize with BAK1 (Chinchilla et al., 2009; Roux et al., 2011; Sun et al., 2013). BAK1 phosphorylate BOTRYTIS-INDUCED KINASE1 (BIK1), a receptor-like cytoplasmic kinase that positively regulates plant immunity (Lu et al., 2010; Zhang et al., 2010). However, the BIK1 acts as a negative regulator of BR signaling. The *bik1* mutant plants show enhancement in

dephosphorylation of BZR1 and BES1 (Lin et al., 2013), Thus activation of BIK1 by pathogens may inactivate BZR1 (through cytoplasmic retention) and thereby induce expression of FLD (**Figure 3**).

Chromatin Remodeling

Eukaryotic DNA is packed into nucleosomes, which must transiently unpack during transcription. Alteration of nucleosome density, also known as chromatin remodeling, affects transcription of genes. In nucleosomes, DNA is wrapped around histone octamers consisting of two copies each of H2A, H2B, H3 and H4 (Kamakaka and Biggins, 2005). Post-translational modifications of histones as well as methylation of cytosine residues in DNA affect chromatin composition. The modifications of histones include methylation, acetylation, ubiquitination, and phosphorylation (Geiman and Robertson, 2002; Nowak and Corces, 2004). Usually, DNA methylation leads to suppression of transcriptional activity, whereas, acetylation of histones, especially in H3 and H4, activates transcription (Vaillant and Paszkowski, 2007). In contrast, methylation of histones can affect transcription both positively and negatively, depending on the histone protein and position of the modification (Zhang, 2008). Histone replacement, a process of substitution of canonical histones with histone variants, is also associated with chromatin remodeling (Kamakaka and Biggins, 2005; March-Diaz et al., 2008). Higher eukaryotes including plants possess machinery to initiate and maintain both DNA and histone modifications. Evidence suggests that both flowering and SAR are regulated by epigenetic modifications; interestingly, with machinery shared by both the pathways.

Pathogen- and SA- Induced Histone Modification

Expression of several SA responsive genes is epigenetically regulated. Exogenous application of benzothiadiazole (BTH), a chemical analog of SA and potential SAR inducer, induces accumulation of modified histones that favor transcription, such as acetylated histone 3 (H3Ac), and di- and tri- methylated histone 3 at lysine 4 (H3K4me2 and H3K4me3) in the promoters of several *WRKY* genes, whose functions are associated with SAR activation (Jaskiewicz et al., 2011). Exogenous application of SA also induces such modifications in the *PR-1* promoter (Mosher et al., 2006). During SAR activation upon primary infection, the systemic tissues undergo similar epigenetic modifications, which is associated with robust expression of these defense related genes during challenge inoculation (Conrath, 2011; Singh et al., 2014b). Under stress-free condition, SUPPRESSOR OF PR-1 INDUCIBLE 1 (SNI1), a negative regulator of SAR, is thought to contribute to maintaining the basal expression of *PR-1* and *WRKY* genes by reducing these histones marks (Mosher et al., 2006).

Histone Modifications; Flowering and SAR

FLD codes for an *Arabidopsis* ortholog of human LYSINE SPECIFIC DEMETHYLASE 1 (LSD1; Liu et al., 2007). FLD, an approximately 96 KDa protein contains a small DNA binding SWRIM domain, and a large polyamine oxidase (PAO) domain (He et al., 2003). Transcriptional co-repressor complexes containing PAO domains are one of the major regulators of gene expression in animals (Jepsen and Rosenfeld, 2002). LSD1 is a component of a co-repressor complex, with histone demethylase activity (Shi et al., 2004). The biochemical function of FLD has not been ascertained. However, the *fld* loss-of-function mutants show increased occupancy of methylated H3K4 in *FLC* locus as might be expected based on its structural similarity to LSD1, which is a histone demethylase (Liu et al., 2007; Singh et al., 2014b). In addition, the FLC locus also shows increased accumulation of acetylated H3 in the *fld* mutant background, both of which support the observation of increased expression of *FLC* in *fld* mutants (He et al., 2003; Singh et al., 2014b). However, in contrast to the *FLC* locus, promoters of *WRKY6* and *WRKY29* genes show reduced accumulation of methylated H3K4 and acetylated H3 (Singh et al., 2014b). Nevertheless, experiments suggest that the histone demethylase activity of FLD is important for SAR activation and flowering. For example, exogenous application of histone demethylase inhibitor *trans*-2-phenylcyclopropylamine (2-PCPA) results in an *fld* loss-of-function phenotype in terms of both flowering and SAR activation (Singh et al., 2014a). Application of 2-PCPA results in impairment of SAR activation, suppression of accumulation of methylated H3K4 in WRKY promoters and delays flowering (Singh et al., 2014a). FLD targets for SAR induction remain unidentified. It is postulated that the effect of FLD on histone modification of WRKY genes is indirect and may be mediated through other factors, functions of which are modulated by FLD (Singh et al., 2013, 2014a,b).

Histone deacetylases (HDACs) are often found in multi-protein co-repressor complexes. HISTONE DEACETYLASE 19 (HDA19) of *Arabidopsis* is a yeast REDUCED POTASSIUM DEFICIENCY 3 (RPD3)-like protein that affects both flowering and SAR (Zhou et al., 2005; Choi et al., 2012; Krogan et al., 2012; Wang et al., 2014). HDA19 interacts with LEUNIG/SEUSS co-repressor complex and negatively regulates expression of the floral patterning gene AGAMOUS (AG; Gonzalez et al., 2007). The *hda*19 mutant accumulates SA and has increased expression of SA-inducible genes such as *EDS1*, *PAD4*, *ICS1* as well as *PR* genes, providing resistance against *P. syringae* (Choi et al., 2012). HDA19, a putative corepressor has been found to directly associate with and deacetylate histones at the *PR-1* and *PR-2* promoters and repress their expression by modifying histones (Choi et al., 2012).

Histone Replacement in SAR and Flowering

The mutants that constitutively activate SAR, show disease resistance, accumulation of SA and expression of PR genes without pathogen challenge, and also often develop microscopic cell death (Jirage et al., 2001; Nandi et al., 2005; Swain et al., 2011). Substitution of canonical histone H2A with H2A.Z variant is a mechanism of chromatin remodeling that is associated with early flowering and activation of constitutive SAR. Replacement of histone H2A with H2A.Z requires a multi-subunit complex, SWI2/SNF2-RELATED 1 (SWR1) in yeast

and SNF2-RELATED CBP ACTIVATOR PROTEIN (SRCAP) in humans (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004). *Arabidopsis* proteins PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), ACTIN-RELATED PROTEIN 6 and SERRATED LEAVES AND EARLY FLOWERING (SEF) are related to SWR1 and SRCAP protein complex components (Noh and Amasino, 2003; March-Diaz et al., 2007, 2008). The PIE/ARP6/SEF complex is functional equivalent of yeast SWRI1 complex, components of which are required for deposition of H2A.Z variant in genes like FLC that negatively regulates flowering (Martin-Trillo et al., 2006; Deal et al., 2007; March-Diaz et al., 2007). Mutations in these genes result in down regulation of FLC and early flowering (**Figure 3**). Mutations in *Arabidopsis HTA9* and *HTA11* genes that code for H2A.Z also result in developmental abnormalities and early flowering (**Figure 3**), very similar to the mutants of PEI/ARP6/SEF complex (March-Diaz et al., 2008). The double mutant *hta9hta11* shows reduction in *FLC* expression and concomitant induction of *FT* expression similar to *sef* and *pie1* mutants (Amasino, 1996). As an interesting correlation between SAR and flowering, the *pie1*, *sef,* and *hta9hta11* mutants show activation of constitutive SAR (March-Diaz et al., 2008). Consequently, the *pie1*, *sef,* and *hta9hta11* mutants show spontaneous cell death, and support less bacterial growth than wild-type plants (March-Diaz et al., 2008). The *pie1* mutants also show constitute activation of *PR1*, *WRKY38* and *WRKY18*, expression of which are associated with SAR activation (Wang et al., 2006).

Sumoylation Regulators Connect SAR and Flowering

Ubiquitin and SMALL UBIQUITIN-LIKE MODIFIER (SUMO) attach to a wide range of proteins, and alter their function and longevity in cells (Miura et al., 2007). SUMO covalently attaches to lysine residues of target proteins through E3 SUMO ligase (Wilkinson and Henley, 2010). SUMO conjugation modifies the conformation of target proteins and influences their interaction with other proteins (Hickey et al., 2012). SUMO modifications have been implicated in many biological processes including, nutrition metabolism, abiotic stress response, flowering, and immunity (Miura et al., 2005; Catala et al., 2007; Lee et al., 2007a; Zhang et al., 2013a). *Arabidopsis* SIZ1 (AtSIZ1) is an ortholog of mammalian and yeast E3 SUMO ligase (Miura et al., 2005). AtSIZ1 negatively regulates SAR activation (Lee et al., 2007a). The mutants of *AtSIZ1*, show enhanced expression of PHYTOALEXIN DEFICIENT 4 (PAD4) and ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) that positively regulate SA biosynthesis. The *atsiz1* mutants also accumulate SA and SA-glucoside conjugates (SAG), express SAR marker *PR-1* constitutively, and are resistant to pathogens. All these phenotypes of *atsiz1* are dependent on SA accumulation, as *NahG* expression in the *atsiz1* mutant background, abolishes all SAR-associated responses (Lee et al., 2007a). As a very interesting cross-connection between SAR and flowering, it has been reported that AtSIZ1 promotes FLC expression and thereby negatively regulates the flowering transition (**Figure 3**; Jin et al., 2008). Very recently, it has also been shown that AtSIZ1 physically interacts with FLC and influences its stability (**Figure 3**; Son et al., 2014).

Reversal of SUMO conjugation is carried out by SUMO protease. The *early in short days 4* (*esd4*) mutant has a mutation in the SUMO protease and thus accumulates SUMO conjugates (Villajuana-Bonequi et al., 2014). Though, the exact cause of early flowering in *esd4* mutant is not known, it is believed that physiological stress caused by hyper-accumulation of SUMO conjugates may result in early flowering. Interestingly, a genetic screen for suppressors of *esd4*, identified a mutant of the SA biosynthetic gene *isochorismate synthase 1* (*ICS1*/*SID2*; Villajuana-Bonequi et al., 2014). Early flowering of *esd4* is partially rescued by the reduced SA levels in the *esd4 sid2* double mutant (Villajuana-Bonequi et al., 2014). Thus early flowering in *esd4* mutant is also associated with SA signaling activation, an integral event of SAR activation.

Reverse Association between Flowering and SAR

The studies described above, provided evidence to support the idea that the flowering and SAR signaling pathways are highly integrated. However, there are reports that contradict this idea. For example, the *HOPW1-1-INTERACTING3* (*WIN3; alias PBS3, GDG1, GH3.12*) gene of *Arabidopsis* is a positive regulator for SAR and negative regulator of flowering (Wang et al., 2011). The *WIN3* gene product codes for an enzyme that conjugates aminoacids to 4-aminobenzoate or 4-hydroxybenzoate; a process which is required for SA biosynthesis (Okrent et al., 2009). WIN3 expression is induced by SA, and its function is needed for pathogenesis associated SA responses (Nobuta et al., 2007; Wang et al., 2011). However, mutation in *WIN3* results in early flowering under long-day conditions (Lee et al., 2007b; Wang et al., 2011). The reverse-association is also observed in plants undergoing the shade avoidance response. Since plants absorb more red than far-red light, the red:far-red ratio gets reduced under dense vegetation. The reduced red:far-red ratio enhances early flowering in *Arabidopsis*, a component of the shade-avoidance response (Halliday et al., 1994, 2003). In contrast, plants grown in low red:far-red ratio exhibit compromised defense response in the form of reduced SA dependent PR1 and WRKY expression (de Wit et al., 2013). The lack of an association between SAR and flowering is observed in mutants such as *npr1* and *sfd1*, which are defective in SAR but not in flowering. Therefore, these reports indicate that the SAR and flowering pathways are genetically separable, even though they share common inducers, such as SA.

Conclusion

It appears that some of the molecular machinery that regulates flowering time is shared by the SAR activation processes in plants. A number of studies suggest that retention of infection

memory in plants is mostly mediated through epigenetic mechanisms. While generating resistance against invading pathogens, plant tissues generate signals that are capable of long distance transport to carry infection information to distant tissues. Upon arrival in distal tissues, mobile signals are perceived, leading to biosynthesis and accumulation of SA, which is required for SAR activation as well as the floral transition. In addition, the perception of mobile signals also initiates epigenetic modification of certain key genes which contribute to infection memory development and SAR associated priming defense responses. Alteration of histone methylation and acetylation, and histone replacement influence flowering and SAR. Suppression of SAR in healthy plants and flowering during vegetative growth, are highly important for overall growth, development and productivity of plants. Emerging data strongly suggest common genetic and epigenetic regulators for flowering and SAR.

Author contributions

Both the authors equally contributed to the manuscript.

Acknowledgments

We acknowledge Supriya Chakraborty for critical reading of the manuscript. This work is supported by Department of Biotechnology (DBT grant No. BT/PR14656/BRB/10/864/2010) to AN and ICMR fellowship to ZB.

References

Amasino, R. M. (1996). Control of flowering time in plants. *Curr. Opin. Genet. Dev.* 6, 480–487. doi: 10.1016/S0959-437X(96)80071-2

Aukerman, M. J., and Amasino, R. M. (1996). Molecular genetic analysis of flowering time in *Arabidopsis*. *Semin. Cell Dev. Biol.* 7, 427–433. doi: 10.1006/scdb.1996.0053

Ballare, C. L., Mazza, C. A., Austin, A. T., and Pierik, R. (2012). Canopy light and plant health. *Plant Physiol.* 160, 145–155. doi: 10.1104/pp.112.200733

Caruso, F. L., and Kuc, J. (1977). Protection of watermelon and muskmelon against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. *Phytopathology* 67, 1285–1289. doi: 10.1094/Phyto-67-1285

Catala, R., Ouyang, J., Abreu, I. A., Hu, Y., Seo, H., Zhang, X., et al. (2007). The *Arabidopsis* E3 SUMO ligase SIZ1 regulates plant growth and drought responses. *Plant Cell* 19, 2952–2966. doi: 10.1105/tpc.106.049981

Chailakhyan, M. K. (1936). New facts in support of the hormonal theory of plant development. *C. R. Acad. Sci. URSS* 13, 79–83.

Champigny, M. J., Isaacs, M., Carella, P., Faubert, J., Fobert, P. R., and Cameron, R. K. (2013). Long distance movement of DIR1 and investigation of the role of DIR1-like during systemic acquired resistance in *Arabidopsis*. *Front. Plant. Sci.* 4:230. doi: 10.3389/fpls.2013.00230

Chanda, B., Xia, Y., Mandal, M. K., Yu, K., Sekine, K. T., Gao, Q. M., et al. (2011). Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nat. Genet.* 43, 421–427. doi: 10.1038/ng.798

Chandra-Shekara, A. C., Gupte, M., Navarre, D., Raina, S., Raina, R., Klessig, D., et al. (2006). Light-dependent hypersensitive response and resistance signaling against turnip crinkle virus in *Arabidopsis*. *Plant J.* 45, 320–334. doi: 10.1111/j.1365-313X.2005.02618.x

Chaturvedi, R., Krothapalli, K., Makandar, R., Nandi, A., Sparks, A. A., Roth, M. R., et al. (2008). Plastid omega3-fatty acid desaturase-dependent accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. *Plant J.* 54, 106–117. doi: 10.1111/j.1365-313X.2007.03400.x

Chaturvedi, R., Venables, B., Petros, R. A., Nalam, V., Li, M., Wang, X., et al. (2012). An abietane diterpenoid is a potent activator of systemic acquired resistance. *Plant J.* 71, 161–172. doi: 10.1111/j.1365-313X.2012.04981.x

Chinchilla, D., Shan, L., He, P., De Vries, S., and Kemmerling, B. (2009). One for all: the receptor-associated kinase BAK1. *Trends Plant Sci.* 14, 535–541. doi: 10.1016/j.tplants.2009.08.002

Choi, S. M., Song, H. R., Han, S. K., Han, M., Kim, C. Y., Park, J., et al. (2012). HDA19 is required for the repression of salicylic acid biosynthesis and salicylic acid-mediated defense responses in *Arabidopsis*. *Plant J.* 71, 135–146. doi: 10.1111/j.1365-313X.2012.04977.x

Cleland, C. F., and Ajami, A. (1974). Identification of the flower-inducing factor isolated from aphid honeydew as being salicylic acid. *Plant Physiol.* 54, 904–906. doi: 10.1104/pp.54.6.904

Clouse, S. D. (2011). Brassinosteroid signal transduction: from receptor kinase activation to transcriptional networks regulating plant development. *Plant Cell* 23, 1219-1230. doi: 10.1105/tpc.111.084475

Conrath, U. (2011). Molecular aspects of defence priming. *Trends Plant Sci.* 16, 524–531. doi: 10.1016/j.tplants.2011.06.004

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., et al. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* 316, 1030–1033. doi: 10.1126/science.1141752

Deal, R. B., Topp, C. N., Mckinney, E. C., and Meagher, R. B. (2007). Repression of flowering in *Arabidopsis* requires activation of FLOWERING LOCUS C expression by the histone variant H2A.Z. *Plant Cell* 19, 74–83. doi: 10.1105/tpc.106.048447

Dempsey, D. A., and Klessig, D. F. (2012). SOS - too many signals for systemic acquired resistance? *Trends Plant Sci.* 17, 538–545. doi: 10.1016/j.tplants.2012.05.011

de Wit, M., Spoel, S. H., Sanchez-Perez, G. F., Gommers, C. M., Pieterse, C. M., Voesenek, L. A., et al. (2013). Perception of low red:far-red ratio compromises both salicylic acid- and jasmonic acid-dependent pathogen defences in *Arabidopsis*. *Plant J.* 75, 90–103. doi: 10.1111/tpj.12203

Dong, X. (2004). NPR1, all things considered. *Curr. Opin. Plant Biol.* 7, 547–552. doi: 10.1016/j.pbi.2004.07.005

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

Eulgem, T., Tsuchiya, T., Wang, X. J., Beasley, B., Cuzick, A., Tor, M., et al. (2007). EDM2 is required for RPP7-dependent disease resistance in *Arabidopsis* and affects RPP7 transcript levels. *Plant J.* 49, 829–839. doi: 10.1111/j.1365-313X.2006.02999.x

Fragniere, C., Serrano, M., Abou-Mansour, E., Metraux, J. P., and L'haridon, F. (2011). Salicylic acid and its location in response to biotic and abiotic stress. *FEBS Lett.* 585, 1847–1852. doi: 10.1016/j.febslet.2011.04.039

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fujioka, S., Sakurai, A., Yamaguchi, I., Murofushi, N., Takahashi, N., Kaihara, S., et al. (1987). Isolation and identification of L-Pipecolic acid and nicotinamide as flower-inducing substances in *Lemna*. *Plant Cell Physiol.* 28, 995–1003.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., et al. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261, 754–756. doi: 10.1126/science.261.5122.754

Geiman, T. M., and Robertson, K. D. (2002). Chromatin remodeling, histone modifications, and DNA methylation-how does it all fit together? *J. Cell Biochem.* 87, 117–125. doi: 10.1002/jcb.10286

Gonzalez, D., Bowen, A. J., Carroll, T. S., and Conlan, R. S. (2007). The transcription corepressor LEUNIG interacts with the histone deacetylase

HDA19 and mediator components MED14 (SWP) and CDK8 (HEN3) to repress transcription. *Mol. Cell. Biol.* 27, 5306–5315. doi: 10.1128/MCB.01912-1916

Griebel, T., and Zeier, J. (2008). Light regulation and daytime dependency of inducible plant defenses in *Arabidopsis*: phytochrome signaling controls systemic acquired resistance rather than local defense. *Plant Physiol.* 147, 790–801. doi: 10.1104/pp.108.119503

Guedes, M. E. M., Richmond, S., and Kuc, J. (1980). Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with *Colletotrichum lagenarium* and the onset of flowering and fruiting physiol. *Plant Pathol.* 17, 229–233.

Halliday, K. J., Koornneef, M., and Whitelam, G. C. (1994). Phytochrome B and at least one other phytochrome mediate the accelerated flowering response of *Arabidopsis thaliana* L. to low Red/Far-Red ratio. *Plant Physiol.* 104, 1311–1315.

Halliday, K. J., Salter, M. G., Thingnaes, E., and Whitelam, G. C. (2003). Phytochrome control of flowering is temperature sensitive and correlates with expression of the floral integrator FT. *Plant J.* 33, 875–885. doi: 10.1046/j.1365-313X.2003.01674.x

He, Y., and Amasino, R. M. (2005). Role of chromatin modification in flowering-time control. *Trends Plant Sci.* 10, 30–35. doi: 10.1016/j.tplants.2004.11.003

He, Y. H., Michaels, S. D., and Amasino, R. M. (2003). Regulation of flowering time by histone acetylation in *Arabidopsis*. *Science* 302, 1751–1754. doi: 10.1126/science.1091109

Helliwell, C. A., Wood, C. C., Robertson, M., James Peacock, W., and Dennis, E. S. (2006). The *Arabidopsis* FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. *Plant J.* 46, 183–192. doi: 10.1111/j.1365-313X.2006.02686.x

Henderson, I. R., and Dean, C. (2004). Control of *Arabidopsis* flowering: the chill before the bloom. *Development* 131, 3829–3838. doi: 10.1242/dev.01294

Hickey, C. M., Wilson, N. R., and Hochstrasser, M. (2012). Function and regulation of SUMO proteases. *Nat. Rev. Mol. Cell Biol.* 13, 755–766. doi: 10.1038/nrm3478

Iriti, M., and Faoro, F. (2007). Review of innate and specific immunity in plants and animals. *Mycopathologia* 164, 57–64. doi: 10.1007/s11046-007-9026-9027

Islam, S. Z., Babadoost, M., Bekal, S., and Lambert, K. (2008). Red Light-induced systemic disease resistance against Root-knot nematode *Meloidogyne javanica* and *Pseudomonas syringae* pv. *tomato* DC 3000. *J. Phytopathol.* 156, 708–714. doi: 10.1111/j.1439-0434.2008.01435.x

Jaskiewicz, M., Conrath, U., and Peterhansel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* 12, 50–55. doi: 10.1038/embor.2010.186

Jepsen, K., and Rosenfeld, M. G. (2002). Biological roles and mechanistic actions of co-repressor complexes. *J. Cell Sci.* 115, 689–698.

Jin, J. B., Jin, Y. H., Lee, J., Miura, K., Yoo, C. Y., Kim, W. Y., et al. (2008). The SUMO E3 ligase, AtSIZ1, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on FLC chromatin structure. *Plant J.* 53, 530–540. doi: 10.1111/j.1365-313X.2007.03359.x

Jirage, D., Zhou, N., Cooper, B., Clarke, J. D., Dong, X., and Glazebrook, J. (2001). Constitutive salicylic acid-dependent signaling in cpr1 and cpr6 mutants requires PAD4. *Plant J.* 26, 395–407. doi: 10.1046/j.1365-313X.2001.2641040.x

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91. doi: 10.1126/science.1170025

Kamakaka, R. T., and Biggins, S. (2005). Histone variants: deviants? *Genes Dev.* 19, 295–310. doi: 10.1101/gad.1272805

Kangasjarvi, S., Neukermans, J., Li, S., Aro, E. M., and Noctor, G. (2012). Photosynthesis, photorespiration, and light signalling in defence responses. *J. Exp. Bot.* 63, 1619–1636. doi: 10.1093/jxb/err402

Khurana, J. P., and Cleland, C. F. (1992). Role of salicylic acid and benzoic acid in flowering of a Photoperiod-Insensitive strain, *Lemna paucicostata* LP6. *Plant Physiol.* 100, 1541–1546. doi: 10.1104/pp.100.3.1541

Kim, T. W., Guan, S., Burlingame, A. L., and Wang, Z. Y. (2011). The CDG1 kinase mediates brassinosteroid signal transduction from BRI1 receptor kinase to BSU1 phosphatase and GSK3-like kinase BIN2. *Mol. Cell* 43, 561–571. doi: 10.1016/j.molcel.2011.05.037

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.1105/tpc.12.12.2339

Knott, J. E. (1934). Effect of localized photoperiod on spinach. *Proc. Am. Soc. Hortic. Sci.* 31, 152–154.

Kobor, M. S., Venkatasubrahmanyam, S., Meneghini, M. D., Gin, J. W., Jennings, J. L., Link, A. J., et al. (2004). A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. *PLoS Biol.* 2:E131. doi: 10.1371/journal.pbio.0020131

Krogan, N. J., Keogh, M. C., Datta, N., Sawa, C., Ryan, O. W., Ding, H., et al. (2003). A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. *Mol. Cell* 12, 1565–1576. doi: 10.1016/S1097-2765(03)00497-0

Krogan, N. T., Hogan, K., and Long, J. A. (2012). APETALA2 negatively regulates multiple floral organ identity genes in *Arabidopsis* by recruiting the co-repressor TOPLESS and the histone deacetylase HDA19. *Development* 139, 4180–4190. doi: 10.1242/dev.085407 dev.085407

Kuc, J., and Richmond, S. (1977). Aspects of protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. *Phytopathology* 67, 533–536. doi: 10.1094/Phyto-67-533

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., et al. (1996). Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10, 71–82. doi: 10.1046/j.1365-313X.1996.10010071.x

Lee, J., Nam, J., Park, H. C., Na, G., Miura, K., Jin, J. B., et al. (2007a). Salicylic acid-mediated innate immunity in *Arabidopsis* is regulated by SIZ1 SUMO E3 ligase. *Plant J.* 49, 79–90. doi: 10.1111/j.1365-313X.2006.02947.x

Lee, M. W., Lu, H., Jung, H. W., and Greenberg, J. T. (2007b). A key role for the *Arabidopsis* WIN3 protein in disease resistance triggered by *Pseudomonas syringae* that secrete AvrRpt2. *Mol. Plant Microbe Interact.* 20, 1192–1200. doi: 10.1094/MPMI-20-10-1192

Li, C., Luo, L., Fu, Q., Niu, L., and Xu, Z. F. (2014). Isolation and functional characterization of JcFT, a FLOWERING LOCUS T (FT) homologous gene from the biofuel plant *Jatropha curcas*. *BMC Plant Biol.* 14:125. doi: 10.1186/1471-2229-14-125

Lin, W., Lu, D., Gao, X., Jiang, S., Ma, X., Wang, Z., et al. (2013). Inverse modulation of plant immune and brassinosteroid signaling pathways by the receptor-like cytoplasmic kinase BIK1. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12114–12119. doi: 10.1073/pnas.1302154110

Liu, F., Quesada, V., Crevillen, P., Baurle, I., Swiezewski, S., and Dean, C. (2007). The *Arabidopsis* RNA-binding protein FCA requires a lysine-specific demethylase 1 homolog to downregulate FLC. *Mol. Cell* 28, 398–407. doi: 10.1016/j.molcel.2007.10.018

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. (2010). A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 496–501. doi: 10.1073/pnas.0909705107

Luna, E., Bruce, T. J., Roberts, M. R., Flors, V., and Ton, J. (2012). Next-generation systemic acquired resistance. *Plant Physiol.* 158, 844–853. doi: 10.1104/pp.111.187468

March-Diaz, R., Garcia-Dominguez, M., Florencio, F. J., and Reyes, J. C. (2007). SEF, a new protein required for flowering repression in *Arabidopsis*, interacts with PIE1 and ARP6. *Plant Physiol.* 143, 893–901. doi: 10.1104/pp.106.092270

March-Diaz, R., Garcia-Dominguez, M., Lozano-Juste, J., Leon, J., Florencio, F. J., and Reyes, J. C. (2008). Histone H2A.Z and homologues of components of the SWR1 complex are required to control immunity in *Arabidopsis*. *Plant J.* 53, 475–487. doi: 10.1111/j.1365-313X.2007.03361.x

Martin-Trillo, M., Lázaro, A., Poethig, R. S., Gómez-Mena, C., Piñeiro, M. A., Martinez-Zapater, J. M., et al. (2006). EARLY IN SHORT DAYS 1 (ESD1) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates FLC accumulation in *Arabidopsis*. *Development* 133, 1241–1252. doi: 10.1242/dev.02301

Martinez, C., Pons, E., Prats, G., and Leon, J. (2004). Salicylic acid regulates flowering time and links defence responses and reproductive development. *Plant J.* 37, 209–217. doi: 10.1046/j.1365-313X.2003.01954.x

Metraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006. doi: 10.1126/science.250.4983.1004

Michaels, S. D., and Amasino, R. M. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* 11, 949–956. doi: 10.1105/tpc.11.5.949

Miura, K., Jin, J. B., and Hasegawa, P. M. (2007). Sumoylation, a post-translational regulatory process in plants. *Curr. Opin. Plant Biol.* 10, 495–502. doi: 10.1016/j.pbi.2007.07.002

Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A. S., Raghothama, K. G., et al. (2005). The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7760–7765. doi: 10.1073/pnas.0500778102

Mizuguchi, G., Shen, X., Landry, J., Wu, W. H., Sen, S., and Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science* 303, 343–348. doi: 10.1126/science.1090701

Mosher, R. A., Durrant, W. E., Wang, D., Song, J., and Dong, X. (2006). A comprehensive structure-function analysis of *Arabidopsis* SNI1 defines essential regions and transcriptional repressor activity. *Plant Cell* 18, 1750–1765. doi: 10.1105/tpc.105.039677

Nandi, A., Moeder, W., Kachroo, P., Klessig, D. F., and Shah, J. (2005). *Arabidopsis* ssi2-conferred susceptibility to *Botrytis cinerea* is dependent on EDS5 and PAD4. *Mol. Plant Microbe Interact.* 18, 363–370. doi: 10.1094/MPMI-18-0363

Nandi, A., Welti, R., and Shah, J. (2004). The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene SUPPRESSSOR OF FATTY ACID DESATURASE DEFICIENCY1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell* 16, 465–477. doi: 10.1105/tpc.016907

Navarova, H., Bernsdorff, F., Doring, A. C., and Zeier, J. (2012). Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *Plant Cell* 24, 5123–5141. doi: 10.1105/tpc.112.103564

Nimchuk, Z., Eulgem, T., Holt, B. F. III, and Dangl, J. L. (2003). Recognition and response in the plant immune system. *Annu. Rev. Genet.* 37, 579–609. doi: 10.1146/annurev.genet.37.110801.142628

Nobuta, K., Okrent, R. A., Stoutemyer, M., Rodibaugh, N., Kempema, L., Wildermuth, M. C., et al. (2007). The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent defense responses in *Arabidopsis*. *Plant Physiol.* 144, 1144–1156. doi: 10.1104/pp.107.097691

Noh, Y. S., and Amasino, R. M. (2003). PIE1, an ISWI family gene, is required for FLC activation and floral repression in *Arabidopsis*. *Plant Cell* 15, 1671–1682. doi: 10.1105/tpc.012161

Nowak, S. J., and Corces, V. G. (2004). Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet.* 20, 214–220. doi: 10.1016/j.tig.2004.02.007

Okrent, R. A., Brooks, M. D., and Wildermuth, M. C. (2009). *Arabidopsis* GH3.12 (PBS3) conjugates amino acids to 4-substituted benzoates and is inhibited by salicylate. *J. Biol. Chem.* 284, 9742–9754. doi: 10.1074/jbc.M806662200

Ross, A. F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14, 340–358. doi: 10.1016/0042-6822(61)90319-1

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., et al. (2011). The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell* 23, 2440–2455. doi: 10.1105/tpc.111.084301

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., and Hunt, M. D. (1996). Systemic acquired resistance. *Plant Cell* 8, 1809–1819. doi: 10.1105/tpc.8.10.1809

Sano, S., Aoyama, M., Nakai, K., Shimotani, K., Yamasaki, K., Sato, M. H., et al. (2014). Light-dependent expression of flg22-induced defense genes in *Arabidopsis*. *Front. Plant Sci.* 5:531. doi: 10.3389/fpls.2014.00531

Schwessinger, B., and Ronald, P. C. (2012). Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* 63, 451–482. doi: 10.1146/annurev-arplant-042811-105518

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., et al. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941–953. doi: 10.1016/j.cell.2004.12.012

Simpson, G. G., and Dean, C. (2002). *Arabidopsis*, the rosetta stone of flowering time? *Science* 296, 285–289. doi: 10.1126/science.296.5566.285 296/5566/285

Simpson, G. G., Gendall, A. R., and Dean, C. (1999). When to switch to flowering. *Annu. Rev. Cell Dev. Biol.* 15, 519–550. doi: 10.1146/annurev.cellbio.15.1.519

Singh, V., Banday, Z. Z., and Nandi, A. K. (2014a). Exogenous application of histone demethylase inhibitor trans-2-phenylcyclopropylamine mimics FLD loss-of-function phenotype in terms of systemic acquired resistance in *Arabidopsis thaliana*. *Plant Signal. Behav.* doi: 10.4161/psb.29658 [Epub ahead of print].

Singh, V., Roy, S., Singh, D., and Nandi, A. K. (2014b). *Arabidopsis* flowering locus D influences systemic-acquired-resistance- induced expression and histone modifications of WRKY genes. *J. Biosci.* 39, 119–126. doi: 10.1007/s12038-013-9407-7

Singh, V., Roy, S., Giri, M. K., Chaturvedi, R., Chowdhury, Z., Shah, J., et al. (2013). *Arabidopsis thaliana* FLOWERING LOCUS D is required for systemic acquired resistance. *Mol. Plant Microbe Interact.* 26, 1079–1088. doi: 10.1094/MPMI-04-13-0096-R

Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B., and Mauch-Mani, B. (2012). Descendants of primed *Arabidopsis* plants exhibit resistance to biotic stress. *Plant Physiol.* 158, 835–843. doi: 10.1104/pp.111.191593 pp.111.191593

Smith, H. (2000). Phytochromes and light signal perception by plants–an emerging synthesis. *Nature* 407, 585–591. doi: 10.1038/35036500

Son, G. H., Park, B. S., Song, J. T., and Seo, H. S. (2014). FLC-mediated flowering repression is positively regulated by sumoylation. *J. Exp. Bot.* 65, 339–351. doi: 10.1093/jxb/ert383 ert383

Sticher, L., Mauch-Mani, B., and Metraux, J. P. (1997). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 35, 235–270. doi: 10.1146/annurev.phyto.35.1.235

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). CONSTANS mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* 410, 1116–1120. doi: 10.1038/35074138

Sun, Y., Li, L., Macho, A. P., Han, Z., Hu, Z., Zipfel, C., et al. (2013). Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science* 342, 624–628. doi: 10.1126/science.1243825

Swain, S., Roy, S., Shah, J., Van Wees, S., Pieterse, C. M., and Nandi, A. K. (2011). *Arabidopsis thaliana* cdd1 mutant uncouples the constitutive activation of salicylic acid signalling from growth defects. *Mol. Plant Pathol.* 12, 855–865. doi: 10.1111/j.1364-3703.2011.00717.x

Tamaki, S., Matsuo, S., Wong, H. L., Yokoi, S., and Shimamoto, K. (2007). Hd3a protein is a mobile flowering signal in rice. *Science* 316, 1033–1036. doi: 10.1126/science.1141753

Tang, W., Kim, T. W., Oses-Prieto, J. A., Sun, Y., Deng, Z., Zhu, S., et al. (2008). BSKs mediate signal transduction from the receptor kinase BRI1 in *Arabidopsis*. *Science* 321, 557–560. doi: 10.1126/science.1156973

Tang, W., Yuan, M., Wang, R., Yang, Y., Wang, C., Oses-Prieto, J. A., et al. (2011). PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1. *Nat. Cell Biol.* 13, 124–131. doi: 10.1038/ncb2151

Tsuchiya, T., and Eulgem, T. (2010). The *Arabidopsis* defense component EDM2 affects the floral transition in an FLC-dependent manner. *Plant J.* 62, 518–528. doi: 10.1111/j.1365-313X.2010.04169.x TPJ4169

Tuzun, S., and Kuc, J. (1985). Movement of a factor in tobacco infected with *Peronospora tabacina* Adam which systemically protect against blue mold. *Physiol. Plant Pathol.* 26, 321–330. doi: 10.1016/0048-4059(85)90007-4

Vaillant, I., and Paszkowski, J. (2007). Role of histone and DNA methylation in gene regulation. *Curr. Opin. Plant Biol.* 10, 528–533. doi: 10.1016/j.pbi.2007.06.008

van Loon, L. C., Rep, M., and Pieterse, C. M. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162. doi: 10.1146/annurev.phyto.44.070505.143425.

Varkonyi-Gasic, E., Moss, S. M., Voogd, C., Wang, T., Putterill, J., and Hellens, R. P. (2013). Homologs of FT, CEN and FD respond to developmental and environmental signals affecting growth and flowering in the perennial vine kiwifruit. *New Phytol.* 198, 732–746. doi: 10.1111/nph.12162

Verhage, A., Van Wees, S. C., and Pieterse, C. M. (2010). Plant immunity: it's the hormones talking, but what do they say? *Plant Physiol.* 154, 536–540. doi: 10.1104/pp.110.161570

Villajuana-Bonequi, M., Elrouby, N., Nordstrom, K., Griebel, T., Bachmair, A., and Coupland, G. (2014). Elevated salicylic acid levels conferred by increased expression of ISOCHORISMATE SYNTHASE 1 contribute to hyperaccumulation of SUMO1 conjugates in the *Arabidopsis* mutant early in short days 4. *Plant J.* 79, 206–219. doi: 10.1111/tpj.12549

Vlot, A. C., Klessig, D. F., and Park, S. W. (2008). Systemic acquired resistance: the elusive signal(s). *Curr. Opin. Plant Biol.* 11, 436–442. doi: 10.1016/j.pbi.2008.05.003

Wada, K. C., Mizuuchi, K., Koshio, A., Kaneko, K., Mitsui, T., and Takeno, K. (2014). Stress enhances the gene expression and enzyme activity of phenylalanine ammonia-lyase and the endogenous content of salicylic acid to induce flowering in pharbitis. *J. Plant Physiol.* 171, 895–902. doi: 10.1016/j.jplph.2014.03.008

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2:e123. doi: 10.1371/journal.ppat.0020123

Wang, G. F., Seabolt, S., Hamdoun, S., Ng, G., Park, J., and Lu, H. (2011). Multiple roles of WIN3 in regulating disease resistance, cell death, and flowering time in *Arabidopsis*. *Plant Physiol.* 156, 1508–1519. doi: 10.1104/pp.111.176776

Wang, H., Jiang, Y. P., Yu, H. J., Xia, X. J., Shi, K., Zhou, Y. H., et al. (2010). Light quality affects incidence of powdery mildew, expression of defence-related genes and associated metabolism in cucumber plants. *Eur. J. Plant Pathol.* 127, 125–135. doi: 10.1007/s10658-009-9577-9571

Wang, Z., Cao, H., Chen, F., and Liu, Y. (2014). The roles of histone acetylation in seed performance and plant development. *Plant Physiol. Biochem.* 84C, 125–133. doi: 10.1016/j.plaphy.2014.09.010

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., et al. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3, 1085–1094. doi: 10.1105/tpc.3.10.1085

Wilkinson, K. A., and Henley, J. M. (2010). Mechanisms, regulation and consequences of protein SUMOylation. *Biochem. J.* 428, 133–145. doi: 10.1042/BJ20100158

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Yalpani, N., Silverman, P., Wilson, T. M., Kleier, D. A., and Raskin, I. (1991). Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell* 3, 809–818. doi: 10.1105/tpc.3.8.809

Yang, C. J., Zhang, C., Lu, Y. N., Jin, J. Q., and Wang, X. L. (2011). The mechanisms of brassinosteroids' action: from signal transduction to plant development. *Mol. Plant* 4, 588–600. doi: 10.1093/mp/ssr020

Zeevaart, J. A. (2006). Florigen coming of age after 70 years. *Plant Cell* 18, 1783–1789. doi: 10.1105/tpc.106.043513

Zeier, J., Pink, B., Mueller, M. J., and Berger, S. (2004). Light conditions influence specific defence responses in incompatible plant-pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. *Planta* 219, 673–683. doi: 10.1007/s00425-004-1272-z

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., et al. (2010). Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe* 7, 290–301. doi: 10.1016/j.chom.2010.03.007

Zhang, S., Qi, Y., Liu, M., and Yang, C. (2013a). SUMO E3 ligase AtMMS21 regulates drought tolerance in *Arabidopsis thaliana*(F). *J. Integr. Plant Biol.* 55, 83–95. doi: 10.1111/jipb.12024

Zhang, Y., Li, B., Xu, Y., Li, H., Li, S., Zhang, D., et al. (2013b). The cyclophilin CYP20-2 modulates the conformation of BRASSINAZOLE-RESISTANT1, which binds the promoter of FLOWERING LOCUS D to regulate flowering in *Arabidopsis*. *Plant Cell* 25, 2504–2521. doi: 10.1105/tpc.113.110296

Zhang, X. (2008). The epigenetic landscape of plants. *Science* 320, 489–492. doi: 10.1126/science.1153996

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wu, K. (2005). HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. *Plant Cell* 17, 1196–1204. doi: 10.1105/tpc.104.028514

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 16 December 2014
doi: 10.3389/fpls.2014.00701

The phosphate transporter *PHT4;1* is a salicylic acid regulator likely controlled by the circadian clock protein CCA1

Guoying Wang†, Chong Zhang†, Stephanie Battle and Hua Lu*

Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD, USA

Edited by:
Xin Li, University of British Columbia, Canada

Reviewed by:
Brad Day, Michigan State University, USA
Keiko Yoshioka, University of Toronto, Canada

***Correspondence:**
Hua Lu, Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250, USA
e-mail: hualu@umbc.edu

† Guoying Wang and Chong Zhang have contributed equally to this work.

The small phenolic compound salicylic acid (SA) plays a critical role in plant defense against broad-spectrum of pathogens. The phosphate transporter gene *PHT4;1* was previously shown to affect SA-mediated defense and its expression is regulated by the circadian clock. To further understand how *PHT4;1* affects SA accumulation, here we analyzed the genetic interactions between the gain-of-function mutant *pht4;1-1* and several known SA mutants, including *sid2-1*, *ald1-1*, *eds5-3*, and *pad4-1*. The genetic analysis was conducted in the *acd6-1* background since the change of *acd6-1* dwarfism can be used as a convenient readout for the change of defense levels caused by impairments in some SA genes. We found that compared with the corresponding double mutants, the triple mutants *acd6-1pht4;1-1ald1-1*, *acd6-1pht4;1-1eds5-3*, and *acd6-1pht4;1-1pad4-1* accumulated lower levels of SA and *PR1* transcripts, suggesting that *PHT4;1* contributes to *acd6-1*-conferred defense phenotypes independently of these known SA regulators. Although some triple mutants had wild type (wt)-like levels of SA and *PR1* transcripts, these plants were smaller than wt and displayed minor cell death, suggesting that additional regulatory pathways contribute to *acd6-1*-conferred dwarfism and cell death. Our data further showed that circadian expression of *PHT4;1* was dependent on CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), a central oscillator component of *Arabidopsis* circadian clock. Recombinant CCA1 protein was demonstrated to bind to the *PHT4;1* promoter in electrophoretic mobility shift assays, suggesting a direct transcriptional regulation of *PHT4;1* by CCA1. Together these results indicate that PHT4;1 is a SA regulator acting independently of several known SA genes and they also implicate a role of the circadian clock mediated by CCA1 in regulating phosphate transport and/or innate immunity in *Arabidopsis*.

Keywords: circadian clock, defense signaling, programmed cell death, disease resistance, phosphate transporter

INTRODUCTION

Successful defense against pathogen attacks is critical to plant growth and development. In addition to pre-formed physical and chemical barriers, plants can monitor the presence of pathogens and subsequently activate defense responses to restrict further proliferation and spreading of pathogens. However, it remains challenging to identify genes that control plant defense, understand their mechanisms of action, and determine how they interact to form complex defense networks to orchestrate resistance to invaders.

The small phenolic compound salicylic acid (SA) plays a central role in plant defense signaling (Hammond-Kosack and Jones, 1996; Ryals et al., 1996; Tsuda et al., 2008). Genes that positively regulate SA-mediated defense have been identified in *Arabidopsis*. These genes can be grouped into three types based on their potential biochemical and molecular functions (Lu, 2009; Lu et al., 2009a). The type I SA genes encode enzymes directly involved in SA biosynthesis, which is proposed to take place in the chloroplast and cytoplasm of a cell, involving multiple pathways (Chen et al., 2009; Dempsey et al., 2011). The type I SA gene *ISOCHORISMATE SYNTHASE 1* (*ICS1*), also called SA *Induction-Deficient 2* (*SID2*) and *ENHANCED DISEASE SUSCEPTIBILITY 16* (*EDS16*), contributes to the bulk SA biosynthesis (Nawrath and Metraux, 1999; Wildermuth et al., 2001; Ng et al., 2011). ICS1/SID2/EDS16 protein was shown to be chloroplast-localized (Strawn et al., 2007), indicating that the major SA biosynthetic pathway likely occurs in the chloroplast. To support this notion, the bacterial gene *nahG* that encodes SA hydroxylase to convert SA to the breakdown product catechol (Friedrich et al., 1995), when expressed in the chloroplast, abolishes SA accumulation in the transgenic plants challenged with pathogens or UV light (Fragniere et al., 2011).

Protein products of type II SA genes may not be directly involved in SA biosynthesis. But like SA biosynthetic enzymes (type I), they can influence SA accumulation, possibly through indirect ways, for instance, chemically modifying SA precursors, affecting availability of SA precursors and/or products, influencing expression of type I SA genes, and/or changing activities of SA biosynthetic enzymes. One example of the type II SA regulators is SID1/EDS5, which was localized to the chloroplast membrane and was proposed to transport SA from the chloroplast to the cytoplasm in a cell (Nawrath et al., 2002; Serrano et al., 2013;

Yamasaki et al., 2013). The lack of such a SA-transport activity in the *eds5* mutants likely leads to SA accumulation in the chloroplast that feedback-inhibits SA biosynthesis under defense conditions. Indeed, like *sid2* mutants, *eds5* mutants accumulate much reduced SA levels under defense conditions (Nawrath et al., 2002; Ng et al., 2011). Thus these observations further support the idea that the chloroplast is the major site for SA biosynthesis. Additional examples of type II SA regulators include *Accelerated Cell Death (ACD6), AGD2-LIKE DEFENSE 1 (ALD1), EDS1,* and *PHYTOALEXIN DEFICIENT 4* (*PAD4*) (Falk et al., 1999; Jirage et al., 1999; Lu et al., 2003; Song et al., 2004). Loss of function mutations in these SA regulators often lead to EDS and partially reduced SA accumulation upon pathogen insults, compared to wild type (wt) plants. However, the mechanisms by which many of the type II SA regulators act have not been well understood.

Activation of SA signaling often leads to enhanced disease resistance in plants. The type III SA genes act downstream of SA, including SA receptors and signaling transducers. *Non-expressor of PR GENES 1* (*NPR1*) is an example of type III SA genes that has been elegantly studied for its mechanism of action (Dong, 2004; Fu and Dong, 2013). The NPR1 protein has been shown as a key component for SA signaling, overexpression of which confers enhanced disease resistance to a range of pathogens in *Arabidopsis* and some crop plants (Chern et al., 2001; Ekengren et al., 2003; Fitzgerald et al., 2004; Lin et al., 2004; Makandar et al., 2006; Malnoy et al., 2007; Yuan et al., 2007; Quilis et al., 2008; Sandhu et al., 2009; Zhang et al., 2010). Two close homologs of NPR1, NPR3 and NPR4 were recently shown to be SA receptors with different binding affinities to SA (Fu et al., 2012; Fu and Dong, 2013). However, whether or not NPR1 itself is also an SA receptor remains controversial (Wu et al., 2012; Yan and Dong, 2014).

Recent studies showed that two members of a phosphate transporter family, the PHT4 family, were involved in SA regulation. The PHT4 family has six members, five of which (PHT4;1-4;5) are plastid-localized, and one (PHT4;6) is Golgi-localized (Roth et al., 2004; Guo et al., 2008a; Pavon et al., 2008; Cubero et al., 2009). Recombinant proteins of PHT4 family members were demonstrated to have phosphate transport activities (Guo et al., 2008a; Pavon et al., 2008; Cubero et al., 2009). However, only a loss of function mutation in the *PHT4;6* gene but not in other five genes confers enhanced disease resistance to *Pseudomonas syringae* infection and high levels of SA besides reduced salt tolerance (Cubero et al., 2009; Hassler et al., 2012). These results suggest that *PHT4;6* is a negative regulator of SA-mediated defense and is also involved in salt stress response.

The lack of defense and salt tolerance phenotypes in loss of function mutants of other five *PHT4* members is possibly due to functional redundancy among these members. To further support roles of the *PHT4* family members in defense control, we identified a gain of function mutant of the *PHT4;1* gene, *pht4;1-1*, in a genetic screen for *acd6-1* suppressors with a goal to uncover new defense genes (Wang et al., 2011b). *ACD6* encodes an ankyrin repeat protein with transmembrane domain and has been shown as a major determinant of fitness in *Arabidopsis* ecotypes (Lu et al., 2003; Todesco et al., 2010, 2014). *acd6-1* is a small gain-of-function mutant that displays extreme dwarfism, constitutive defense, and spontaneous cell death phenotypes (Rate et al., 1999; Lu et al., 2003). The small size of *acd6-1* is largely in an inverse correlation with the defense level of the plant. This characteristics of *acd6-1* has proven useful in genetic screens to identify novel genes critical for plant defense (Lu et al., 2009a) and in genetic analyses to interrogate interactions between known defense genes (Song et al., 2004; Ng et al., 2011; Wang et al., 2011a). The *pht4;1-1* mutation suppressed high SA accumulation in *acd6-1* and conferred EDS to *P. syringae* infection in the absence of *acd6-1*, which could be rescued by exogenous SA treatment (Wang et al., 2011b). This mutation was caused by a T-DNA insertion that resulted in expression of truncated *PHT4;1* transcripts. Since increasing *PHT4;1* expression by introducing extra copies of *PHT4;1* transgene into wt also conferred EDS (Wang et al., 2011b), we conclude that *pht4;1-1* is a gain of function allele and both PHT4;1 and PHT4;1-1 proteins act similarly as negative regulators of *Arabidopsis* defense. Genetic analysis further indicated that *pht4;1-1* possibly contributed to both SID2-dependent and – independent pathways in regulating *acd6-1*-conferred dwarfism and cell death phenotypes. In addition, *PHT4;1* expression was shown to be regulated by the circadian clock (Guo et al., 2008a; Wang et al., 2011b). Thus we propose that *PHT4;1* is a type II SA regulator, the function of which implicates the circadian clock.

In this report, we further investigated the role of *PHT4;1* in SA regulation and the mechanism of circadian regulation of *PHT4;1*. We examined genetic interactions between *pht4;1-1* and several type II mutants, *ald1-1, eds5-3*, and *pad4-1*, besides the type I SA mutant *sid2-1*. The genetic analysis was done in the *acd6-1* background because the change of *acd6-1* size can be used as a convenient visual readout of functional interactions between the mutants. Our results show that *pht4;1-1* acts additively with *sid2-1*, *ald1-1*, *eds5-3*, and *pad4-1* to regulate *acd6-1* dwarfism, cell death, and/or defense responses, suggesting that *PHT4;1* has distinct function from these other SA regulators. To elucidate the mechanism by which *PHT4;1* is circadian clock-regulated, we tested the hypothesis that *PHT4;1* is a direct target of the core component of *Arabidopsis* circadian clock CIRCADIAN CLOCK ASSOCIATED 1 (CCA1). Our data support the hypothesis and underscore a possible role of the circadian clock mediated by CCA1 in regulating the function of *PHT4;1* in phosphate transport and/or innate immunity control in *Arabidopsis*.

MATERIALS AND METHODS

PLANT MATERIALS

All *Arabidopsis* plants used in this report are in Columbia-0 background. Plants were grown in growth chambers with a 12 h light/12 h dark cycle, light intensity at 200 μmol m^{-2} s^{-1}, 60% humidity, and 22°C. The triple mutant *acd6-1pht4;1-1sid2-1* was previously described (Wang et al., 2011b). Additional triple mutants were made by crossing *acd6-1pht4;1-1* with *acd6-1ald1-1*, *acd6-1eds5-3*, or *acd6-1pad4-1* and selected for homozygotes by polymerase chain reaction (PCR) with appropriate primers (Ng et al., 2011).

RNA ANALYSIS

Whole plants of each genotype at 25-day old were harvested at ZT1 (1 h after lights on) for RNA extraction. For circadian clock-regulated gene expression, plants grown in 12 h L/12 h D were

transferred to constant light (LL) and harvested starting at ZT1 at a 4 h interval for 48 h. RNA extraction and northern blotting were performed as described (Ng et al., 2011). Radioactive probes were made by PCR with antisense primers specific to individual gene fragments in the presence of [^{32}P] dCTP. Primers used for making the *PR1* probe are PR1_sense 5′ GTAGGTGCTCTTGTTCTTCCC 3′ and PR1_antisense 5′ CACATAATTCCCACGAGGATC 3′ and for making the *PHT4;1* probe are PHT4;1_sense 5′ ATGAACGCGAGAGCTCTTCTTTGCTC 3′ and PHT4;1_antisense 5′ AATCGATTATCTTCTCTCCGGTTG 3′.

SA MEASUREMENT

SA was extracted from 25-day old plants and quantified by a high-performance liquid chromatography (HPLC) instrument as previously described (Ng et al., 2011; Wang et al., 2011a).

CELL DEATH STAINING

The sixth or seventh leaves of 25-day old plants were harvested for trypan blue staining as described (Ng et al., 2011). Stained leaves were washed with 50% ethanol and mounted on glass slides with cover slips for photographing with a complementary metal–oxide–semiconductor (CMOS) camera connected to a dissecting microscope (Leica M205 FA, Leica Microsystems, Germany).

PURIFICATION OF CCA1-GST RECOMBINANT PROTEIN FROM *Escherichia coli*

The pGEX-CCA1 construct containing CCA1-GST in the pGEX-3X vector was a kind gift from Steve Kay at University of South California. pGEX-CCA1 was transformed into the *Escherichia coli* strain BL21(DE3)-pLysS to express the recombinant protein. A single colony was picked for overnight culture in 5 ml LB media, which was subsequently added into 500 ml LB media for further culture. At OD_{600} = 0.5, the culture was treated with 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 3 h followed by harvesting by centrifugation at 8000 g for 10 min at 4°C. The pellet was resuspended in 25 ml ice–cold 1 X PBS containing 1% Triton X-100 and 2x protease inhibitor (Roche, LOT# 14549800) and lysed by sonication on ice. The sonication condition was 30 s on followed by 30 s off at 30% amplitude for 20 cycles, using Virsonic Cell Disruptor (Model 16-850, The Virtis Co., New York). Cell lysates were collected by centrifugation at 8000 g for 10 min at 4°C. The supernatant was loaded onto a 2 ml glutathione spin column (Pierce, Product # 16107), incubated at 4°C for 30 min on a rocking platform. The column was washed with 10x bed volumes of equilibration/wash buffer (125 mM Tris, 150 mM NaCl, pH 8.0). The CCA1-GST recombinant proteins were eluted with elution buffer (10 mM glutathione, 125 mM Tris, 150 mM NaCl, pH 8.0), according to manufacturer's instruction (Pierce, Product # 16107). Purified CCA1-GST protein was verified on a 6% SDS-PAGE gel and aliquoted into 30 μl per microcentrifuge tube for storage at −80°C.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS

Three DNA fragments (probes) from the *PHT4;1* promoter were generated by PCR amplification, purified, and used for CCA1-GST binding assays. Probe 1 (396 bp) covers from −348 to +48 bp relative to the ATG start site of the *PHT4;1* promoter (primers 5′ TTGTTATTGGTATTGCCGTATTATTGTA 3′, and 5′ GTAGAGAGAGTGAATATTTGAAGA 3′). Probe 2 (118 bp) covers from −348 to −230 bp relative to the ATG start site of the *PHT4;1* promoter (primers 5′ TTGTTATTGGTATTGCCGTATTATTGTA 3′, and 5′ GTTAGCTTACGAGCATAAATTGC 3′). Probe 3 (117 bp) covers from −69 to +48 bp relative to the ATG start site of the *PHT4;1* promoter (primers 5′ AATCAATTCCTCTCTCTTAAAACAAA 3′, and 5′ GTAGAGAGAGTGAATATTTGAAGA 3′). The negative probe *PHT4;1-NC* (without CCA1 binding site) was generated by PCR amplification of the region from +134 to +668 of the *PHT4;1* gene (primers 5′ CTACCCGCGAAATAGGTCCAGTG 3′, and 5′ ATCAACAAACCACTGATTCAACTACACTT 3′). Probes (60 ng each) were end-labeled with γ-[^{32}P]-dATP, using T4 polynucleotide kinase (Thermo Scientific, product # EK0031) in the following reaction: 2 pmol DNA fragment, 2 μl 10x forward reaction buffer, 4 pmol γ-[^{32}P]-dATP, 1 μl T4 PNK, in a total volume of 20 μl. The reaction was carried out at 37°C for 30 min, then added 1 μl of 0.5 M EDTA (pH 8.0) and incubated at 75°C for 10 min to terminate the reaction. Labeled DNA probes were purified by using a PCR purification kit (Qiagen, cat#28104) and eluted with 30 μl sterile water. Binding reactions were carried out as following: 2 μl 5X electrophoretic mobility shift assays (EMSA) buffer [125 mM HEPES-KOH (pH 7.5), 12.5 mM DTT, 5 mM PMSF, 250 mM KCl], 2 μl 50% glycerol, 1 μl 1 μg/ul poly-dIdC, 30–90 ng CCA1-GST recombinant protein, 1 μl labeled probe, in a total volume of 10 μl. For a competition assay, excessive amount of a corresponding cold probe or the negative probe *PHT4;1-NC* at the indicated concentrations was added to a binding reaction. Both binding and competition reactions were incubated on ice for 20 min before being immediately loaded onto a 6% non-denaturing polyacrylamide gel, prepared in 0.5X TBE buffer [40 mM Tris-Cl (pH 8.3), 45 mM boric acid, 1 mM EDTA]. Electrophoresis was conducted at 100 V for ∼1 h at room temperature to separate free probes from DNA-protein complexes. The gels were dried on a gel dryer (Hoefer, model SE1160) at 80°C for 1 h followed by exposure to X-ray film for 2–4 days.

RESULTS

PHT4;1 INTERACTS ADDITIVELY WITH MULTIPLE SA REGULATORS TO AFFECT *ACD6-1* DWARFISM

Our previous data suggest that the *PHT4;1* gene acts upstream of SA to regulate SA accumulation (Wang et al., 2011b). To further investigate the role of *PHT4;1* in SA regulation, we sought to examine genetic interactions between the gain of function mutant *pht4;1-1* and mutants disrupting type II SA genes, *ADL1*, *EDS5*, and *PAD4* (Jirage et al., 1999; Nawrath et al., 2002; Song et al., 2004). We crossed *pht4;1-1* to these mutants in the *acd6-1* background because the small size of *acd6-1* is sensitized to the change of defense levels and thus *acd6-1* can be conveniently used to dissect the functional relationship among SA genes (Song et al., 2004; Ng et al., 2011). A previous similar experiment showed that *pht4;1-1* acts additively with the type I SA mutant *sid2-1* in affecting *acd6-1* dwarfism (Wang et al., 2011b). We found here that similar to *acd6-1pht4;1-1sid2-1*, the triple mutants *acd6-1pht4;1-1ald1-1*, *acd6-1pht4;1-1eds5-3*, and *acd6-1pht4;1-1pad4-1*

were significantly larger than their corresponding double mutants (**Figures 1A,B**), suggesting that *PHT4;1* acts additively with multiple SA regulators in influencing *acd6-1* dwarfism.

PHT4;1 INTERACTS ADDITIVELY WITH MULTIPLE SA REGULATORS TO AFFECT DEFENSE PHENOTYPES AND CELL DEATH IN *ACD6-1*

Since previous studies showed that the dwarfism of *acd6-1* is grossly in reverse correlation with the defense level of the plant (Song et al., 2004; Lu et al., 2009a; Ng et al., 2011; Wang et al., 2011a), the increased size of the triple mutants shown in **Figure 1** suggests reduced defense of the plants. To further test this, we measured SA levels and expression of the defense marker gene *PR1* in these plants. Indeed we found that *acd6-1pht4;1-1ald1-1* and *acd6-1pht4;1-1eds5-3* accumulated near wt-level of SA and *PR1* transcripts (**Figures 2A,B**). *acd6-1pht4;1-1pad4-1*, on the other hand, had much reduced SA level than the two parental double mutants but this level was still significantly higher than that seen in wt. Expression of *PR1* was only slightly reduced in *acd6-1pht4;1-1pad4-1*, compared with the corresponding double

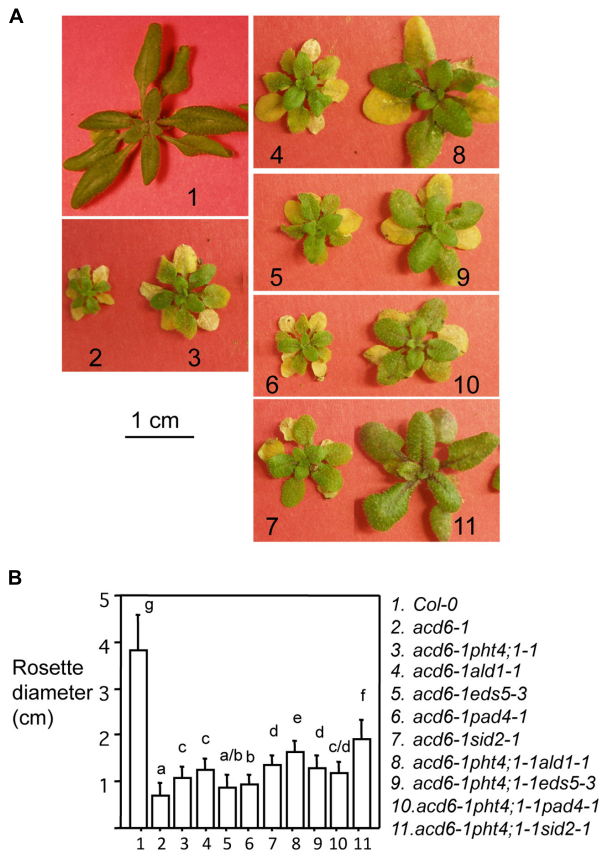


FIGURE 1 | *pht4;1-1* acts additively with SA mutants to suppress *acd6-1* dwarfism. (A) Pictures of 25-day old plants. The single mutants *pht4;1-1*, *ald1-1*, *eds5-3*, *pad4-1*, and *sid2-1* are morphologically similar to Col-0 (not shown). The scale bar represents 1 cm and applies to all panels. **(B)** Plant size measurement. Plants shown in **(A)** were measured for their rosette diameters. Statistical analysis was performed with Student's *t*-test (StatView 5.0.1). Different letters indicate significant difference among the samples ($P < 0.05$; $n = 10$).

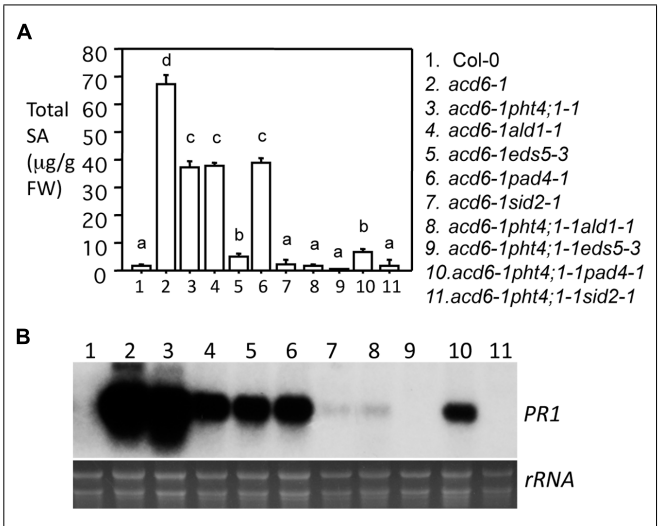


FIGURE 2 | *pht4;1-1* acts additively with SA mutants to suppress SA accumulation and *PR1* expression in *acd6-1*. Twenty five-day old plants were harvested for SA extraction followed by HPLC analysis and RNA preparation followed by northern blotting. **(A)** SA quantitation. Statistical analysis was performed with Student's *t*-test (StatView 5.0.1). Different letters indicate significant difference among the samples ($P < 0.05$; $n = 3$). **(B)** *PR1* expression. rRNA was shown as a loading control.

mutants (**Figure 2B**). These results indicate that *PHT4;1* has distinct function from these type II SA genes in regulating SA accumulation and *PR1* expression. Consistent with a major role of *SID2* in SA biosynthesis (Wildermuth et al., 2001; Ng et al., 2011), we found that SA accumulation and *PR1* expression in *acd6-1pht4;1-1sid2-1* were comparable to those of *acd6-1sid2-1* and wt. We also noticed that the near-wt level of SA in some triple mutants (*acd6-1pht4;1-1ald1-1*, *acd6-1pht4;1-1eds5-3*, and *acd6-1pht4;1-1sid2-1*) was not correlated with a complete suppression of *acd6-1* dwarfism, suggesting there are additional pathways independent of SA contributing to plant size regulation in *acd6-1*.

Besides dwarfism and enhanced defense phenotypes, the *acd6-1* mutant displays severe cell death, even in the absence of pathogen challenge. Suppression of *acd6-1*-conferred dwarfism and defense phenotypes is usually associated with reduced cell death (Song et al., 2004; Lu et al., 2009a; Ng et al., 2011; Wang et al., 2011a). Consistent with this previous observation, we found that triple mutants *acd6-1pht4;1-1ald1-1*, *acd6-1pht4;1-1eds5-3*, and *acd6-1pht4;1-1sid2-1* had substantially reduced but not abolished cell death on their leaves when the plants were stained with trypan blue to visualize cell death (**Figure 3**). Since these mutants accumulated wt-level SA, like plant-size regulation, cell death formation in these plants could be influenced by additional SA-independent pathway(s). Interestingly *acd6-1pht4;1-1pad4-1* displayed similar cell death as *acd6-1pht4;1-1*. This could be due to the relatively high level of SA presented in the triple mutant. Alternatively *PHT4;1* and *PAD4* could act in the same pathway to affect cell death of *acd6-1*.

CIRCADIAN EXPRESSION OF *PHT4;1* IS CCA1-DEPENDENT

Expression of *PHT4;1* was previously shown to be regulated by the circadian clock (Guo et al., 2008a; Wang et al., 2011b).

Such a circadian expression pattern persisted in *pht4;1-1* and *acd6-1* mutants and in the presence of *P. syringae* challenge (Wang et al., 2011b). Consistent with being regulated by the circadian clock, the *PHT4;1* promoter has two *cis*-elements (the CBS motifs), starting at −17 and −281 bp positions, respectively, that are putative binding sites for the core clock component CCA1 (Alabadi et al., 2002; Green and Tobin, 2002; Michael and McClung, 2002). Thus we hypothesized that CCA1 directly targets *PHT4;1* promoter for expression regulation. To test this hypothesis, we first examined *PHT4;1* expression in *CCA1* overexpressing (*CCA1ox*) plants, which display arrhythmic clock activity in both constant light (LL) and light/dark (LD) conditions (Wang and Tobin, 1998; Zhang et al., 2013). We found that when the plants were transferred from LD to LL, *PHT4;1* demonstrated a circadian expression pattern in wt Col-0. However, *CCA1ox* disrupted this expression pattern of *PHT4;1* (**Figure 4**). This result indicates a role of CCA1 in controlling *PHT4;1* expression but could not pinpoint whether such an effect is direct or indirect.

To further test if CCA1 directly binds to the *PHT4;1* promoter, we conducted EMSA with CCA1-GST recombinant protein and *PHT4;1* promoter fragments. The probe 1 is a *PHT4;1* fragment containing two CBS motifs (**Figure 5A**). We found that probe 1 was bound by recombinant CCA1-GST protein, resulting in slower moving bands containing protein-DNA complexes (**Figure 5B**, lane 2–4). Unlabeled probe 1 could compete with isotope-labeled probe 1 for CCA1-GST binding in a dose-dependent manner (**Figure 5B**, lane 5–7). However, excess amount of a negative fragment (PHT4;1-negative) from *PHT4;1* without a CBS motif did not compete with isotope-labeled probe 1 in CCA1-GST binding (**Figure 5B**, lane 8–10). These results suggest that the binding between probe 1 and CCA1-GST protein is specific. We also noticed that there were two shifted bands in most lanes from probe 1 and CCA1-GST binding reactions (**Figure 5B**, lane 3–5 and 8–10). We speculated that both CBS motifs in probe 1 can be bound by CCA1-GST when the protein is present in abundance. To test this, we incubated two shorter *PHT4;1* promoter fragments (probe 2 and probe 3), containing only one CBS motif each, with CCA1-GST (**Figure 5C**). Indeed, both probe 2 and 3 were bound by CCA1-GST, forming a single DNA-protein complex that separated from the free probes. Thus these *in vitro* binding assays support our hypothesis that *PHT4;1* is a direct target of CCA1.

DISCUSSION

In this study, we took biochemical, genetic, and molecular approaches to further investigate the function of the phosphate transporter gene *PHT4;1*. Our results show that *PHT4;1* genetically interacts with several SA genes, including *SID2, ALD1*, *EDS5*, and *PAD4*, in regulating defense responses. In addition, we show that circadian expression of *PHT4;1* is dependent on the circadian clock protein CCA1, which could directly bind to the *PHT4;1* promoter. These results corroborate the role of *PHT4;1* in defense regulation and also suggest that the circadian clock gene *CCA1* regulates phosphate transport and/or defense responses, possibly through influencing *PHT4;1*-mediated pathway.

Our previous study indicated that *PHT4;1* is a negative defense regulator acting upstream of SA (Wang et al., 2011b). Genetic analysis conducted here further showed that *pht4;1-1* acts additively with SA mutants *ald1-1*, *eds5-3*, and *pad4-1* to suppress high levels of SA accumulation and *PR1* expression in *acd6-1* (**Figure 2**). Thus *PHT4;1* likely functions in a separate pathway from *ALD1, EDS5*, and *PAD4* in regulating these defense outputs. Consistent with this notion, expression of *ACD6*, *ALD1*, and *PAD4* are inducible by SA treatment, suggesting that these genes are involved in signal amplification loops with SA (Nawrath et al., 2002; Lu et al., 2003; Song et al., 2004). However, expression of *PHT4;1* is not affected by SA treatment (data not shown), suggesting that unlike *ACD6*, *ALD1*, and *PAD4*, *PHT4;1* is not part of SA-signal amplification loop. Together these results further support a previous notion that there are multiple pathways affecting SA-mediated defense in *Arabidopsis* (Song et al., 2004; Ng et al., 2011; Wang et al., 2011a). Interestingly although some triple mutants show wt-like levels of SA and *PR1* expression, none of these triple mutants revert to wt-like phenotypes in terms of plant size and cell death (**Figures 1** and **3**). These results suggest that the regulation of plant size and cell death can be uncoupled from that of some defense phenotypes in *acd6-1*. Additional SA-independent pathway(s) could contribute to the regulation of plant size and cell death formation in *acd6-1*.

While the gain of function mutant *pht4;1-1* displayed compromised defense phenotypes, the loss of function alleles of *PHT4;1* did not show altered defense responses (Wang et al., 2011b). This can be explained by possible functional redundancy among some PHT4 family members. Indeed, PHT4;1 and four other members in the family share high levels of homology and are all plastid-localized. Functional redundancy among these members could prevent manifestation of defense phenotypes in single loss of function mutants. So far only one disrupted member, *PHT4;2*, showed small effects on plant growth (Irigoyen et al., 2011). Besides *pht4;1* loss of function mutants, available single loss of function mutants of *PHT4;4* and *PHT4;5* are indistinguishable from wt in morphology and defense responses (data not shown).

While five plastid-localized PHT4 family members could share redundant function, the sixth member of the family, PHT4;6, might be functionally divergent from other members in the family. PHT4.6 is localized to the Golgi and was shown to have Pi transport activity in the Golgi (Guo et al., 2008a; Cubero et al., 2009). A single loss of function mutation in *PHT4;6* results in enhanced disease resistance to *P. syringae* infection, dwarfism, and reduced salt tolerance (Cubero et al., 2009; Hassler et al., 2012). The *pht4;6* mutant also accumulates modestly higher levels of SA than wt. Thus like PHT4;1, PHT4;6 is also a negative regulator of plant defense.

The involvement of two members of the PHT4 family in defense suggests a possibility that phosphate transport is critical for host-pathogen interactions. Phosphorus (P) is essential for plant growth and development. However, plants do not produce P but take up inorganic phosphate ion (Pi) from the soil to the root, reallocate Pi to different tissue and cell types, and redistribute Pi to different organelles within a cell in order to fulfill the Pi requirement for cellular functions. These processes are mediated by phosphate transporters to maintain phosphate homeostasis and

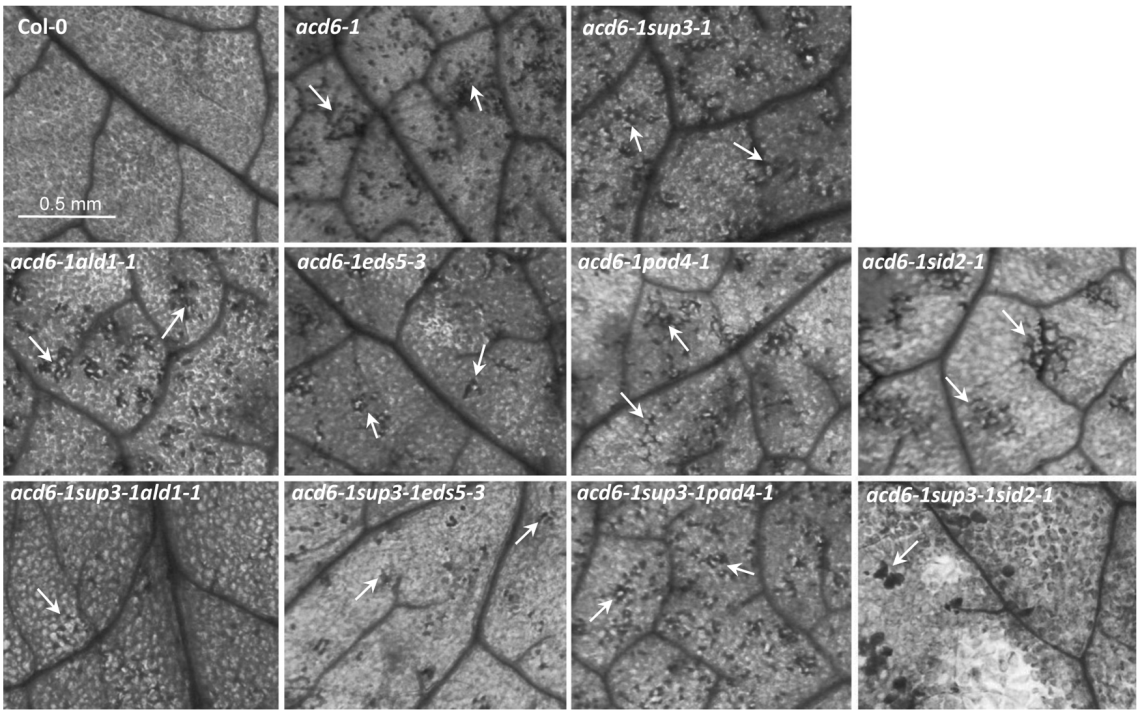


FIGURE 3 | *pht4;1-1* acts additively with SA mutants to suppress cell death in *acd6-1*. The sixth or seventh leaves of 25-day old plants were harvested for trypan blue staining as described (Ng et al., 2011). Stained leaves were photographed with a CMOS camera connected to a dissecting microscope (Leica M205 FA, Leica Microsystems, Germany). Cell death is shown in the dark stained spots or patches on a leaf (arrows). At least four leaves of each genotype were stained and examined for cell death. No cell death was detected in *ald1-1*, *eds5-3*, *pad4-1*, *sid2-1*, and *pht4;1-1* (data not shown). The scale bar represents 0.5 mm and applies to all panels.

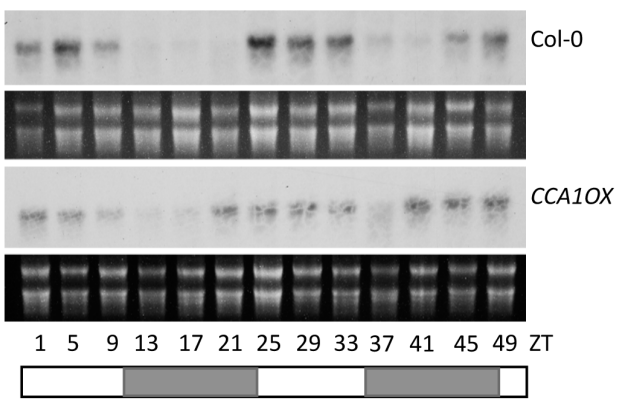


FIGURE 4 | Circadian expression of *PHT4;1* is CCA1-dependent. Twenty five-day-old Col-0 and *CCA1ox* plants grown in a chamber with a 12 h light/12 h dark cycle and 22°C were transferred to LL at 22°C. Starting at ZT1, plants were harvested at every 4 h for 48 h for RNA extraction followed by northern blotting. White boxes indicate subjective light periods and gray boxes indicate subjective dark periods in LL. rRNA was shown as a loading control.

the normal function of cells. At least five phosphate transporter families, PHT1, PHT2, PHT3, PHT4, and pPT, have been reported in *Arabidopsis* (Poirier and Bucher, 2002; Guo et al., 2008b). Among these phosphate transporter families, only mutations in some *PHT1* genes and one *PHT2* gene resulted in alterations in Pi concentration *in planta* (Versaw and Harrison, 2002; Shin et al., 2004; Gonzalez et al., 2005). The *PHT1* genes encode plasma membrane-localized high affinity Pi/H^+ symporters and are expressed abundantly in the root (Karthikeyan et al., 2002; Mudge et al., 2002). The *PHT2* gene encodes a chloroplast-localized phosphate transporter and is highly expressed in the green tissue (Versaw and Harrison, 2002). Based on these tissue- and cell-specific expression patterns, *PHT1* was proposed to acquire Pi from the root whereas *PHT2* was proposed to influence the reallocation of phosphate within different tissues of a plant. PHT4 and other phosphate transporter families have not been reported to have a major effect on phosphate concentration at the whole plant level. Except two members of the *PHT4* family (*PHT4;1* and *PHT4;6*), none of the other phosphate transporter genes have been demonstrated a role in defense regulation. Therefore it is currently unknown whether perturbation of phosphate concentration *in planta* could result in altered defense responses. However, there is evidence to support a connection between altered phosphate signaling and defense control. One example is the *SIZ1* gene encoding a SUMO E3 ligase that targets PHR1, a MYB transcriptional activator critical for phosphate response. A *siz1* mutant demonstrated reduced phosphate response and enhanced disease resistance (Rubio et al., 2001; Miura et al., 2005; Lee et al., 2007; Jin et al., 2008).

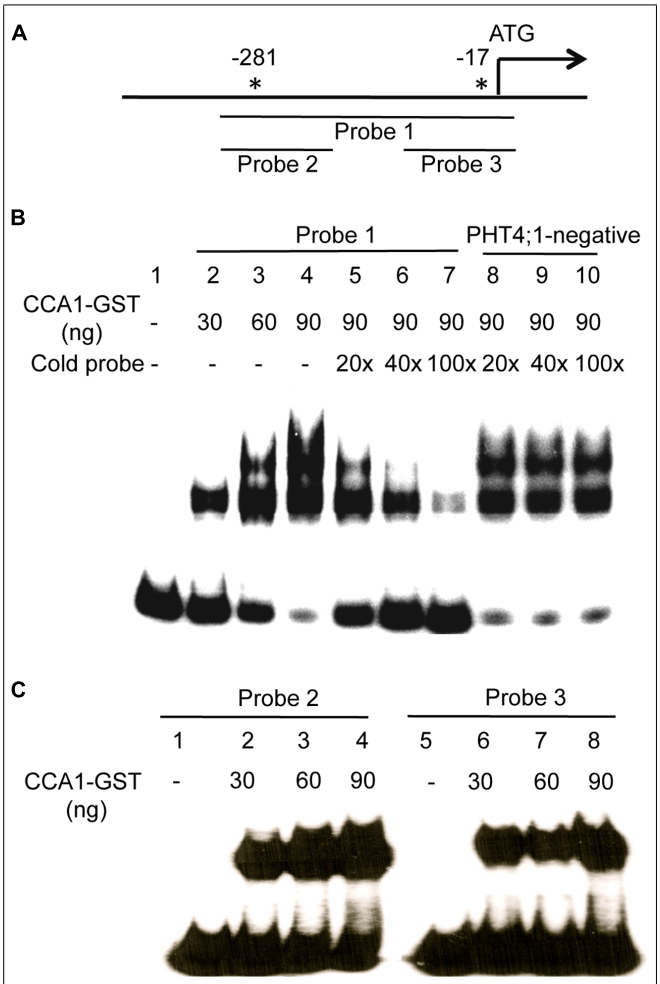


FIGURE 5 | CCA1 binds to the *PHT4;1* promoter in EMSA. (A) Positions of the *PHT4;1* promoter fragments (probes) and the CBS motifs (asterisks). Positions are relative to the translation start site (ATG). **(B)** EMSA with probe 1. **(C)** EMSA with probe 2 and probe 3. The probes were end-labeled with γ-^{32}P and incubated with purified recombinant CCA1-GST protein. For competition assays in **(B)**, unlabeled fragments (probe 1 or PHT4;1-negative) at the indicated folds more than the input (isotope-labeled probe 1) were added to the binding reactions. The reactions were resolved on 6% native PAGE gels followed by gel drying and exposure to X-ray film.

Since PHT4;1 is not known to perturb phosphate concentration at the whole plant level, the defense phenotypes observed in the *pht4;1-1* mutant could be caused by altered PHT4;1 transport activity at the subcellular level. *PHT4;1* is mainly expressed in the shoot tissue (Guo et al., 2008a,b). Pavon et al. (2008) showed that the PHT4;1 protein was localized to the thylakoid member of the chloroplast and thus proposed that PHT4;1 transports Pi across thylakoid lumen and stroma in the chloroplast, using its Na^+ and/or H^+- dependent phosphate transporter activity (Guo et al., 2008b). In another study, Roth et al. (2004) localized PHT4;1 to the inner membrane of the plastid. Although the precise localization of PHT4;1 remains to be determined, these studies pointed to the connection of PHT4;1 with the chloroplast, the central organelle for photosynthesis and many secondary and primary metabolisms, including SA biosynthesis. It is conceivable that Pi transported by PHT4;1 could directly or indirectly affect SA biosynthetic pathways or proteins/processes that affect SA accumulation. Such function of PHT4;1 could be shared by other four plastid-localized PHT4 family members (PHT4;2-4;5). However, Golgi-localized PHT4;6 may influence SA accumulation and SA-mediated defense through a different mechanism from that used by PHT4;1.

The observation of circadian clock regulated *PHT4;1* expression has prompted us to elucidate the role of the circadian clock in defense control (Guo et al., 2008a; Wang et al., 2011b; Zhang et al., 2013). The circadian clock is an internal time measuring machinery important for development and fitness of plants (Green et al., 2002; Michael et al., 2003; Dodd et al., 2005; Ni et al., 2009; Graf et al., 2010; Dong et al., 2011). Increasing evidence supports a role of the circadian clock in defense regulation. First, like *PHT4;1*, expression of some defense genes were reported to be under the circadian clock control (Wang et al., 2001, 2011b; Sauerbrunn and Schlaich, 2004; Weyman et al., 2006; Roden and Ingle, 2009). Second, wt *Arabidopsis* shows temporal variations in a day in its susceptibility to *P. syringae* infection and such variations can be disrupted by overexpression of *CCA1* (Bhardwaj et al., 2011). Third, misexpression of several core clock genes, including *CCA1*, its close homolog *LATE ELONGATED HYPOCOTYL* (*LHY*) (Alabadi et al., 2002; Mizoguchi et al., 2002; Lu et al., 2009b), and *TIME FOR COFFEE* (Hall et al., 2003; Ding et al., 2007), leads to compromised resistance to the bacterial pathogen *P. syringae* and/or to the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) (Bhardwaj et al., 2011; Wang et al., 2011c; Shin et al., 2012; Zhang et al., 2013). Data from our study further indicate that defense activation can reciprocally regulate clock activity, suggesting crosstalk between the circadian clock and plant innate immunity (Zhang et al., 2013).

Both experimental studies and *in silico* analysis of circadian clock-regulated gene expression indicate that *PHT4;1* is the only member in the *PHT4* family that demonstrates a robust circadian expression pattern (Mockler et al., 2007; Guo et al., 2008a, and data not shown). We presented here evidence to further demonstrate that *PHT4;1* could be a direct transcriptional target of the circadian clock protein CCA1 (**Figures 4** and **5**). Interestingly, while we show here that *PHT4;1* is an SA regulator that acts independently of several known SA genes, our previous study indicated that the clock genes *CCA1* and *LHY* acted in a SA-independent manner in defense regulation (Zhang et al., 2013). The *cca1-1lhy-20* double mutations suppressed *acd6-1*-conferred constitutive defense but not its dwarfism and high SA accumulation. Such a discrepancy in terms of SA regulation by PHT4;1 and CCA1 suggest that CCA1-regulation of *PHT4;1* might be important for phosphate transport activity of PHT4;1 but may not be directly related to the role of PHT4;1 in SA regulation. It is also possible that there are additional factor(s) affecting circadian expression of the *PHT4;1* gene and/or phosphate transport activity of the PHT4;1 protein. Alternatively, CCA1 and its close homolog LHY could regulate expression of multiple defense genes, including both positive and negative SA regulators. Thus in the CCA1 and LHY loss of function background, the effect on SA accumulation could be negated by the changes of these two opposing groups of SA genes. Additional biochemical, genetic, and molecular studies are required to further elucidate the biological relevance of CCA1

binding on the *PHT4;1* promoter in terms of phosphate transport and defense regulation.

ACKNOWLEDGMENTS

We thank members in the Lu laboratory for assistance with this work. We thank N. S. Alberola and D. Eisenmann for suggestions in the EMSA experiments. This work was partly supported by a grant from Citrus Research and Development Foundation and a grant from National Science Foundation (NSF RIG-0818651) to Hua Lu and a scholarship from China Scholarship Council to Chong Zhang.

REFERENCES

Alabadi, D., Yanovsky, M. J., Mas, P., Harmer, S. L., and Kay, S. A. (2002). Critical role for CCA1 and LHY in maintaining circadian rhythmicity in *Arabidopsis*. *Curr. Biol.* 12, 757–761. doi: 10.1016/S0960-9822(02)00815-1

Bhardwaj, V., Meier, S., Petersen, L. N., Ingle, R. A., and Roden, L. C. (2011). Defence responses of *Arabidopsis thaliana* to infection by *Pseudomonas syringae* are regulated by the circadian clock. *PLoS ONE* 6:e26968. doi: 10.1371/journal.pone.0026968

Chen, Z., Zheng, Z., Huang, J., Lai, Z., and Fan, B. (2009). Biosynthesis of salicylic acid in plants. *Plant Signal. Behav.* 4, 493–496. doi: 10.4161/psb.4.6.8392

Chern, M. S., Fitzgerald, H. A., Yadav, R. C., Canlas, P. E., Dong, X., and Ronald, P. C. (2001). Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in *Arabidopsis*. *Plant J.* 27, 101–113. doi: 10.1046/j.1365-313x.2001.01070.x

Cubero, B., Nakagawa, Y., Jiang, X., Miura, K., Li, F., Raghothama, K. G., et al. (2009). The phosphate transporter PHT4; 6 Is a determinant of salt tolerance that is localized to the Golgi apparatus of *Arabidopsis*. *Mol. Plant* 2, 535–552. doi: 10.1093/mp/ssp013

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arabidopsis Book* 9:e0156. doi: 10.1199/tab.0156

Ding, Z., Millar, A. J., Davis, A. M., and Davis, S. J. (2007). TIME FOR COFFEE encodes a nuclear regulator in the *Arabidopsis thaliana* circadian clock. *Plant Cell* 19, 1522–1536. doi: 10.1105/tpc.106.047241

Dodd, A. N., Salathia, N., Hall, A., Kevei, E., Toth, R., Nagy, F., et al. (2005). Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* 309, 630–633. doi: 10.1126/science.1115581

Dong, M. A., Farre, E. M., and Thomashow, M. F. (2011). Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7241–7246. doi: 10.1073/pnas.1103741108

Dong, X. (2004). NPR1, all things considered. *Curr. Opin. Plant Biol.* 7, 547–552. doi: 10.1016/j.pbi.2004.07.005

Ekengren, S., Liu, Y., Schiff, M., Dinesh-Kumar, S., and Martin, G. (2003). Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. *Plant J.* 36, 905–917. doi: 10.1046/j.1365-313X.2003.01944.x

Falk, A., Feys, B. J., Frost, L. N., Jones, J. D., Daniels, M. J., and Parker, J. E. (1999). EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3292–3297. doi: 10.1073/pnas.96.6.3292

Fitzgerald, H. A., Chern, M. S., Navarre, R., and Ronald, P. C. (2004). Overexpression of (At)NPR1 in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. *Mol. Plant Microbe Interact.* 17, 140–151. doi: 10.1094/MPMI.2004.17.2.140

Fragniere, C., Serrano, M., Abou-Mansour, E., Metraux, J. P., and L'haridon, F. (2011). Salicylic acid and its location in response to biotic and abiotic stress. *FEBS Lett.* 585, 1847–1852. doi: 10.1016/j.febslet.2011.04.039

Friedrich, L., Vernooij, B., Gaffney, T., Morse, A., and Ryals, J. (1995). Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Mol. Biol.* 29, 959–968. doi: 10.1007/BF00014969

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gonzalez, E., Solano, R., Rubio, V., Leyva, A., and Paz-Ares, J. (2005). PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in *Arabidopsis*. *Plant Cell* 17, 3500–3512. doi: 10.1105/tpc.105.036640

Graf, A., Schlereth, A., Stitt, M., and Smith, A. M. (2010). Circadian control of carbohydrate availability for growth in *Arabidopsis* plants at night. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9458–9463. doi: 10.1073/pnas.0914299107

Green, R. M., Tingay, S., Wang, Z. Y., and Tobin, E. M. (2002). Circadian rhythms confer a higher level of fitness to *Arabidopsis* plants. *Plant Physiol.* 129, 576–584. doi: 10.1104/pp.004374

Green, R. M., and Tobin, E. M. (2002). The role of CCA1 and LHY in the plant circadian clock. *Dev. Cell* 2, 516–518. doi: 10.1016/S1534-5807(02)00184-3

Guo, B., Irigoyen, S., Fowler, T. B., and Versaw, W. K. (2008a). Differential expression and phylogenetic analysis suggest specialization of plastid-localized members of the PHT4 phosphate transporter family for photosynthetic and heterotrophic tissues. *Plant Signal. Behav.* 3, 784–790. doi: 10.4161/psb.3.10.6666

Guo, B., Jin, Y., Wussler, C., Blancaflor, E. B., Motes, C. M., and Versaw, W. K. (2008b). Functional analysis of the *Arabidopsis* PHT4 family of intracellular phosphate transporters. *New Phytol.* 177, 889–898. doi: 10.1111/j.1469-8137.2007.02331.x

Hall, A., Bastow, R. M., Davis, S. J., Hanano, S., Mcwatters, H. G., Hibberd, V., et al. (2003). The TIME FOR COFFEE gene maintains the amplitude and timing of *Arabidopsis* circadian clocks. *Plant Cell* 15, 2719–2729. doi: 10.1105/tpc.013730

Hammond-Kosack, K. E., and Jones, J. D. (1996). Resistance gene-dependent plant defense responses. *Plant Cell* 8, 1773–1791. doi: 10.1105/tpc.8.10.1773

Hassler, S., Lemke, L., Jung, B., Mohlmann, T., Kruger, F., Schumacher, K., et al. (2012). Lack of the Golgi phosphate transporter PHT4;6 causes strong developmental defects, constitutively activated disease resistance mechanisms and altered intracellular phosphate compartmentation in *Arabidopsis*. *Plant J.* 72, 732–744. doi: 10.1111/j.1365-313X.2012.05106.x

Irigoyen, S., Karlsson, P. M., Kuruvilla, J., Spetea, C., and Versaw, W. K. (2011). The sink-specific plastidic phosphate transporter PHT4;2 influences starch accumulation and leaf size in *Arabidopsis*. *Plant Physiol.* 157, 1765–1777. doi: 10.1104/pp.111.181925

Jin, J. B., Jin, Y. H., Lee, J., Miura, K., Yoo, C. Y., Kim, W. Y., et al. (2008). The SUMO E3 ligase, AtSIZ1, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on FLC chromatin structure. *Plant J.* 53, 530–540. doi: 10.1111/j.1365-313X.2007.03359.x

Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., et al. (1999). *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13583–13588. doi: 10.1073/pnas.96.23.13583

Karthikeyan, A. S., Varadarajan, D. K., Mukatira, U. T., D'Urzo, M. P., Damsz, B., and Raghothama, K. G. (2002). Regulated expression of *Arabidopsis* phosphate transporters. *Plant Physiol.* 130, 221–233. doi: 10.1104/pp.020007

Lee, J., Nam, J., Park, H. C., Na, G., Miura, K., Jin, J. B., et al. (2007). Salicylic acid-mediated innate immunity in *Arabidopsis* is regulated by SIZ1 SUMO E3 ligase. *Plant J.* 49, 79–90. doi: 10.1111/j.1365-313X.2006.02947.x

Lin, W. C., Lu, C. F., Wu, J. W., Cheng, M. L., Lin, Y. M., Yang, N. S., et al. (2004). Transgenic tomato plants expressing the *Arabidopsis* NPR1 gene display enhanced resistance to a spectrum of fungal and bacterial diseases. *Transgenic Res.* 13, 567–581. doi: 10.1007/s11248-004-2375-9

Lu, H. (2009). Dissection of salicylic acid-mediated defense signaling networks. *Plant Signal. Behav.* 4, 713–717. doi: 10.4161/psb.4.8.9173

Lu, H., Rate, D. N., Song, J. T., and Greenberg, J. T. (2003). ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the *Arabidopsis* defense response. *Plant Cell* 15, 2408–2420. doi: 10.1105/tpc.015412

Lu, H., Salimian, S., Gamelin, E., Wang, G., Fedorowski, J., Lacourse, W., et al. (2009a). Genetic analysis of acd6-1 reveals complex defense networks and leads to identification of novel defense genes in *Arabidopsis*. *Plant J.* 58, 401–412. doi: 10.1111/j.1365-313X.2009.03791.x

Lu, S. X., Knowles, S. M., Andronis, C., Ong, M. S., and Tobin, E. M. (2009b). CIRCADIAN CLOCK ASSOCIATED1 and LATE ELONGATED HYPOCOTYL function synergistically in the circadian clock of *Arabidopsis*. *Plant Physiol.* 150, 834–843. doi: 10.1104/pp.108.133272

Makandar, R., Essig, J. S., Schapaugh, M. A., Trick, H. N., and Shah, J. (2006). Genetically engineered resistance to *Fusarium* head blight in wheat by expression of *Arabidopsis* NPR1. *Mol. Plant Microbe Interact.* 19, 123–129. doi: 10.1094/MPMI-19-0123

Malnoy, M., Jin, Q., Borejsza-Wysocka, E. E., He, S. Y., and Aldwinckle, H. S. (2007). Overexpression of the apple MpNPR1 gene confers increased disease resistance in Malus × domestica. *Mol. Plant Microbe Interact.* 20, 1568–1580. doi: 10.1094/MPMI-20-12-1568

Michael, T. P., and McClung, C. R. (2002). Phase-specific circadian clock regulatory elements in *Arabidopsis*. *Plant Physiol.* 130, 627–638. doi: 10.1104/pp.004929

Michael, T. P., Salome, P. A., Yu, H. J., Spencer, T. R., Sharp, E. L., and Mcpeek, M. A. (2003). Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science* 302, 1049–1053. doi: 10.1126/science.1082971

Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A. S., Raghothama, K. G., et al. (2005). The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7760–7765. doi: 10.1073/pnas.0500778102

Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H. R., et al. (2002). LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. *Dev. Cell* 2, 629–641. doi: 10.1016/S1534-5807(02)00170-3

Mockler, T. C., Michael, T. P., Priest, H. D., Shen, R., Sullivan, C. M., Givan, S. A., et al. (2007). The DIURNAL project: DIURNAL and circadian expression profiling, model-based pattern matching, and promoter analysis. *Cold Spring Harb. Symp. Quant. Biol.* 72, 353–363. doi: 10.1101/sqb.2007.72.006

Mudge, S. R., Rae, A. L., Diatloff, E., and Smith, F. W. (2002). Expression analysis suggests novel roles for members of the PHT1 family of phosphate transporters in *Arabidopsis*. *Plant J.* 31, 341–353. doi: 10.1046/j.1365-313X.2002.01356.x

Nawrath, C., Heck, S., Parinthawong, N., and Metraux, J. P. (2002). EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell* 14, 275–286. doi: 10.1105/tpc.010376

Nawrath, C., and Metraux, J. P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11, 1393–1404.

Ng, G., Seabolt, S., Zhang, C., Salimian, S., Watkins, T. A., and Lu, H. (2011). Genetic dissection of salicylic acid-mediated defense signaling networks in *Arabidopsis*. *Genetics* 189, 851–859. doi: 10.1534/genetics.111.132332

Ni, Z., Kim, E. D., Ha, M., Lackey, E., Liu, J., Zhang, Y., et al. (2009). Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. *Nature* 457, 327–331. doi: 10.1038/nature07523

Pavon, L. R., Lundh, F., Lundin, B., Mishra, A., Persson, B. L., and Spetea, C. (2008). *Arabidopsis* ANTR1 is a thylakoid Na+-dependent phosphate transporter: functional characterization in *Escherichia coli*. *J. Biol. Chem.* 283, 13520–13527. doi: 10.1074/jbc.M709371200

Poirier, Y., and Bucher, M. (2002). Phosphate transport and homeostasis in *Arabidopsis*. *Arabidopsis Book* 1:e0024. doi: 10.1199/tab.0024

Quilis, J., Penas, G., Messeguer, J., Brugidou, C., and San Segundo, B. (2008). The *Arabidopsis* AtNPR1 inversely modulates defense responses against fungal, bacterial, or viral pathogens while conferring hypersensitivity to abiotic stresses in transgenic rice. *Mol. Plant Microbe Interact.* 21, 1215–1231. doi: 10.1094/MPMI-21-9-1215

Rate, D. N., Cuenca, J. V., Bowman, G. R., Guttman, D. S., and Greenberg, J. T. (1999). The gain-of-function *Arabidopsis* acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. *Plant Cell* 11, 1695–1708. doi: 10.1105/tpc.11.9.1695

Roden, L. C., and Ingle, R. A. (2009). Lights, rhythms, infection: the role of light and the circadian clock in determining the outcome of plant-pathogen interactions. *Plant Cell* 21, 2546–2552. doi: 10.1105/tpc.109.069922

Roth, C., Menzel, G., Petetot, J. M., Rochat-Hacker, S., and Poirier, Y. (2004). Characterization of a protein of the plastid inner envelope having homology to animal inorganic phosphate, chloride and organic-anion transporters. *Planta* 218, 406–416. doi: 10.1007/s00425-003-1121-5

Rubio, V., Linhares, F., Solano, R., Martin, A. C., Iglesias, J., Leyva, A., et al. (2001). A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes Dev.* 15, 2122–2133. doi: 10.1101/gad.204401

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H. Y., et al. (1996). Systemic acquired resistance. *Plant Cell* 8, 1809–1819. doi: 10.1105/tpc.8.10.1809

Sandhu, D., Tasma, I. M., Frasch, R., and Bhattacharyya, M. K. (2009). Systemic acquired resistance in soybean is regulated by two proteins, orthologous to *Arabidopsis* NPR1. *BMC Plant Biol.* 9:105. doi: 10.1186/1471-2229-9-105

Sauerbrunn, N., and Schlaich, N. L. (2004). PCC1: a merging point for pathogen defence and circadian signalling in *Arabidopsis*. *Planta* 218, 552–561. doi: 10.1007/s00425-003-1143-z

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., et al. (2013). Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiol.* 162, 1815–1821. doi: 10.1104/pp.113.218156

Shin, H., Shin, H. S., Dewbre, G. R., and Harrison, M. J. (2004). Phosphate transport in *Arabidopsis*: Pht1; 1 and Pht1; 4 play a major role in phosphate acquisition from both low- and high-phosphate environments. *Plant J.* 39, 629–642. doi: 10.1111/j.1365-313X.2004.02161.x

Shin, J., Heidrich, K., Sanchez-Villarreal, A., Parker, J. E., and Davis, S. J. (2012). TIME FOR COFFEE represses accumulation of the MYC2 transcription factor to provide time-of-day regulation of jasmonate signaling in *Arabidopsis*. *Plant Cell* 24, 2470–2482. doi: 10.1105/tpc.111.095430

Song, J. T., Lu, H., Mcdowell, J. M., and Greenberg, J. T. (2004). A key role for ALD1 in activation of local and systemic defenses in *Arabidopsis*. *Plant J.* 40, 200–212. doi: 10.1111/j.1365-313X.2004.02200.x

Strawn, M. A., Marr, S. K., Inoue, K., Inada, N., Zubieta, C., and Wildermuth, M. C. (2007). *Arabidopsis* isochorismate synthase functional in pathogen-induced salicylate biosynthesis exhibits properties consistent with a role in diverse stress responses. *J. Biol. Chem.* 282, 5919–5933. doi: 10.1074/jbc.M605193200

Todesco, M., Balasubramanian, S., Hu, T. T., Traw, M. B., Horton, M., Epple, P., et al. (2010). Natural allelic variation underlying a major fitness trade-off in *Arabidopsis thaliana*. *Nature* 465, 632–636. doi: 10.1038/nature09083

Todesco, M., Kim, S. T., Chae, E., Bomblies, K., Zaidem, M., Smith, L. M., et al. (2014). Activation of the *Arabidopsis thaliana* immune system by combinations of common ACD6 alleles. *PLoS Genet.* 10:e1004459. doi: 10.1371/journal.pgen.1004459

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J.* 53, 763–775. doi: 10.1111/j.1365-313X.2007.03369.x

Versaw, W. K., and Harrison, M. J. (2002). A chloroplast phosphate transporter, PHT2; 1, influences allocation of phosphate within the plant and phosphate-starvation responses. *Plant Cell* 14, 1751–1766. doi: 10.1105/tpc.002220

Wang, G. F., Seabolt, S., Hamdoun, S., Ng, G., Park, J., and Lu, H. (2011a). Multiple roles of WIN3 in regulating disease resistance, cell death, and flowering time in *Arabidopsis*. *Plant Physiol.* 156, 1508–1519. doi: 10.1104/pp.111.176776

Wang, G. Y., Shi, J. L., Ng, G., Battle, S. L., Zhang, C., and Lu, H. (2011b). Circadian clock-regulated phosphate transporter PHT4; 1 plays an important role in *Arabidopsis* defense. *Mol. Plant* 4, 516–526. doi: 10.1093/mp/ssr016

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tor, M., Caldelari, D., et al. (2011c). Timing of plant immune responses by a central circadian regulator. *Nature* 470, 110–114. doi: 10.1038/nature09766

Wang, Z. X., Yamanouchi, U., Katayose, Y., Sasaki, T., and Yano, M. (2001). Expression of the Pib rice-blast-resistance gene family is up-regulated by environmental conditions favouring infection and by chemical signals that trigger secondary plant defences. *Plant Mol. Biol.* 47, 653–661. doi: 10.1023/A:1012457113700

Wang, Z. Y., and Tobin, E. M. (1998). Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93, 1207–1217. doi: 10.1016/S0092-8674(00)81464-6

Weyman, P. D., Pan, Z., Feng, Q., Gilchrist, D. G., and Bostock, R. M. (2006). A circadian rhythm-regulated tomato gene is induced by Arachidonic acid and *Phythophthora* infestans infection. *Plant Physiol.* 140, 235–248. doi: 10.1104/pp.105.068874

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Yamasaki, K., Motomura, Y., Yagi, Y., Nomura, H., Kikuchi, S., Nakai, M., et al. (2013). Chloroplast envelope localization of EDS5, an essential factor for salicylic acid biosynthesis in *Arabidopsis thaliana*. *Plant Signal. Behav.* 8:e23603. doi: 10.4161/psb.23603

Yan, S., and Dong, X. (2014). Perception of the plant immune signal salicylic acid. *Curr. Opin. Plant Biol.* 20C, 64–68. doi: 10.1016/j.pbi.2014.04.006

Yuan, Y., Zhong, S., Li, Q., Zhu, Z., Lou, Y., Wang, L., et al. (2007). Functional analysis of rice NPR1-like genes reveals that OsNPR1/NH1 is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. *Plant Biotechnol. J.* 5, 313–324. doi: 10.1111/j.1467-7652.2007.00243.x

Zhang, C., Xie, Q., Anderson, R. G., Ng, G., Seitz, N. C., Peterson, T., et al. (2013). Crosstalk between the circadian clock and innate immunity in *Arabidopsis*. *PLoS Pathog.* 9:e1003370. doi: 10.1371/journal.ppat.1003370

Zhang, X., Francis, M. I., Dawson, W. O., Graham, J. H., Orbovic, V., Triplett, E. W., et al. (2010). Over-expression of the *Arabidopsis* NPR1 gene in citrus increases resistance to citrus canker. *Eur. J. Plant Pathol.* 128, 91–100. doi: 10.1007/s10658-010-9633-x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 October 2014; accepted: 25 November 2014; published online: 16 December 2014.

Citation: Wang G, Zhang C, Battle S and Lu H (2014) The phosphate transporter PHT4;1 is a salicylic acid regulator likely controlled by the circadian clock protein CCA1. Front. Plant Sci. ***5****:701. doi: 10.3389/fpls.2014.00701*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

PERSPECTIVE ARTICLE
published: 09 January 2015
doi: 10.3389/fpls.2014.00775

Some things get better with age: differences in salicylic acid accumulation and defense signaling in young and mature *Arabidopsis*

Philip Carella [†]***, Daniel C. Wilson*** [†] ***and Robin K. Cameron****

Department of Biology, McMaster University, Hamilton, ON, Canada

Edited by:
Hua Lu, University of Maryland at Baltimore County, USA

Reviewed by:
Saskia C. M. Van Wees, Utrecht University, Netherlands
Dale R. Walters, Scottish Agricultural College, Scotland

***Correspondence:**
Robin K. Cameron, Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada
e-mail: rcamero@mcmaster.ca

[†] *These authors have contributed equally to this work.*

In *Arabidopsis*, much of what we know about the phytohormone salicylic acid (SA) and its role in plant defense comes from experiments using young plants. We are interested in understanding why young plants are susceptible to virulent strains of *Pseudomonas syringae*, while mature plants exhibit a robust defense response known as age-related resistance (ARR). SA-mediated signaling is important for defense in young plants, however, ARR occurs independently of the defense regulators NPR1 and WHY1. Furthermore, intercellular SA accumulation is an important component of ARR, and intercellular washing fluids from ARR-competent plants exhibit antibacterial activity, suggesting that SA acts as an antimicrobial agent in the intercellular space. Young plants accumulate both intracellular and intercellular SA during PAMP- and effector-triggered immunity, however, virulent *P. syringae* promotes susceptibility by suppressing SA accumulation using the phytotoxin coronatine. Here we outline the hypothesis that mature, ARR-competent *Arabidopsis* alleviates coronatine-mediated suppression of SA accumulation. We also explore the role of SA in other mature-plant processes such as flowering and senescence, and discuss their potential impact on ARR.

Keywords: *Arabidopsis thaliana*, *Pseudomonas syringae* pv. *tomato*, age-related resistance, salicylic acid, antimicrobial, flowering, senescence, intercellular space

INTRODUCTION

The phenolic phytohormone salicylic acid (SA) contributes to a number of developmental and physiological responses in plants. SA is predominately known for its role in initiating defense responses against pathogens such as *Pseudomonas syringae* (reviewed in Vlot et al., 2009; An and Mou, 2011), a hemibiotrophic bacterial pathogen. Seminal research established SA as an essential player in plant defense. Wild-type plants respond to microbial attack by accumulating high levels of SA, which induces expression of PATHOGENESIS-RELATED (PR) proteins, ultimately allowing the plant to respond in a resistant manner (Malamy et al., 1990; Métraux et al., 1990). Importantly, plants with reduced SA levels due to ectopic expression of a bacterial SA-hydroxylase gene (*NahG*) are unable to activate defense responses and are highly susceptible to pathogen attack (Gaffney et al., 1993; Delaney et al., 1994). The level of pathogen-induced SA accumulation is correlated with the magnitude of pathogen resistance, where high levels of SA are associated with resistance and low levels of SA are associated with susceptibility. Thus, SA is a focal point in the tug-of-war between plants and pathogens, with each side attempting to regulate SA levels for its own benefit. Not surprisingly, plant and pathogen genotypes play a large role in dictating the outcome of this tug-of-war, however, an often-overlooked aspect in this struggle is the developmental stage of the plant. In this perspective, we outline the profound impact that developmental age has on SA-mediated plant-pathogen interactions in *Arabidopsis*.

GENERAL PLANT DEFENSE RESPONSES

Plant defense is comprised of several overlapping layers that include PAMP-triggered immunity (PTI), effector-triggered susceptibility (ETS), and effector-triggered immunity (ETI; reviewed in Jones and Dangl, 2006). Basal defenses such as PTI are induced upon the recognition of common microbial epitopes or PAMPs (pathogen-associated molecular patterns) such as flagellin or chitin by cognate pattern-recognition receptors. The PTI response includes accumulation of SA (reviewed in Boller and Felix, 2009; Meng and Zhang, 2013). SA is synthesized through two distinct metabolic routes. It can be generated from L-phenylalanine via the PAL (PHENYLALANINE AMMONIA LYASE) pathway or from chorismate via ICS1/SID2 (ISOCHORISMATE SYNTHASE1/SALICYLIC ACID INDUCTION DEFICIENT2) the latter of which is responsible for the bulk of chloroplast-derived SA produced during pathogen infection in *Arabidopsis* (reviewed in Vlot et al., 2009; Dempsey et al., 2011). *Arabidopsis sid2* mutants produce little SA and are defective in basal/PTI responses (Nawrath and Métraux, 1999; Wildermuth et al., 2001). To overcome PTI, adapted pathogens employ virulence effector proteins that translocate into plant cells via the type 3 secretion system (T3SS), as well as small diffusible phytotoxins such as coronatine. Once inside the cell, some effector proteins and phytotoxins target

host proteins to interfere with PTI, resulting in host susceptibility or enhanced pathogenicity. The mechanisms by which effectors and phytotoxins suppress defense vary, however many suppress plant defenses such as SA accumulation and *PR* gene expression (Xin and He, 2013). To overcome the suppression of plant defense by effector proteins, plants employ ETI. To initiate ETI, an effector protein is first recognized by a highly specific Resistance (R) receptor protein, either directly or indirectly. Recognition of an effector or "avirulence" protein by its cognate R receptor initiates a signaling cascade that results in SA accumulation, *PR* gene expression, and a form of programmed cell death known as the hypersensitive response (Jones and Dangl, 2006). This form of resistance is highly specific and affords a high degree of resistance. Both ETI and PTI also initiate systemic acquired resistance (SAR), a defense response in which an initial local infection induces long-distance signaling to protect distant uninfected leaves against future pathogen attack (reviewed in Champigny and Cameron, 2009; Shah and Zeier, 2013). Much like PTI and ETI, plants defective in SA accumulation are defective in SAR. Although SA itself is not the long-distance SAR signal (Rasmussen et al., 1991; Vernooij et al., 1994), the SA conjugate methyl salicylate (MeSA) participates in SAR (Park et al., 2007; Vlot et al., 2008; Liu et al., 2011).

MECHANISM OF SA SIGNAL TRANSDUCTION

Salicylic acid accumulation initiates a complex signaling cascade that includes hallmark *PR* gene expression. Early genetic screens for mutants defective in SA signaling discovered NPR1 (NON-EXPRESSOR OF PR1), a transcriptional co-activator important for plant defense (Cao et al., 1997). Our current understanding of SA signaling places NPR1 in a central role as the master-regulator of SA-induced signal transduction (reviewed in Vlot et al., 2009; An and Mou, 2011; Yan and Dong, 2014). In brief, SA accumulation leads to a change in cellular redox status that facilitates the monomerization of a cytosolic oligomer pool of NPR1, which translocate to the nucleus and interact with TGA transcription factors to regulate gene expression (Mou et al., 2003). Although NPR1 plays a central role in signaling, its inability to reliably bind SA in conventional ligand-binding assays suggests that it is not the SA receptor. A search for the SA receptor demonstrated that NPR1 homologs NPR3 and NPR4 bind SA and regulate NPR1 protein stability to mediate SA-signaling (Fu et al., 2012). Based on their findings, the authors depict a model wherein SA levels affect the ability of NPR3 or NPR4 to target NPR1 for ubiquitinylation and degradation via the proteasome. At the lowest and highest levels of SA, the NPR1 homologs direct NPR1 degradation, preventing SA signaling. At intermediate SA levels, NPR1 is no longer targeted for degradation and can participate in SA signaling (reviewed in Yan and Dong, 2014). This regulatory module ensures that SA induces defense gene expression only when necessary and prevents constitutive SA-mediated immune signaling, which is generally detrimental to growth and development (reviewed in Durrant and Dong, 2004; Rivas-San Vicente and Plasencia, 2011).

MATURITY AND DEFENSE—UNCONVENTIONAL DISEASE RESISTANCE

Much of what we know about SA signaling and its impact on induced resistance comes from experiments using young plants. In the *P. syringae–Arabidopsis* pathosystem, young plants inoculated with virulent *P. syringae* pv. *tomato* (*Pst*) support high levels of *in planta* bacterial growth and are susceptible to disease, while mature plants support low levels of *in planta* bacterial growth and are resistant (Kus et al., 2002). This phenomenon, known as age-related resistance (ARR), is a highly robust form of developmentally regulated resistance. The focus of this perspective is ARR in *Arabidopsis*, however, developmentally regulated disease resistance has been observed in a variety of other plants (reviewed in Whalen, 2005; Develey-Rivière and Galiana, 2007). Much like defense in young plants, the ability to accumulate SA in response to pathogen infection is required for ARR in *Arabidopsis*. Plants defective in SA biosynthesis or accumulation (*sid2*, *eds1*, *eds5/sid1*, *NahG*) are ARR-defective such that mature plants remain susceptible to *Pst* at later stages of development (Kus et al., 2002; Carviel et al., 2009, 2014). Unlike defense in young plants, NPR1 is not required for ARR (Kus et al., 2002; Cameron and Zaton, 2004), suggesting that although SA accumulation is critical, NPR1-dependent SA signaling is dispensable during ARR. This led us to speculate that ARR may employ NPR1-independent SA signaling. Our knowledge of NPR1-independent SA signaling is less extensive in comparison to NPR1-dependent responses, however, the ssDNA-binding transcription factor WHIRLY1 (WHY1) is among a small number of genes thought to be involved in NPR1-independent SA signaling and defense (reviewed in Desveaux et al., 2005; An and Mou, 2011). WHY1 is required for SA and pathogen-induced *PR1* expression irrespective of NPR1. Moreover, ssDNA-binding activity of WHY1 is induced by SA treatment in both wild-type and *npr1-1* plants, suggesting that WHY1 functions to induce *PR* expression independent of NPR1 (Desveaux et al., 2004). To investigate the requirement of NPR1-independent SA signaling for ARR, we compared the ARR phenotypes of two independent *why1* T-DNA insertion mutants (*why1-1, why1-2*) to wild-type Col-0 and the SA-deficient *sid2-1* mutant. Plants were inoculated with 10^6 colony-forming units per ml (cfu ml^{-1}) of virulent *Pst* (DC3000) at 4 and 7 weeks post-germination (wpg) followed by determination of *in planta* bacterial density 3 days later (**Figure 1**). For both wild-type Col-0 and the *why1* mutants, young plants supported high *in planta* bacterial densities (2–5 $\times$ 10^6 cfu per leaf disk [cfu ld^{-1}]), whereas mature plants displayed reduced bacterial densities (3–6 $\times$ 10^4 cfu ld^{-1}) consistent with a strong ARR response. In comparison, the SA-deficient *sid2-1* mutant displayed a characteristic ARR-defective phenotype, with high *in planta* bacterial densities ($>1 \times 10^7$ cfu ld^{-1}) at 4 and 7 wpg. These data suggest that WHY1 function is not required for ARR. Given that WHY1 and NPR1 are not required for ARR competence, we suggest that SA signaling through these proteins is not an important component of ARR. Indeed, we previously demonstrated that ARR-competent plants express less *PR1* in response to virulent *Pst* compared to young plants (Kus et al., 2002; Rusterucci et al., 2005), indicating that ARR represents an unconventional SA-dependent defense response that occurs in older plants. Although it is possible that SA plays an NPR1- and WHY1-independent signaling role that is not associated with *PR1* expression, we propose that SA may play a different role altogether during ARR.

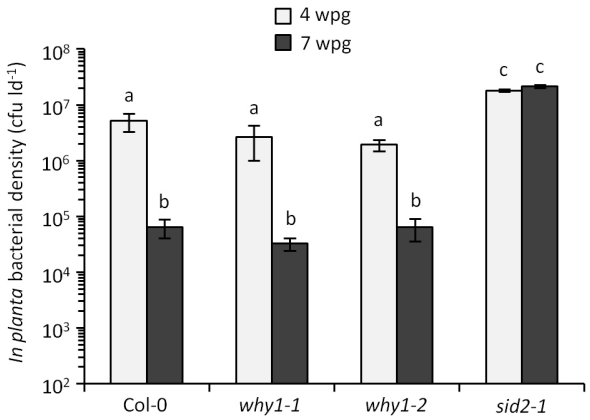


FIGURE 1 | WHIRLY1 is not required for ARR. Plants were inoculated with 10^6 cfu ml^{-1} of virulent *Pst* DC3000 at 4 and 7 weeks post-germination (wpg). *In planta* bacterial density [colony-forming units per leaf disk (cfu ld^{-1})] was determined 3 days later and is presented as the mean ± standard deviation of three sample replicates. Different letters indicate statistically significant differences between means (ANOVA, Tukey's HSD, $P < 0.05$). This experiment was performed three times with similar results. Plant growth, inoculations, and quantification of bacterial levels were performed as described previously (Carviel et al., 2014). The T-DNA mutants *why1-1* (SALK_023713C) and *why1-2* (SALK_147680C) were obtained from TAIR and have been characterized previously (Isemer et al., 2012).

A POTENTIAL NON-SIGNALING ROLE FOR SA IN PLANT DEFENSE RESPONSES

An alternative, non-signaling role for SA during ARR was explored by Cameron and Zaton (2004), who hypothesized that SA may act as an antimicrobial agent in the intercellular space (apoplast) during ARR. This hypothesis arose from the observation that intercellular washing fluids (IWFs) collected from mature (ARR-competent) plants inoculated with *Pst* possessed antimicrobial activity that was not present in corresponding IWFs from young (ARR-incompetent) plants (Kus et al., 2002). Moreover, antimicrobial activity was absent in IWFs from mature *NahG* plants, suggesting that SA accumulation is required for the antimicrobial activity observed in mature wild-type plants. Further investigation revealed that SA accumulated in IWFs from mature plants but not young plants following inoculation with *Pst* (Cameron and Zaton, 2004). Infiltration of exogenous SA into the intercellular space rescued the ARR-defect in *sid2-1* but not *NahG*. Conversely, addition of the SA-degrading salicylate hydroxylase enzyme to the intercellular space impaired the ARR response of wild-type plants. Together these data suggest that SA accumulation in the intercellular space is a key aspect of the ARR response. The antimicrobial effect of SA on *Pst in vitro* (Cameron and Zaton, 2004) suggests that SA itself could be acting as an antimicrobial agent *in planta* during ARR. Moreover, SA and structurally related compounds possess antimicrobial activity against a variety of other phytopathogens *in vitro* (Prithiviraj et al., 1997; Georgiou et al., 2000; Amborabé et al., 2002; El-Mougy, 2002; Martín et al., 2010).

Mature plants accumulate high levels of intercellular SA in response to virulent *Pst*, while young plants accumulate relatively little (Cameron and Zaton, 2004; Carviel et al., 2014). We therefore propose that pathogen-mediated suppression of intercellular SA accumulation contributes to disease susceptibility in young plants, and that mature plants are able to overcome this virulence strategy. In young plants the *P. syringae* phytotoxin coronatine has been shown to suppress SA accumulation at the whole-leaf level (deTorresZabala et al., 2009; Zheng et al., 2012) as well as in the intercellular space (Carviel et al., 2014). Young plants inoculated with a coronatine-deficient *Pst* mutant accumulated higher levels of intracellular and intercellular SA, and supported lower bacterial levels compared to plants inoculated with wild-type *Pst* (Carviel et al., 2014). This suggests that intercellular SA accumulation is a component of the basal defense response that is suppressed by *Pst*. A specific signaling pathway for coronatine-mediated suppression of SA accumulation in young plants has recently been uncovered (Zheng et al., 2012), and we hypothesize that ARR involves the activity of one or more developmentally regulated gene products that alleviate coronatine-mediated suppression of defense (Wilson et al., 2014). Similar to mature plants responding to virulent *Pst*, young plants responding to avirulent *Pst* also accumulated high levels of SA in IWFs (Carviel et al., 2014). Thus, intercellular SA accumulation may also contribute to ETI.

SA-ASSOCIATED MATURE-PLANT PROCESSES AND ARR COMPETENCE

Our ARR research has revealed novel aspects of SA-mediated defense in both young and mature plants. However, the fundamental question, "how do mature plants become competent for ARR?," remains to be answered. In *Arabidopsis*, several mature-plant developmental processes have been associated with SA accumulation (reviewed in Rivas-San Vicente and Plasencia, 2011). We speculate that these SA-dependent processes may contribute to ARR competence. Below, we briefly describe two major developmental processes, the transition to flowering and leaf senescence, and our efforts to understand their contribution to SA accumulation and ARR.

IMPACT OF LEAF SENESCENCE AND SA CATABOLISM ON ARR

Leaf senescence is an actively regulated developmental process that coordinates the reallocation of metabolic resources from leaves to reproductive tissues in older plants (reviewed in Lim et al., 2007). As a mature-plant process, leaf senescence could contribute to ARR competence. In a recent study, Zhang et al. (2013) identified the *Arabidopsis* S3H (SA-3-HYDROXYLASE) protein, which is responsible for the catabolism of SA to 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA. *Arabidopsis s3h* mutants accumulated high levels of SA and underwent leaf senescence early, whereas transgenic *Arabidopsis* overexpressing *S3H* had low levels of SA, high levels of 2,3-DHBA sugar conjugates, and were delayed in senescence (Zhang et al., 2013). This study demonstrates the strong positive correlation between SA levels and the induction of leaf senescence. The authors also determined that 2,3-DHBA and its xyloside conjugate 2,3-DHB3X accumulated with age (Zhang et al., 2013). In a previous study, 2,3-DHBA was identified as an EDS1-dependent metabolite that accumulated in response to *P. syringae* infection and with age (Bartsch et al., 2010). Although 2,3-DHBA does not possess a

strong capacity to induce *PR1* gene expression, the authors propose that it may contribute to EDS1-dependent defense. We agree with the authors' idea that 2,3-DHBA, an isochorismate-derived metabolite that accumulates with age and is dependent on EDS1, may contribute to ARR. Their finding that 2,3-DHBA was a poor inducer of *PR1* expression is in agreement with our observations that ARR-competent plants do not express *PR1* to high levels and that ARR doesn't require NPR1 or WHY1. Whether 2,3-DHBA plays a role in ARR could be addressed by quantifying 2,3-DHBA and 2,3-DHB3X in IWFs collected from young and mature plants inoculated with *Pst*, and by determining if DHBA contributes to the antimicrobial activity of IWFs from ARR-competent plants. However, ARR competence is not associated with early-stage senescence marker gene expression (*SAG-13*) or senescence-induced leaf tip chlorosis (Kus et al., 2002), suggesting that senescence is not a developmental cue for ARR competence. Rather, aspects of leaf aging such as an increase in SA catabolism and DHBA accumulation may contribute to ARR competence in *Arabidopsis* independent of leaf senescence.

THE TRANSITION TO FLOWERING IS ASSOCIATED WITH ARR

The transition from vegetative to reproductive growth is a highly regulated process that relies on multiple endogenous and environmental cues (reviewed in Amasino, 2010). Interestingly, SA appears to act as a positive regulator of flowering in *Arabidopsis*, as SA-deficient mutants flower later than wild-type plants (Martínez et al., 2004). Detailed genetic analyses indicated that the promotion of flowering by SA appears to proceed through several independent mechanisms, involving components of the autonomous and photoperiod flowering pathways (Martínez et al., 2004). In both short- and long-day-grown *Arabidopsis* the floral transition occurs at approximately the same time as the onset of

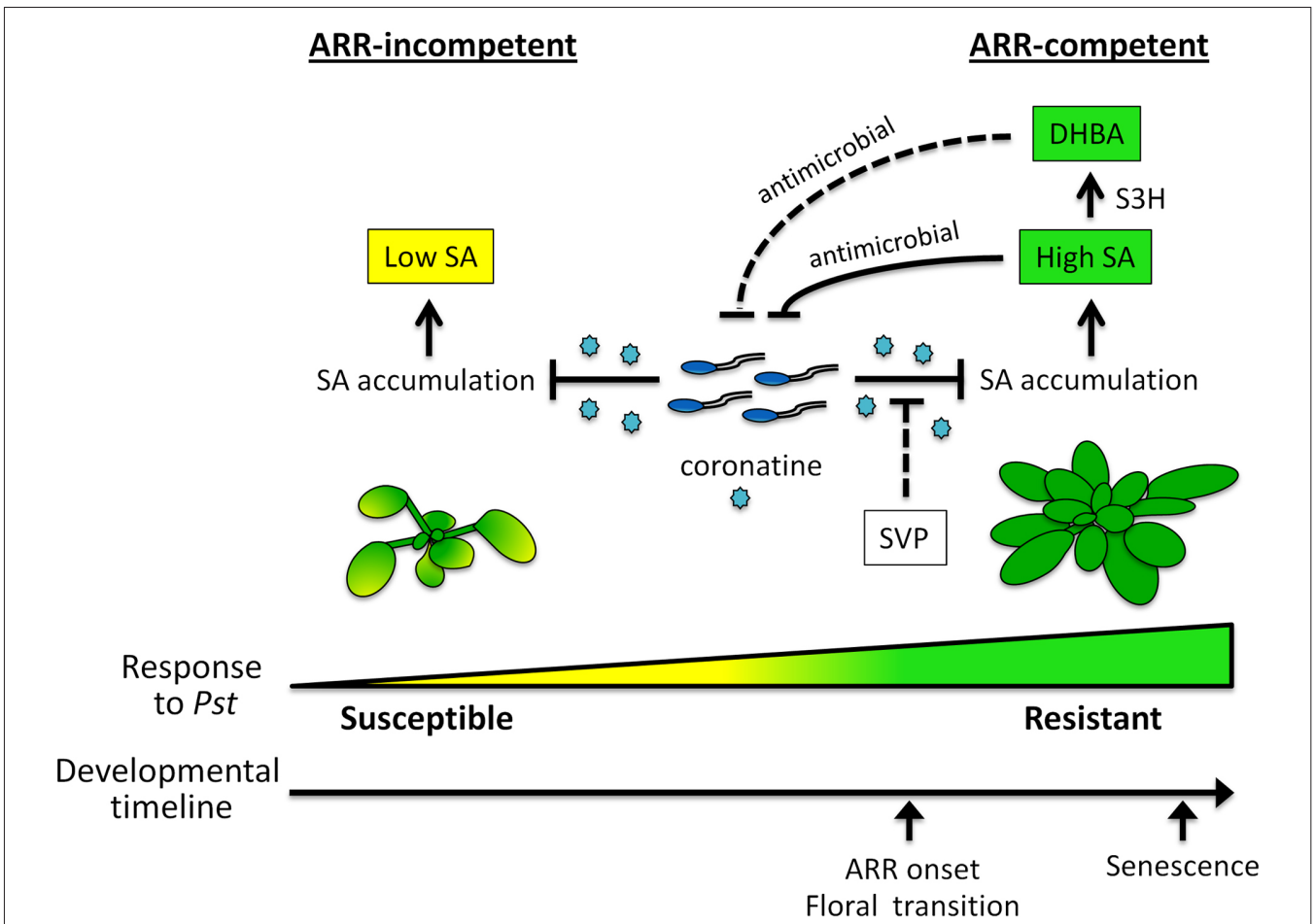


FIGURE 2 | Salicylic acid-mediated disease resistance in young and mature *Arabidopsis*. The model illustrates key aspects of the *Arabidopsis* age-related resistance (ARR) response to *Pseudomonas syringae* pv. *tomato (Pst)* with a focus on salicylic acid (SA) accumulation in young and mature plants. In young plants, coronatine produced by *Pst* suppresses the accumulation of SA to dampen defense, resulting in susceptibility to disease. At later stages of development, plants acquire ARR competence and become resistant to *Pst*. Mature plants infected with virulent *Pst* accumulate high levels of SA despite the presence of coronatine. Our accumulated evidence supports the idea that intercellular SA acts as an antimicrobial agent to limit *Pst* growth. The onset of ARR competence coincides with the transition to flowering whereas leaf senescence occurs well after. We hypothesize that the floral repressor SHORT VEGETATIVE PHASE (SVP) contributes to ARR by alleviating coronatine-mediated suppression of SA. SA-3-HYDROXYLASE (S3H) converts SA to 2,3-dihydroxybenzoic acid (DHBA), which accumulates with age and contributes to leaf senescence. We hypothesize that DHBA contributes to ARR as an antimicrobial agent in the intercellular space. Dashed bar—hypothesized relationship, solid bar—relationship supported by evidence.

ARR (Rusterucci et al., 2005). This led us to speculate that the transition to flowering could be a developmental cue for ARR competence. However, further investigation effectively separated the transition to flowering from ARR competence (Wilson et al., 2013). Early-flowering mutants and wild-type plants forced to flower early by transient exposure to long days did not exhibit early ARR, nor did late-flowering mutants display delayed ARR. Together these data suggest that the transition to flowering is neither sufficient nor required for the onset of ARR competence.

Unexpectedly, our analysis of flowering-time mutants revealed that early-flowering *svp-31* was ARR-incompetent. SVP (SHORT VEGETATIVE PHASE) is a MADS-domain transcription factor that acts as a negative regulator of the floral transition (Hartmann et al., 2000). A genome-wide ChIP-chip study (Tao et al., 2012) identified many SVP target genes including three NAC transcription factors that have been shown to mediate the suppression of SA accumulation by coronatine (Zheng et al., 2012). Our current efforts are focused on elucidating the role of SVP in ARR and determining whether SVP suppresses *NAC* gene expression to prevent coronatine-mediated suppression of SA accumulation in mature plants.

CONCLUSION—DEVELOPMENTAL DIFFERENCES IN SA-MEDIATED DEFENSE

It is clear that SA plays a central role in immune responses to *Pst* in both young and mature *Arabidopsis*. Moreover, *Arabidopsis* ARR is also effective against the biotrophic pathogen *Hyaloperonospora arabidopsidis* (Hpa; Rusterucci et al., 2005; Carviel et al., 2009). Since several *Hpa* effectors have been shown to suppress SA-mediated immunity in young plants, (Anderson et al., 2012; Caillaud et al., 2013; Asai et al., 2014) we speculate that suppression of SA-mediated defense by *Hpa* is also alleviated in mature ARR-competent plants. Our current model of ARR and the role that SA plays in mature versus young plants is illustrated in **Figure 2**. At earlier developmental stages, plants support high levels of bacterial growth and are susceptible to *Pst*. The phytotoxin coronatine contributes to the suppression of SA accumulation in young plants to prevent SA-mediated immune signaling, thus promoting disease susceptibility. At later stages of development, plants gain competence for ARR and are resistant to *Pst* infection. This is associated with the accumulation of high levels of SA, which may act as an antimicrobial agent in the intercellular space. The transition to flowering overlaps with the onset of ARR, however, it is not the developmental cue for ARR competence. Interestingly, our recent studies with SVP, a negative regulator of the transition to flowering, suggest that this transcription factor may contribute to ARR by limiting coronatine-mediated suppression of SA accumulation. Further, we hypothesize that the SA-catabolite 2,3-DHBA, acts as an antimicrobial agent in the intercellular space similar to SA. Future research is required to address the key questions posed by our model and clarify the role of SA during plant-pathogen interactions in mature versus young *Arabidopsis*.

ACKNOWLEDGMENTS

This research was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) grant to Robin K. Cameron, an NSERC graduate scholarship to Daniel C. Wilson, and an Ontario Graduate Scholarship to Philip Carella.

REFERENCES

Amasino, R. (2010). Seasonal and developmental timing of flowering. *Plant J.* 61, 1001–1013. doi: 10.1111/j.1365-313X.2010.04148.x

Amborabé, B., Fleurat-Lessard, P., Chollet, J., and Roblin, G. (2002). Antifungal effects of salicylic acid and other benzoic acid derivatives towards *Eutypa lata*: structure–activity relationship. *Plant Physiol. Biochem.* 40, 1051–1060. doi: 10.1016/S0981-9428(02)01470-5

An, C., and Mou, Z. (2011). Salicylic acid and its function in plant immunity. *J. Integer. Plant Biol.* 53, 412–428. doi: 10.1111/j.1744-7909.2011.01043.x

Anderson, R. G., Casady, M. S., Fee, R. A., Vaughan, M. M., Deb, D., Fedkenheuer, K., et al. (2012). Homologous RXLR effectors from *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* suppress immunity in distantly related plants. *Plant J.* 72, 882–893. doi: 10.1111/j.1365-313X.2012.05079.x

Asai, S., Rallapalli, G., Piquerez, S. J. M., Caillaud, M.-C., Furzer, O. J., Ishaque, N., et al. (2014). Expression profiling during *Arabidopsis*/downy mildew interaction reveals a highly-expressed effector that attenuates responses to salicylic acid. *PLoS Pathog.* 10:e1004443. doi: 10.1371/journal.ppat.1004443

Bartsch, M., Bednarek, P., Vivancos, P. D., Schneider, B., von Roepenack-Lahaye, E., Foyer, C. H., et al. (2010). Accumulation of isochorismate-derived 2,3-dihydroxybenzoic 3-O-β-D-xyloside in *Arabidopsis* resistance to pathogens and ageing of leaves. *J. Biol. Chem.* 285, 25654–25665. doi: 10.1074/jbc.M109.092569

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by patter-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Caillaud, M.-C., Asai, S., Rallapalli, G., Piquerez, S., Fabro, G., and Jones, D. G. J. (2013). A downy mildew effector attenuates salicylic acid-triggered immunity in *Arabidopsis* by interacting with the host mediator complex. *PLoS Biol.* 11:e1001732. doi: 10.1371/journal.pbio.1001732

Cameron, R. K., and Zaton, K. (2004). Intercellular salicylic acid accumulation is important for age-related resistance in *Arabidopsis* to *Pseudomonas syringae*. *Physiol. Mol. Plant Pathol.* 66, 197–209. doi: 10.1016/j.pmpp.2005.02.002

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., and Dong, X. (1997). The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57–63. doi: 10.1016/S0092-8674(00)81858-9

Carviel, J. L., Al-Daoud, F., Neumann, M., Mohammad, A., Provart, N. J., Moeder, W., et al. (2009). Forward and reverse genetics to identify genes involved in the age-related resistance response in *Arabidopsis thaliana*. *Mol. Plant Pathol.* 10, 621–634. doi: 10.1111/J.1364-3703.2009.00557.X

Carviel, J. L., Wilson, D. C., Isaacs, M., Carella, P., Catana, V., Golding, B., et al. (2014). Investigation of intercellular salicylic acid accumulation during compatible and incompatible *Arabidopsis-Pseudomonas syringae* interactions using a fast neutron-generated mutant allele of EDS5 identified by genetic mapping and whole-genome sequencing. *PLoS ONE* 9:e88608. doi: 10.1371/journal.pone.0088608

Champigny, M. J., and Cameron, R. K. (2009). "Action at a distance: long-distance signals in induced resistance," in *Plant Innate Immunity*, Vol. 51, ed. L. C. Van Loon (London: Academic Press), 123–171.

de Torres Zabala, M., Bennett, M. H., Truman, W. H., and Grant, M. R. (2009). Antagonism between salicylic acid and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. *Plant J.* 59, 375–386. doi: 10.1111/j.1365-313X.2009.03875.x

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. (1994). Central role of salicylic acid in plant disease resistance. *Science* 266, 1247–1250. doi: 10.1126/science.266.5188.1247

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arabidopsis Book* 9, 1–24. doi: 10.1199/tab.0156

Desveaux, D., Subramaniam, R., Despres, C., Mess, J. N., Levesque, C., Fobert, P. R., et al. (2004). A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in *Arabidopsis*. *Dev. Cell* 6, 229–240. doi: 10.1016/S1534-5807(04)00028-0

Desveaux, D., Marechal, A., and Brisson, N. (2005). Whirly transcription factors: defense gene regulation and beyond. *Trends Plant Sci.* 10, 95–102. doi: 10.1016/j.tplants.2004.12.008

Develey-Rivière, M., and Galiana, E. (2007). Resistance to pathogens and host developmental stage: a multifaceted relationship within the plant kingdom. *New Phytol.* 175, 405–416. doi: 10.1111/j.1469-8137.2007.02130.x

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

El-Mougy, N. S. (2002). In vitro studies on antimicrobial activity of salicylic acid and acetylsalicylic acid as pesticidal alternatives against some soilborne plant pathogens. *Egypt. J. Phytopathol.* 30, 41–55.

Fu, Z. Q., Yan, S. P., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., et al. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261, 754–756. doi: 10.1126/science.261.5122.754

Georgiou, C. D., Tairis, N., and Sotiropoulou, A. (2000). Hydroxyl radical scavengers inhibit lateral-type sclerotial differentiation and growth in phytopathogenic fungi. *Mycologia* 95, 825–834. doi: 10.2307/3761577

Hartmann, U., Höhmann, S., Nettesheim, K., Wisman, E., Saedler, H., and Huijser, P. (2000). Molecular cloning of SVP: a negative regulator of the floral transition in *Arabidopsis*. *Plant J.* 21, 351–360. doi: 10.1046/j.1365-313x.2000.00682.x

Isemer, R., Krause, K., Grabe, N., Kitahata, N., Asami, T., and Krupinska, K. (2012). Plastid located WHIRLY1 enhances the responsiveness of *Arabidopsis* seedlings toward abscisic acid. *Front. Plant Sci.* 3:283. doi: 10.3389/fpls.2012.00283

Jones, J. D. J., and Dangl, J. F. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kus, J. V., Zaton, K., Sarkar, R., and Cameron, R. K. (2002). Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. *Plant Cell* 14, 479–490. doi: 10.1105/tpc.010481

Lim, P. K., Kim, H. J., and Nam, H. G. (2007). Leaf senescence. *Annu. Rev. Plant Biol.* 58, 115–136. doi: 10.1146/annurev.arplant.57.032905.105316

Liu, P. P., von Dahl, C. C., and Klessig, D. F. (2011). The extent to which methyl salicylate is required for signaling systemic acquired resistance is dependent on exposure to light after infection. *Plant Physiol.* 157, 2216–2226. doi: 10.1104/pp.111.187773

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250, 1002–1004. doi: 10.1126/science.250.4983.1002

Martín, J. A., Solla, A., Witzell, J., Gil, L., and García-Vallejo, M. C. (2010). Antifungal effect and reduction of *Ulmus minor* symptoms to *Ophiostoma novo-ulmi* by carvacrol and salicylic acid. *Eur. J. Plant Pathol.* 127, 21–32. doi: 10.1007/s10658-009-9567-3

Martínez, C., Pons, E., Prats, G., and León, J. (2004). Salicylic acid regulates flowering time and links defense responses and reproductive development. *Plant J.* 37, 209–217. doi: 10.1046/j.1365-313X.2003.01954.x

Meng, X., and Zhang, S. (2013). MAPK cascades in plant disease resistance signaling. *Annu. Rev. Phytopathol.* 51, 245–266. doi: 10.1146/annurev-phyto-082712-102314

Métraux, J.-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gandin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006. doi: 10.1126/science.250.4983.1004

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Nawrath, C., and Métraux, J.-P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 8, 1393–1404. doi: 10.1105/tpc.11.8.1393

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Prithiviraj, B., Manickam, M., Singh, U. P., and Ray, A. B. (1997). Antifungal activity of anacardic acid, a naturally occurring derivative of salicylic acid. *Can. J. Bot.* 75, 207–211. doi: 10.1139/b97-021

Rasmussen, J. B., Hammerschmidt, R., and Zook, M. N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* 97, 1342–1347. doi: 10.1104/pp.97.4.1342

Rivas-San Vicente, M., and Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *J. Exp. Bot.* 62, 3321–3338. doi: 10.1093/jxb/err031

Rusterucci, C., Zhao, Z., Haines, K., Mellersh, D., Neumann, M., and Cameron, R. K. (2005). Age-related resistance to *Pseudomonas syringae* pv. *tomato* is associated with the transition to flowering in *Arabidopsis* and is effective against *Peronospora parasitica*. *Physiol. Mol. Plant Pathol.* 66, 222–231. doi: 10.1016/j.pmpp.2005.08.004

Shah, J., and Zeier, J. (2013). Long-distance communication and signal amplification in systemic acquired resistance. *Front. Plant Sci.* 4:30. doi: 10.3389/fpls.2013.00030

Tao, Z., Shen, L., Liu, C., Liu, L., Yan, Y., and Yu, H. (2012). Genome-wide identification of SOC1 and SVP targets during the floral transition in *Arabidopsis*. *Plant J.* 70, 549–561. doi: 10.1111/j.1365-313X.2012.04919.x

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., et al. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* 6, 959–965. doi: 10.1105/tpc.6.7.959

Vlot, A. C., Liu, P. P., Cameron, R. K., Park, S. W., Yang, Y., Kumar, D., et al. (2008). Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic acquired resistance in *Arabidopsis thaliana*. *Plant J.* 56, 445–456. doi: 10.1111/j.1365-313X.2008.03618.x

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Whalen, M. C. (2005). Host defence in a developmental context. *Mol. Plant Pathol.* 6, 347–360. doi: 10.1111/j.1364-3703.2005.00286.x

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Wilson, D. C., Carella, P., and Cameron, R. K. (2014). Intercellular salicylic acid accumulation during compatible and incompatible *Arabidopsis-Pseudomonas syringae* interactions. *Plant Signal. Behav.* 9:e29362. doi: 10.4161/psb.29362

Wilson, D. C., Carella, P., Isaacs, M., and Cameron, R. K. (2013). The floral transition is not the developmental switch that confers competence for the *Arabidopsis* age-related resistance response to *Pseudomonas syringae* pv. *tomato*. *Plant Mol. Biol.* 83, 235–246. doi: 10.1007/s11103-013-0083-7

Xin, X.-F., and He, S.-Y. (2013). *Pseudomonas syringae* pv. *tomato* DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. *Annu. Rev. Phytopathol.* 51, 473–498. doi: 10.1146/annurev-phyto-082712-102321

Yan, S., and Dong, X. (2014). Perception of the plant immune signal salicylic acid. *Curr. Opin. Plant Biol.* 20, 64–68. doi: 10.1016/j.pbi.2014.04.006

Zhang, K., Halitschke, R., Yin, C., Liu, C.-J., and Gan, S.-S. (2013). Salicylic acid 3-hydroxylase regulates *Arabidopsis* leaf longevity by mediating salicylic acid catabolism. *Proc. Natl. Acad. Sci. U.S.A.* 110, 14807–14813. doi: 10.1073/pnas.1302702110

Zheng, X. Y., Spivey, N. W., Zeng, W., Liu, P. P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596. doi: 10.1016/j.chom.2012.04.014

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 October 2014; accepted: 15 December 2014; published online: 09 January 2015.

Citation: Carella P, Wilson DC and Cameron RK (2015) Some things get better with age: differences in salicylic acid accumulation and defense signaling in young and mature Arabidopsis. Front. Plant Sci. 5:775. doi: 10.3389/fpls.2014.00775

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in Plant Science

MINI REVIEW
published: 09 March 2015
doi: 10.3389/fpls.2015.00125

Deciphering the link between salicylic acid signaling and sphingolipid metabolism

*Diana Sánchez-Rangel†, Mariana Rivas-San Vicente, M. Eugenia de la Torre-Hernández, Manuela Nájera-Martínez and Javier Plasencia**

Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, México City, México

The field of plant sphingolipid biology has evolved in recent years. Sphingolipids are abundant in cell membranes, and genetic analyses revealed essential roles for these lipids in plant growth, development, and responses to abiotic and biotic stress. Salicylic acid (SA) is a key signaling molecule that is required for induction of defense-related genes and rapid and localized cell death at the site of pathogen infection (hypersensitive response) during incompatible host–pathogen interactions. Conceivably, while levels of SA rapidly increase upon pathogen infection for defense activation, they must be tightly regulated during plant growth and development in the absence of pathogens. Genetic and biochemical evidence suggest that the sphingolipid intermediates, long-chain sphingoid bases, and ceramides, play a role in regulating SA accumulation in plant cells. However, how signals generated from the perturbation of these key sphingolipid intermediates are transduced into the activation of the SA pathway has long remained to be an interesting open question. At least four types of molecules – MAP kinase 6, reactive oxygen species, free calcium, and nitric oxide – could constitute a mechanistic link between sphingolipid metabolism and SA accumulation and signaling.

Keywords: salicylic acid, sphingolipid, ceramide, sphingoid bases, sphinganine-analog mycotoxin

OPEN ACCESS

Edited by:
Hua Lu, University of Maryland, Baltimore County, USA

Reviewed by:
Shunyuan Xiao, University of Maryland, USA
Robin Katrina Cameron, McMaster University, Canada

****Correspondence:***
Javier Plasencia, Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, 04510 México City, México
javierp@unam.mx

†Present address:
Investigador Cátedras CONACyT adscrita al Instituto de Ecología A. C., Red de Estudios Moleculares Avanzados Carretera Antigua a Coatepec 351, El Haya, 91070 Xalapa, Veracruz, México

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *14 October 2014*
Accepted: *16 February 2015*
Published: *09 March 2015*

Citation:
Sánchez-Rangel D, Rivas-San Vicente M, de la Torre-Hernández ME, Nájera-Martínez M and Plasencia J (2015) Deciphering the link between salicylic acid signaling and sphingolipid metabolism. Front. Plant Sci. 6:125. doi: 10.3389/fpls.2015.00125

Introduction

Salicylic acid (SA) is a phytohormone involved in local and systemic resistance (Vlot et al., 2009), as well as in the response to abiotic stress, growth, and development (Rivas-San Vicente and Plasencia, 2011). The SA signaling pathway requires a functional NPR1 [nonexpressor of pathogenesis-related (PR) genes 1] protein to relay the signal to the nucleus, where it activates *PR* gene expression (Wang et al., 2005; Kumar, 2014; Seyfferth and Tsuda, 2014). SA biosynthesis occurs either through the phenylalanine (PAL) or isochorismate (ICS) pathway, and the relative contribution of each route varies in different species (Chen et al., 2009b; An and Mou, 2011). SA production is controlled by multiple positive and negative regulators (Janda and Ruelland, 2014). Exciting new research reveals that several sphingolipid intermediates induce SA accumulation and affect disease resistance. The objective of this review is to assess the experimental data that link sphingolipid metabolism with SA accumulation and signaling. Such evidence is mainly derived from (1) the phenotypes of *Arabidopsis* and *Nicotiana* plants in which genes involved in sphingolipid metabolism are mutated or silenced, and (2) the effects of sphinganine analog mycotoxins (SAMs, namely AAL and FB1) on sphingolipid metabolism.

Sphingolipid Metabolism

Research in plant sphingolipids has been fostered by the use of novel extraction protocols, followed by mass spectrometry analysis and characterization of *Arabidopsis* mutants. Sphingolipids compose ∼40% of the lipids of the plasma membrane and are also abundant in other endomembranes. Functional genomics of sphingolipid metabolism genes show that these molecules have essential functions in plant growth, development, and stress responses (Chen et al., 2009a; Pata et al., 2010; Berkey et al., 2012). Sphingolipid biosynthesis starts in the endoplasmic reticulum (ER). L-serine is condensed with palmitoyl-CoA to generate a sphingoid long-chain base (LCB) that is reduced and then *N*-acylated to form ceramide. Ceramides are substrates for the production of complex sphingolipids, including inositol phosphorylceramide (IPC), and glucosylceramide. In addition to hydroxylation, LCBs and ceramides can be phosphorylated (**Figure 1**) to yield a wide variety of molecules (Markham et al., 2006).

Disruption of Sphingolipid Metabolism Through Mutation and Silencing Affects Salicylic Acid Levels

In **Table 1**, we summarize the effects of mutation or silencing of genes involved in sphingolipid biosynthesis or metabolism in *Arabidopsis* ecotypes and *Nicotiana benthamiana*, and highlights the effects of altered SA levels and/or signaling on *PR1* gene expression.

Sphingolipid Biosynthesis and Metabolism

Long-Chain Base Biosynthesis

Serine palmitoyl transferase (SPT), a heterodimer formed by LCB1 and LCB2 subunits, catalyzes the first reaction in sphingolipid biosynthesis to form LCBs (**Figure 1**; Chen et al., 2009a). The *Arabidopsis* genome contains one gene encoding the LCB1 subunit and two encoding LCB2. Functional studies using mutant and RNAi suppression lines lacking *LCB1* expression, and double mutants lacking both *LCB2* genes, show that sphingolipids are essential for growth and development (Chen et al., 2006; Dietrich et al., 2008). However, it is unknown whether mutations in any of the *LCB* genes affect the SA pathway. A link between SA and sphingolipid metabolism was established through virus-induced gene silencing (VIGS) of the *N. benthamiana* LCB2 subunit. A 20 to 50% reduction in *NbLCB2* transcript level was sufficient to impair growth and leaf and flower development. Compared to control plants, plants with a ∼50% reduction in *NbLCB2* transcripts display elevated SA levels and constitutive *PR1* expression (**Table 1**), and later, show spontaneous cell death in leaves. These silenced plants are also more susceptible to infection by the fungal necrotroph *Alternaria alternata* f. sp. *lycopersici*. LCB composition in silenced plants is altered with lower trihydroxylated LCB and higher dihydroxylated LCB levels than those of control plants (Rivas-San Vicente et al., 2013). These results suggest that disruption of LCB homeostasis is accompanied by elevated SA levels and induction of cell death. However, the identity of the LCB responsible for this phenotype is unknown.

Ceramide Biosynthesis

Ceramide synthase catalyzes the condensation of a LCB with a fatty acid-CoA to yield ceramide (**Figure 1**). The *Arabidopsis* genome has three ceramide synthase genes –*LOH1*, *LOH2*, and *LOH3*– and each isoform has a selective preference for the type of acyl-CoA and LCB (dihydroxy- or trihydroxy-LCB; Chen et al., 2009a). Mutants for each gene are viable, and only the *loh1* line has a spontaneous cell death phenotype, which occurs late in development. Although SA levels in this mutant are comparable to those in wild type (WT) plants, *PR1* transcription increased 160-fold (**Table 1**). Furthermore, this mutant exhibits modest changes in sphingolipid content, with a 7 and 19% increase in the proportion of species containing a C16 fatty acid in ceramides and GlcCer, respectively, and a fivefold increase in trihydroxy-LCBs (Ternes et al., 2011). These data narrow-down the identity of the bioactive sphingolipids responsible for triggering cell death to free trihydroxy-LCBs, dihydroxy-LCBs, or ceramide species with a C16 fatty acid.

Ceramide Hydroxylation

Ceramide might be hydroxylated in its LCB moiety by a LCB-C4 hydroxylases (SBH) and/or at the fatty acid residues by fatty acid hydroxylases (FAH; Markham et al., 2006). Although double mutants and RNAi suppression lines of *SBH* genes display necrotic lesions in their cotyledons, and constitutively express *PR* genes (Chen et al., 2008), data on SA accumulation and/or signaling is lacking. Conversely, an analysis of double mutants of the two *FAH* genes (*fah1* and *fah2*) demonstrated a link between sphingolipid biosynthesis and SA metabolism. The *fah1/fah2* double mutant displays a 25% reduction in leaf and root growth compared to WT plants, elevated SA levels, and aberrant constitutive *PR1* expression (**Table 1**). Despite elevated SA levels, this mutant lacks a spontaneous cell death phenotype. These plants contain lower levels of ceramides and GlcCer with α-hydroxylated fatty acids, but a 10-fold increase in phytoceramides and a fivefold increase in trihydroxylated LCBs (König et al., 2012). Thus fatty acid hydroxylation of ceramides is required for the biosynthesis of complex sphingolipids and its absence leads to the accumulation of LCBs and ceramides. This elevation activates the SA pathway and supports a link between SA signaling and sphingolipid metabolism.

Ceramide Phosphorylation

ACD5 encodes a 608-amino acid protein with ceramide kinase activity that is located in the ER, Golgi apparatus (GA), and mitochondria (Liang et al., 2003; Bi et al., 2014). In the *Arabidopsis acd5* mutant, a glycine residue is replaced with an arginine and the mutant enzyme retains only 10% of the activity of the WT. These mutant plants develop normally for 5 weeks and then display spontaneous leaf lesions, accumulate free, and conjugated SA along with reactive oxygen species (ROS), and constitutively express *PR1* (**Table 1**). Due to reduced ceramide kinase activity, *acd5* plants accumulate ceramides and hydroxyceramides, with a two- to sixfold increase relative to WT plants. Only ceramides

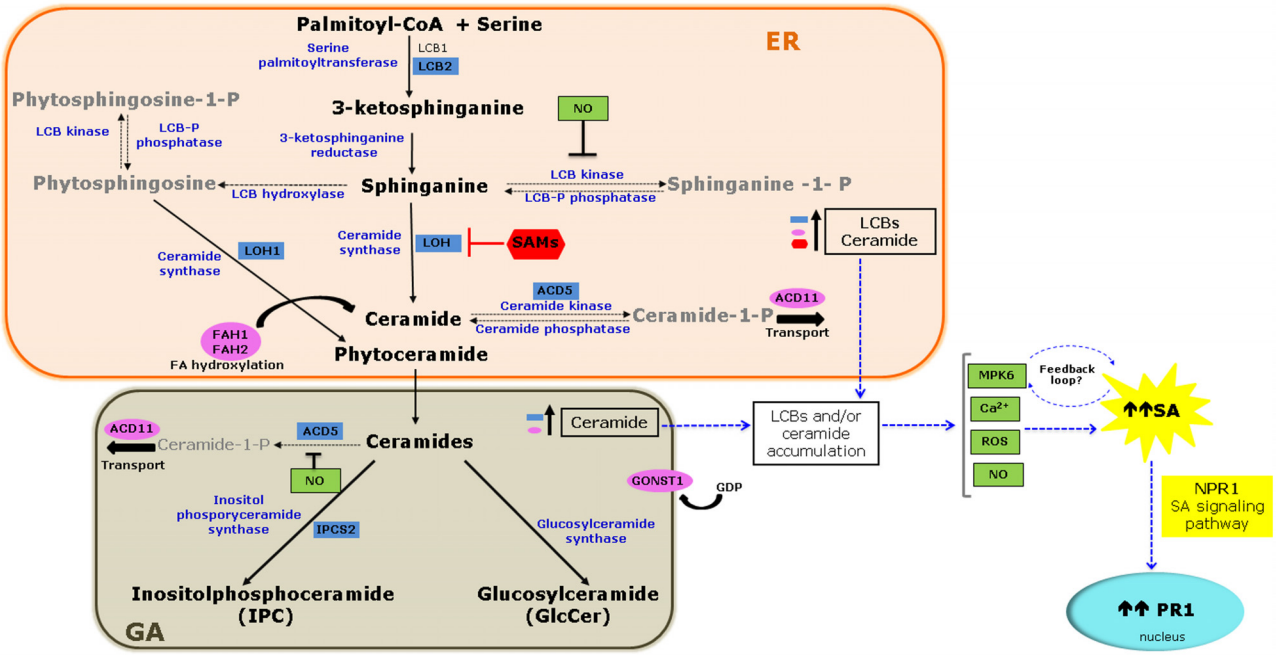


FIGURE 1 | Molecular links between sphingolipid metabolism and the salicylic acid (SA) signaling pathway. *De novo* biosynthesis of sphingolipids starts in the endoplasmic reticulum (ER) and ends in the Golgi apparatus (GA) with the biosynthesis of complex sphingolipids. The diagram highlights mutations or silencing events in *Arabidopsis* and *Nicotiana* that result in loss or reduced function of genes involved in sphingolipid metabolism (blue boxes) or sphingolipid modification or transport (pink ovals) and their effect on SA accumulation and/or signaling as a consequence of the accumulation of long-chain base (LCBs), and/or ceramide (see **Table 1**). Also illustrated is the inhibition of the ceramide synthase by sphinganine analog mycotoxin (SAMs; red) that additionally contributes to LCB accumulation and SA pathway activation through pathogenesis-related *(PR1)* gene 1 expression. Several signaling molecules are candidates connecting sphingolipid metabolism and SA signaling (green boxes). MITOGEN-ACTIVATED PROTEIN KINASE 6 (MPK6) may act in a feedback loop between the two pathways. Solid arrows indicate biosynthetic steps, while dashed arrows indicate modification of LCBs and ceramide. Blue arrows denote proposed steps of convergence between the two pathways.

containing long-chain fatty acids (C16) accumulate while levels of ceramides with very-long-chain fatty acids (C24 and C26) are not altered (Bi et al., 2014).

The *acd5* cell death phenotype is SA-dependent as it is suppressed in the *acd5/NahG* genotype. *Nah*G encodes a salicylate hydroxylase which converts SA to inert catechol such that these plants do not accumulate SA. Moreover, a functional SA signaling pathway is required because *acd5/npr1* double mutants have an attenuated cell death phenotype (Greenberg et al., 2000; Liang et al., 2003; Bi et al., 2014). Mutation in the *ACD5* gene causes an imbalance in the ceramide to ceramide-1-phosphate ratio and ceramide accumulation might activate the SA pathway.

Inositol Phosphorylceramide Biosynthesis

Ceramides serve as substrates for the formation of complex sphingolipids (**Figure 1**). Inositol phosphorylceramide-synthase (IPCS) catalyzes the transfer of phosphorylinositol to phytoceramide to yield IPC. The *Arabidopsis* genome contains three functional *IPCS* genes: *IPCS1*, *IPCS2,* and *IPCS3*. *AtIPCS2* is expressed at higher levels than the other two genes in all organs tested (Mina et al., 2010), and the protein localizes to the *trans*-Golgi network (Wang et al., 2008).

The phenotype associated with *IPCS2* loss of function is only discernible in transgenic plants expressing the resistance gene *RPW8* (*Resistance to powdery mildew*). These plants exhibit spontaneous cell death and were thus named *ehr1* (*enhancing RPW8-mediated hypersensitive response cell death*). *Arabidopsis RPW8* confers broad-spectrum resistance to powdery mildew, and the *ipcs2* mutation in *RPW8* transgenic plants increased sensitivity to fungal infection. The *ipcs2* mutant lines are only 30% the size of the parental line at maturity and exhibit regions of spontaneous cell death. These plants accumulate both free SA and SA conjugates and constitutively express *PR1* (**Table 1**). Spontaneous cell death and constitutive *PR1* expression depend on SA, since both traits are abolished when the *NahG* gene or the *pad4* mutation is introduced. PAD4 is an upstream regulator of the SA signaling pathway. Again, the precise identity of the sphingolipid molecule responsible for this phenotype remains unidentified, because these mutant plants show increased levels of both ceramides and LCBs (Wang et al., 2008).

Sphingolipid Modification

Complex sphingolipids, such as glycosyl IPC (GIPC), are the most abundant lipids in plant cell membranes (Markham et al., 2006). Glycosylation of the inositol head group occurs in the GA. Monosaccharides such as hexuronic acid, galactose, mannose, and arabinose can be attached to GIPC (Buré et al., 2011). Guanidine diphosphate (GDP) sugars serve

TABLE 1 | Association between sphingolipid metabolism and salicylic acid (SA) levels.

Gene name	Gene ID	Gene product	Sphingolipid profile in mutant or silenced plants	SA levels in mutant or silenced plants	*PR1* expression in mutant or silenced plants	Reference
NbSPT	NbLCB2 AM902524	Serine palmitoyl transferase (SPT)	50% reduction of trihydroxylated long-chain base (LCBs). Fourfold increase of dihydroxylated LCBs	1.5-fold increase in total SA levels	Constitutive	Rivas-San Vicente et al. (2013)
AtLOH1	At3g25540	Ceramide synthase	Higher percentage of ceramides (7%) and glucosylceramides (19%) containing C16 fatty acids. Fivefold elevation of trihyhdroxy-LCBs	SA levels are unaffected	Constitutive; 160-fold raise	Ternes et al. (2011)
AtFAH1/ AtFAH2	At2g34770/ At4g20870	Fatty acid hydroxylase (FAH)	Fivefold increase of trihydroxylated LCBs; 100-fold reduction of hydroxyceramides; two- to fourfold reduction of phytoglucosylceramides, 10-fold increase of phytoceramides	3.2-fold higher levels of free SA enriched and 4.3-fold increase in SA conjugates	Constitutive	König et al. (2012)
AtACD5	At3G21630	Ceramide kinase	Two- to sixfold increase in ceramides and hydroxy-ceramides; no changes in glucosylceramides and LCBs levels	Fourfold increase in free SA and ninefold raise in total SA	Constitutive, but impaired induction.	Greenberg et al. (2000), Bi et al. (2014)
AtIPCS2	At2g37940	Inositol –P-ceramide synthase 2	Two- to threefold increase in ceramides and hydroxyceramides. Enriched in trihydroxylated LCBs	Threefold higher levels of free SA and conjugated SA	Constitutive	Wang et al. (2008)
AtGONST1	At2g13650	GDP-D-mannose transporter	3.5-fold decrease in the proportion of Hex_1GIPCs in membranes. Most (75%) of glycosyl inositol phosphorylceramides (GIPCs) found lack hexosylation	Fourfold increase in free SA levels and sixfold increase in total SA	Constitutive; 10-fold raise.	Mortimer et al. (2013)
AtACD11	At2g34690	Ceramide-1-phosphate transfer protein	Sevenfold increase in ceramides; threefold rise in hydroxyceramides; twofold increase in GIPC and GlcCer; twofold increase in LCBs and LCB-P	63-fold increase in total SA	Constitutive	Brodersen et al. (2002, 2005), Simanshu et al. (2014)

Plants with mutated or silenced genes involved in sphingolipid metabolism show alterations in SA levels and/or PR1 gene expression.

as donors for glycosylation and are transported into the GA. GONST1 (GOLGI-LOCALIZED NUCLEOTIDE SUGAR TRANSPORTER) belongs to a family of nucleotide sugar transporters and stimulates GDP-mannose transport (Baldwin et al., 2001). Knock-out of *GONST1* causes severe dwarfing, poor seed set, formation of spontaneous necrotic lesions on the leaves, accumulation of free and conjugated SA, and constitutive *PR1* expression (**Table 1**). Overexpression of *NahG* in the *gonst1* background diminishes SA levels and the number of necrotic lesions, and partially alleviates the growth defect. These data suggest that the ability to accumulate SA is partly responsible for the *gonst1* phenotype. The *gonst1* plants do not differ from WT plants in ceramide or LCB content, but do exhibit changes in sphingolipid sugar decoration; the proportion of Hex_1GIPCs in membranes isolated from *gonst1* is 25%, compared to 90% in the WT (Mortimer et al., 2013).

Sphingolipid Transport

ACD11 encodes a protein homologous to a mammalian glycolipid transfer protein (GLTP) with no predicted transmembrane domains or localization motifs. It was initially characterized as a sphingosine transporter (Brodersen et al., 2002), but a recent study showed that this protein contains a lipid recognition center. ACD11 selectively binds to ceramide-1-phosphate (C1P) and phyto-C1P, but not to related plant sphingolipids such as ceramides, GlcCers, GIPCs, and LCBs (Simanshu et al., 2014).

Arabidopsis acd11 mutant plants show an accelerated cell death phenotype early in development, characterized by ROS generation, necrotic lesions, and constitutive expression of senescence- and defense-related genes (Brodersen et al., 2002). They also accumulate SA and display constitutive *PR1* expression (**Table 1**). Since the cell death phenotype is suppressed in transgenic *NahG* lines that do not accumulate SA, cell death is SA-dependent. Cell death is also blocked by mutations in *PAD4* and *EDS1*, which are upstream regulators of the SA response (Brodersen et al., 2002). Other mutations in key components of the SA biosynthesis and signaling pathway, such as *SID2* and *EDS5,* also diminish SA accumulation in the *acd11* mutant (Brodersen et al., 2005). *SID2* encodes an ICS synthase, suggesting that SA accumulation is partly responsible for the observed phenotype, and ICS precursors might trigger cell death. The *acd11/eds5* mutant exhibits constitutive *PR1* expression and a similar cell death phenotype as the *acd11* mutants, but limited SA accumulation. *EDS5* encodes an extrusion-like transporter involved in SA export from chloroplasts (Serrano et al., 2013). Exogenous application of the SA analog BTH to *acd11/NahG* and *acd11/sid2* plants restores cell death and induces ceramide accumulation, reinforcing SA role in the signaling pathway that results in this phenotype (Brodersen et al., 2002, 2005).

Both LCBs and ceramides could contribute to the *acd11* mutant phenotype, as levels of these sphingolipid intermediates are elevated in these plants. Because of the functional association between sphingolipid metabolism and SA biosynthesis, a feedback loop might regulate these two pathways (Simanshu et al., 2014).

SA Levels are Affected by the Action of Sphinganine Analog Mycotoxins on Sphingolipid Metabolism

SAMs share structural similarity with LCBs and inhibit ceramide synthase activity (**Figure 1**) causing LCB levels to increase (Wang et al., 1991; Abbas et al., 1994). The best-characterized SAMs are the AAL-toxin produced by *A. alternata* f. sp. *lycopersici*, a tomato foliar pathogen, and fumonisin B1 (FB1), produced by *Fusarium verticillioides*, a causal agent of various diseases in maize. Although the hosts and type of diseases caused by these fungi are quite different, genetic evidence supports a role for SAMs in virulence of these fungal pathogens (Sánchez-Rangel and Plasencia, 2010).

Effects of SAMs in *Arabidopsis* Genotypes and Hormonal Crosstalk

Fumonisin B1 causes LCB accumulation in several plant species (Abbas et al., 1994; de la Torre-Hernandez et al., 2010), and in *Arabidopsis*, a 72-h treatment with 10 μM FB1 triggers a 100- to 7000-fold increase in LCBs concentration (Saucedo-García et al., 2011). This dose also triggers lesions reminiscent of those formed in the pathogen-induced HR, accompanied by callose deposition, ROS and camalexin production, and expression of *PR-1*, *PR-2*, and *PR-5* in *Arabidopsis* leaves (Stone et al., 2000). Very low doses (70 nM) of FB1 cause DNA fragmentation and cell death in *Arabidopsis* protoplasts. Because protoplasts from mutant genotypes defective in SA, jasmonic acid (JA), and ethylene (ET) accumulation or signaling are more tolerant to the toxin, it was concluded that all three signaling pathways are required for cell death caused by FB1 (Asai et al., 2000). However, when 50 μM FB1 was infiltrated into rosette leaves of *dde2*, *ein2*, *pad4*, and *sid2* single mutants (defective in JA, ET, SA, and SA signaling pathways, respectively) and the corresponding quadruple mutant, enhanced tolerance to FB1 was not observed (Igarashi et al., 2013). The cell type tested – protoplasts vs. intact leaves – and the ∼700-fold difference in FB1 dose might account for the observed discrepancies.

From the above data, it is clear that FB1 activates the SA pathway, but also other routes that are antagonistic to SA signaling, thus complicating results interpretation. For instance, *Arabidopsis* possesses five ET receptors (ETR1, ETR2, EIN4, ERS1, and ERS2) and genetic studies show that, in absence of ET, the receptors positively regulate CTR1, which acts as a negative regulator of the ET signaling pathway (Ju et al., 2012). FB1 has contrasting effects in mutants of the five ET receptors; while the *etr1-1* mutant shows hypersensitivity to FB1, the *ein4-1* mutant displays diminished cell death and the other three mutants respond similarly to the WT. These results suggest that ET receptors have distinct roles in toxin sensitivity leading to the HR. While ET induces cell death through EIN4, perception of this phytohormone by ETR1 inhibits cell death. Because ET represses *PR1* transcription, mutations in genes encoding ET receptors increase the expression of SA-inducible genes; for instance the *ers1* and the *ein4* mutants display a 29-fold and 115-fold rise in *PR1* expression, respectively (Plett et al., 2009).

Deciphering the Link Between Sphingolipid Metabolism and the SA Pathway

Since both, LCBs and ceramides, serve as signaling molecules in the activation of defense-related PCD (Berkey et al., 2012), it is reasonable to hypothesize that SA acts as an intermediate in this pathway. So far, evidence provided by mutants in sphingolipid biosynthesis and by experiments with SAMs and exogenous LCBs/ceramides, suggests that sphingolipid intermediates act upstream of SA. Expression of *NahG* or negative regulators of the SA pathway in sphingolipid mutants confirms that this phytohormone is required for the cell death phenotype. However, the remaining question is how perturbations in levels of LCBs/ceramides are perceived to induce the SA biosynthesis pathway? Several signaling molecules, upstream and/or downstream of sphingolipid intermediates, are candidates to connect these two pathways (**Figure 1**), will be described briefly.

Both, FB1 and LCBs activate MITOGEN-ACTIVATED PROTEIN KINASE 6 (MPK6) within minutes after the infiltration of *Arabidopsis* rosette leaves. Moreover, *mpk6* mutant seedlings show reduced cell death when exposed to 10 μM FB1, suggesting that MPK6 is a transducer in the pathway leading to LCB-induced PCD in *Arabidopsis* (Saucedo-García et al., 2011). Although MPK6 was characterized as the ortholog of the tobacco SA-induced protein kinase (SIPK), it is rapidly activated by several microbial elicitors (Nühse et al., 2000) with a similar kinetics as with FB1 and LCBs.

Reactive oxygen species elevation is a common feature displayed by several mutants in sphingolipid biosynthesis that show an enhanced cell death phenotype and SA accumulation. Molecules such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$) mediate a variety of cellular responses. In *Arabidopsis*, 10 μM FB1 causes an elevation of *PAL* transcript and activity, which results in a fourfold increase in total SA. This elevation depends on ROS, as inhibitors that disrupt ROS production prevent this response (Xing et al., 2013). Moreover, exogenous LCBs induce ROS production in leaves of *Arabidopsis* seedlings (Shi et al., 2007). Although data on LCB accumulation is lacking in these reports, FB1 biological activity suggests that LCBs elevation mediate ROS generation.

Another hypothesis is that free calcium levels change in response to a sphingolipid imbalance and transduce a signal. In tobacco BY2 cells, exogenous addition of dihydroxy-LCB causes an immediate (∼1 min) dose-dependent elevation of cellular free calcium concentration in the cytosol and 10 min later in the nucleus, followed by H_2O_2 accumulation and cell death (Lachaud et al., 2010, 2011). Calcium also regulates the expression of the SA biosynthesis gene *ICS1* through the Ca^{2+}/calmodulin-binding transcription factor CBP60g (Wang et al., 2009; Zhang et al., 2010). Another Ca^{2+}/calmodulin-binding transcription factor, AtSR1, is a negative regulator of SA signaling, as it controls *EDS1* expression (Du et al., 2009). Because calcium controls SA levels, free cytosolic Ca^{2+} could link sphingolipid metabolism with the SA pathway. Testing the susceptibility of *atsr1* mutants when challenged to an imbalance of sphingolipids levels through exogenous addition of FB1 or LCBs might shed some light on this question.

Finally, nitric oxide (NO), a universal transducer molecule, might play a role in linking sphingolipids and the SA pathway. In *Taxus* sp. cell cultures, a fungal-produced sphingolipid induces rapid and dose-dependent NO production, and because this molecule is a redox regulator of the NPR1/TGA1 system, it promotes NPR1 translocation into the nucleus (Wang et al., 2007; Lindermayr et al., 2010; Guillas et al., 2013). NO might also act upstream of sphingolipids intermediates. Exposure of *Arabidopsis* plants to cold induce NO production that downregulates the synthesis of phytosphingosine-phosphate and ceramide-phosphate. In the *nia1/nia2* nitrate reductase mutant, impaired in NO biosynthesis, such suppression does not occur (Cantrel et al., 2011). Thus NO could participate in the fine-tuning of the balance between certain sphingolipids and their phosphorylated derivatives.

Studies of the phenotypes of *Arabidopsis* mutants in sphingolipid metabolism suggest that imbalance of LCBs and/or ceramides levels activate the SA pathway. However, further research is needed to determine the causality of this relationship and to identify the upstream signal transduction molecule(s) responsible for activating the SA pathway. Additional comparisons of the effects of FB1, LCBs, and ceramides on MPK, ROS, calcium, and NO signaling in relevant *Arabidopsis* WT and mutants will reveal the main players in this complex interaction between the sphingolipid and SA signaling pathways.

Acknowledgments

The authors acknowledge both reviewers for their thorough evaluation of the manuscript and valuable suggestions. Research in JP laboratory is supported by grants from DGAPA-PAPIIT (IN220010) and Programa MASAGRO. DS-R is supported by Cátedras CONACYT program.

References

Abbas, H. K., Tanaka, T., Duke, S. O., Porter, J. K., Wray, E. M., Hodges, L., et al. (1994). Fumonisin- and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. *Plant Physiol.* 106, 1085–1093.

An, C., and Mou, Z. (2011). Salicylic acid and its function in plant immunity. *J. Int. Plant Biol.* 53, 412–428. doi: 10.1111/j.1744-7909.2011.01043.x

Asai, T., Stone, J. M., Heard, J. E., Kovtun, Y., Yorgey, P., Sheen, J., et al. (2000). Fumonisin B1-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. *Plant Cell* 12, 1823–1835. doi: 10.1105/tpc.12.10.1823

Baldwin, T. C., Handford, M. G., Yuseff, M. I., Orellana, A., and Dupree, P. (2001). Identification and characterization of GONST1, a golgi-localized GDP-mannose transporter in *Arabidopsis*. *Plant Cell* 13, 2283–2295. doi: 10.1105/tpc.13.10.2283

Berkey, R., Bendigeri, D., and Xiao, S. (2012). Sphingolipids and plant defense/disease: the "death" connection and beyond. *Front. Plant Sci.* 3:68. doi: 10.3389/fpls.2012.00068

Bi, F. C., Liu, Z., Wu, J. X., Liang, H., Xi, X. L., Sun, T. J., et al. (2014). Loss of ceramide kinase in *Arabidopsis* impairs defenses and promotes ceramide accumulation and mitochondrial H_2O_2 bursts. *Plant Cell* 26, 3449–3467. doi: 10.1105/tpc.114.127050

Brodersen, P., Malinovsky, F. G., Hématy, K., Newman, M. A., and Mundy, J. (2005). The role of salicylic acid in the induction of cell death in *Arabidopsis acd11*. *Plant Physiol.* 138, 1037–1045. doi: 10.1104/pp.105.059303

Brodersen, P., Petersen, M., Pike, H. M., Olszak, B., Skov, S., Ødum, N., et al. (2002). Knockout of *Arabidopsis* accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes Dev.* 16, 490–502. doi: 10.1101/gad.218202

Buré, C., Cacas, J. L., Wang, F., Gaudin, K., Domergue, F., Mongrand, S., et al. (2011). Fast screening of highly glycosylated plant sphingolipids by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 25, 3131–3145. doi: 10.1002/rcm.5206

Cantrel, C., Vazquez, T., Puyaubert, J., Reze, N., Lesch, M., Kaiser, W. M., et al. (2011). Nitric oxide participates in cold-responsive phosphosphingolipid formation and gene expression in *Arabidopsis thaliana*. *New Phytol.* 189, 415–427. doi: 10.1111/j.1469-8137.2010.03500.x

Chen, M., Cahoon, E. B., Saucedo-García, M., Plasencia, J., and Gavilanes-Ruíz, M. (2009a). "Plant sphingolipids: structure, synthesis and function," in *Lipids in Photosynthesis: Essential and Regulatory Functions*, eds H. Wada and N. Murata (Dordrecht: Springer), 77–115.

Chen, Z., Zheng, Z., Huang, J., Lai, Z., and Fan, B. (2009b). Biosynthesis of salicylic acid in plants. *Plant Signal. Behav.* 4, 493–496. doi: 10.4161/psb.4.6.8392

Chen, M., Han, G., Dietrich, C. R., Dunn, T. M., and Cahoon, E. B. (2006). The essential nature of sphingolipids in plants as revealed by the functional identification and characterization of the *Arabidopsis* LCB1 subunit of serine palmitoyltransferase. *Plant Cell* 12, 3576–3593. doi: 10.1105/tpc.105.040774

Chen, M., Markham, J. E., Dietrich, C. R., Jaworski, J. G., and Cahoon, E. (2008). Sphingolipid long-chain base hydroxylation is important for growth and regulation of sphingolipid content and composition in *Arabibopsis*. *Plant Cell* 20, 1862–1878. doi: 10.1105/tpc.107.057851

de la Torre-Hernandez, M. E., Rivas-San Vicente, M., Greaves-Fernandez, N., Cruz-Ortega, R., and Plasencia, J. (2010). Fumonisin B1 induces nuclease activation and salicylic acid accumulation through long-chain sphingoid base build-up in germinating maize. *Physiol. Mol. Plant Pathol.* 74, 337–345. doi: 10.1016/j.pmpp.2010.05.004

Dietrich, C. R., Han, G., Chen, M., Berg, R. H., Dunn, T. M., and Cahoon, E. B. (2008). Loss-of-function mutations and inducible RNAi suppression of *Arabidopsis* LCB2 genes reveal the critical role of sphingolipids in gametophytic and sporophytic cell viability. *Plant J.* 54, 284–298. doi: 10.1111/j.1365-313X.2008.03420.x

Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S., et al. (2009). Ca^{2+}/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* 457, 1154–1159. doi: 10.1038/nature07612

Greenberg, J. T., Silverman, F. P., and Liang, H. (2000). Uncoupling salicylic acid-dependent cell death and defense-related responses from disease resistance in the *Arabidopsis* mutant *acd5*. *Genetics* 156, 341–350.

Guillas, I., Puyaubert, J., and Baudouin, E. (2013). Nitric oxide-sphingolipid interplays in plant signaling: a new enigma from the Sphinx? *Front. Plant Sci.* 4:341. doi: 10.3389/fpls.2013.00341

Igarashi, D., Bethke, G., Xu, Y., Tsuda, K., Glazebrook, J., and Katagiri, F. (2013). Pattern-triggered immunity suppresses programmed cell death triggered by Fumonisin B1. *PLoS ONE* 8:e60769. doi: 10.1371/journal.pone.0060769

Janda, M., and Ruelland, E. (2014). Magical mystery tour: salicylic acid signaling. *Environ. Exp. Bot.* doi: 10.1016/j.envexpbot.2014.07.003

Ju, C., Yoon, G. M., Shemansky, J. M., Lin, D. Y., Ying, Z. I., Chang, J., et al. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19486–19491. doi: 10.1073/pnas.1214848109

König, S., Feussner, K., Schwarz, M., Kaever, A., Iven, T., Landesfeind, M., et al. (2012). *Arabidopsis* mutants of sphingolipid fatty acid α-hydroxylases accumulate ceramides and salicylates. *New Phytol.* 196, 1086–1097. doi: 10.1111/j.1469-8137.2012.04351.x

Kumar, D. (2014). Salicylic acid signaling in disease resistance. *Plant Sci.* 228, 127–124. doi: 10.1016/j.plantsci.2014.04.014

Lachaud, C., Da Silva, D., Amelot, N., Béziat, C., Bière, C., Cotelle, V., et al. (2011). Dihydrosphingosine-induced programmed cell death in tobacco BY-2 cells is independent of H_2O_2 production. *Mol. Plant* 4, 310–318. doi: 10.1093/mp/ssq077

Lachaud, C., Da Silva, D., Cotelle, V., Thuleau, P., Xiong, T. C., Jauneau, A., et al. (2010). Nuclear calcium controls the apoptotic-like cell death induced by D-erythro-sphinganine in tobacco cells. *Cell Calcium* 47, 92–100. doi: 10.1016/j.ceca.2009.11.011

Liang, H., Yao, N., Song, J. T., Luo, S., Lu, H., and Greenberg, J. T. (2003). Ceramides modulate programmed cell death in plants. *Genes Dev.* 17, 2636–2641. doi: 10.1101/gad.1140503

Lindermayr, C., Sell, S., Muller, B., Leister, D., and Durnera, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *Plant Cell* 22, 2894–2907. doi: 10.1105/tpc.109.066464

Markham, J. E., Li, J., Cahoon, E. B., and Jaworski, J. G. (2006). Separation and identification of major plant sphingolipid classes from leaves. *J. Biol. Chem.* 281, 22684–22694. doi: 10.1074/jbc.M604050200

Mina, J. G., Okada, Y., Wansadhipathi, N. K., Pratt, S., Shams-Eldin, H., Schwarz, R. T., et al. (2010). Functional analyses of differentially expressed isoforms of the *Arabidopsis* inositol phosphorylceramide synthase. *Plant Mol. Biol.* 73, 399–407. doi: 10.1007/s11103-010-9626-3

Mortimer, J. C., Yu, X., Albrecht, S., Sicilia, F., Huichalaf, M., Ampuero, D., et al. (2013). Abnormal glycosphingolipid mannosylation triggers salicylic acid-mediated responses in *Arabidopsis*. *Plant Cell* 25, 1881–1894. doi: 10.1105/tpc.113.111500

Nühse, T. S., Peck, S. C., Hirt, H., and Boller, T. (2000). Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK 6. *J. Biol. Chem.* 275, 7521–7526. doi: 10.1074/jbc.275.11.7521

Pata, M. O., Hannun, Y. A., and Ng, C. K.-Y. (2010). Plant sphingolipids: decoding the enigma of the Sphinx. *New Phytol.* 185, 611–630. doi: 10.1111/j.1469-8137.2009.03123.x

Plett, J. M., Cvetkovska, M., Makenson, P., Xing, T., and Regan, S. (2009). *Arabidopsis* ethylene receptors have different roles in Fumonisin B1-induced cell death. *Physiol. Mol. Plant Pathol.* 74, 18–26. doi: 10.1016/j.pmpp.2009.08.004

Rivas-San Vicente, M., Larios-Zarate, G., and Plasencia, J. (2013). Disruption of sphingolipid biosynthesis in *Nicotiana benthamiana* activates salicylic acid-dependent responses and compromises resistance to *Alternaria alternata f.* sp. *lycopersici*. *Planta* 237, 121–136. doi: 10.1007/s00425-012-1758-z

Rivas-San Vicente, M., and Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *J. Exp. Bot.* 62, 3321–3338. doi: 10.1093/jxb/err031

Sánchez-Rangel, D., and Plasencia, J. (2010). The role of sphinganine analog mycotoxins on the virulence of plant pathogenic fungi. *Toxin Rev.* 29, 73–86. doi: 10.3109/15569543.2010.515370

Saucedo-García, M., Guevara-García, A., González-Solís, A., Cruz-García, F., Vázquez-Santana, S., Markham, J. E., et al. (2011). MPK6, sphinganine and the LCB2a gene from serine palmitoyltransferase are required in the signaling pathway that mediates cell death induced by long chain bases in *Arabidopsis*. *New Phytol.* 191, 943–957. doi: 10.1111/j.1469-8137.2011.03727.x

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., et al. (2013). Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiol.* 162, 1815–1821. doi: 10.1104/pp.113.218156

Seyfferth, C., and Tsuda, K. (2014). Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. *Front. Plant Sci.* 5:697. doi: 10.3389/fpls.2014.00697

Shi, L., Bielawski, J., Mu, J., Dong, H., Teng, C., Zhang, J., et al. (2007). Involvement of sphingoid bases in mediating reactive oxygen intermediate production and programmed cell death in *Arabidopsis*. *Cell Res.* 17, 1030–1040. doi: 10.1038/cr.2007.100

Simanshu, D. K., Zhai, X., Munch, D., Hofius, D., Markham, J. E., Bielawski, J., et al. (2014). *Arabidopsis* accelerated cell death 11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels. *Cell Rep.* 6, 388–399. doi: 10.1016/j.celrep.2013.12.023

Stone, J. M., Heard, J. E., Asai, T., and Ausubel, F. M. (2000). Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (fbr) *Arabidopsis* mutants. *Plant Cell* 12, 1811–1822. doi: 10.1105/tpc.12.10.1811

Ternes, P., Feussner, K., Werner, S., Lerche, J., Iven, T., Heilmann, I., et al. (2011). Disruption of the ceramide synthase LOH1 causes spontaneous cell death in *Arabidopsis thaliana*. *New Phytol.* 192, 841–854. doi: 10.1111/j.1469-8137.2011.03852.x

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. (2005). Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 308, 1036–1040. doi: 10.1126/science.1108791

Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H. Jr. (1991). Inhibition of sphingolipid biosynthesis by fumonisins. *J. Biol. Chem.* 266, 14486–14490.

Wang, J., Zheng, L., and Tan, R. (2007). Involvement of nitric oxide in cerebroside-induced defense responses and taxol production in *Taxus yunnanensis* suspension cells. *Appl. Microbiol. Biotechnol.* 75, 1183–1190. doi: 10.1007/s00253-007-0927-7

Wang, L., Tsuda, K., Sato, M., Cohen, J. D., Katagiri, F., and Glazebrook, J. (2009). *Arabidopsis* CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. *PLoS Pathog.* 5:e1000301. doi: 10.1371/journal.ppat.1000301

Wang, W., Yang, X., Tangchaiburana, S., Ndeh, R., Markham, J. E., Tsegaye, Y., et al. (2008). An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*. *Plant Cell* 20, 3163–3179. doi: 10.1105/tpc.108.060053

Xing, F., Li, Z., Sun, A., and Xing, D. (2013). Reactive oxygen species promote chloroplast dysfunction and salicylic acid accumulation in fumonisin B1-induced cell death. *FEBS Lett.* 587, 2164–2172. doi: 10.1016/j.febslet.2013.05.034

Zhang, Y., Xu, S., Ding, P.,Wang, D., Cheng, Y. T., He, J., et al. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18220–18225. doi: 10.1073/pnas.1005225107

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 March 2015
doi: 10.3389/fpls.2015.00186

A systematic simulation of the effect of salicylic acid on sphingolipid metabolism

*Chao Shi, Jian Yin, Zhe Liu †, Jian-Xin Wu, Qi Zhao, Jian Ren and Nan Yao **

State Key Laboratory of Biocontrol, Guangdong Key Laboratory of Plant Resources, Department of Biological Science and Technology, School of Life Sciences, Sun Yat-sen University, Guangzhou, China

OPEN ACCESS

Edited by:
Loreto Holuigue, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Shunyuan Xiao, University of Maryland, USA
Richard Bostock, University of California, Davis, USA
Lee Sweetlove, University of Oxford, UK

***Correspondence:**
Nan Yao, State Key Laboratory of Biocontrol, Guangdong Key Laboratory of Plant Resources, Department of Biological Science and Technology, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China
yaonan@mail.sysu.edu.cn

†Present Address:
Zhe Liu, Guangdong Provincial Center for Disease Control and Prevention, Guangzhou, China

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *20 November 2014*
Accepted: *08 March 2015*
Published: *25 March 2015*

Citation:
Shi C, Yin J, Liu Z, Wu J-X, Zhao Q, Ren J and Yao N (2015) A systematic simulation of the effect of salicylic acid on sphingolipid metabolism. Front. Plant Sci. 6:186. doi: 10.3389/fpls.2015.00186

The phytohormone salicylic acid (SA) affects plant development and defense responses. Recent studies revealed that SA also participates in the regulation of sphingolipid metabolism, but the details of this regulation remain to beexplored. Here, we use *in silico* Flux Balance Analysis (FBA) with published microarray data to construct a whole-cell simulation model, including 23 pathways, 259 reactions, and 172 metabolites, to predict the alterations in flux of major sphingolipid species after treatment with exogenous SA. This model predicts significant changes in fluxes of certain sphingolipid species after SA treatment, changes that likely trigger downstream physiological and phenotypic effects. To validate the simulation, we used ^{15}N-labeled metabolic turnover analysis to measure sphingolipid contents and turnover rate in *Arabidopsis thaliana* seedlings treated with SA or the SA analog benzothiadiazole (BTH). The results show that both SA and BTH affect sphingolipid metabolism, altering the concentrations of certain species and also changing the optimal flux distribution and turnover rate of sphingolipids. Our strategy allows us to estimate sphingolipid fluxes on a short time scale and gives us a systemic view of the effect of SA on sphingolipid homeostasis.

Keywords: ceramides, salicylic acid, sphingolipid

Introduction

Salicylic acid (SA), an important phenolic phytohormone, has well-known roles in pathogen-triggered defense responses including microbe-associated molecular pattern-triggered immunity, effector-triggered immunity, and systemic acquired resistance (Jones and Dangl, 2006; Spoel and Dong, 2012; Yan and Dong, 2014). SA also participates in abiotic stress responses (Vlot et al., 2009; Miura and Tada, 2014) and in plant development, including vegetative and reproductive growth (Vicente and Plasencia, 2011). SA also has indispensible functions in the maintenance of redox homeostasis (Durner and Klessig, 1995, 1996; Slaymaker et al., 2002) and respiratory pathways (Moore et al., 2002). The SA analog benzothiadiazole (BTH) activates the SA signaling pathway, triggers expression of defense genes (Shimono et al., 2007), and produces physiological effects similar to those produced by SA (Lawton et al., 1996).

As a key mediator of defense responses, the SA pathway affects many metabolic pathways. Sphingolipids are a family of complex lipids that have a serine-based head, a fatty acyl chain, and a long-chain base (LCB). Covalent modifications and variability in the length of the fatty acyl chain increase sphingolipid diversity. Sphingolipids are important structural and functional components of the plasma membrane (Hannun and Obeid, 2008) and have important functions in the plant

immune response, abiotic stress responses,and developmental regulation (Chen et al., 2009; Pata et al., 2009; Markham et al., 2013; Bi et al., 2014). In *Arabidopsis*, ceramides, a group of sphingolipids, affect SA-mediated defense responses and programmed cell death (PCD). Some mutants in the sphingolipid metabolic pathway show high levels of expression of defense-related genes, accumulate SA, and undergo PCD. The ceramide kinase-deficient mutant *accelerated cell death 5* (*acd5*) accumulates SA and ceramides late in development, but shows increased susceptibility to pathogens (Greenberg et al., 2000; Liang et al., 2003; Bi et al., 2014). Wang et al. (2008) reported that the insertion knock-out mutant of *Arabidopsis* inositolphosphorylceramide synthase 2 (*erh1*) also spontaneously accumulates SA. Similar increases in SA levels have also been observed in the sphingosine transfer protein mutant *acd11* (Brodersen et al., 2002), the *Arabidopsis* sphingolipid fatty acid hydroxylase mutants *fah1 fah2* (König et al., 2012), and *mips1* (D-myo-inositol 3-phosphate synthase 1) mutants (Meng et al., 2009). Moreover, SA accumulation and PCD signaling mediated by MAPK affect the levels of free LCB (Saucedo-García et al., 2011). However, *fah1 fah2* mutants accumulate SA and have moderate levels of LCB (König et al., 2012). Thus, the SA and sphingolipid pathways have significant but complex crosstalk, particularly in defense and cell death.

Metabolic modeling performs well in prediction of physiological changes and metabolic outcomes resulting from genetic manipulation, where changes in metabolite levels have a strong effect on cellular behavior (Smith and Stitt, 2007; Stitt et al., 2010). The genome of *Arabidopsis thaliana* has been sequenced, making whole-genome metabolic reconstruction feasible (Thiele and Palsson, 2010; Seaver et al., 2012). Much of the early modeling work used steady-state Metabolic Flux Analysis (MFA), based on a steady-state model of the plant metabolic network, and on experiments using isotope labeling to trace metabolites of interest (Libourel and Shachar-Hill, 2008; Allen et al., 2009; Kruger et al., 2012). This method provided insights on metabolic organization and modes, but has difficulty in labeling heterotrophic tissues (Sweetlove and Ratcliffe, 2011), over-relies on manual curation of metabolic pathways (Masakapalli et al., 2010; Sweetlove and Ratcliffe, 2011; Kruger et al., 2012), and uses low-throughput detection, making systematic analysis difficult (Lonien and Schwender, 2009; Sweetlove and Ratcliffe, 2011).

By contrast, Flux Balance Analysis (FBA) overcomes many of the drawbacks of MFA. FBA establishes a model based on a group of ordinary differential equations that formulate a transient quasi-steady state of the metabolic fluxome of target pathways. The transient flux balance calculated by the FBA model has an almost-negligible duration compared to the long-term, fundamental metabolic changes that occur during development or in environmental responses (Varma and Palsson, 1994). In addition, FBA does not require isotopic labeling, suits a variety of trophic modes, and is more flexible than steady-state MFA in handling groups of flux distributions by linear programming and other methods for optimization under constraints (Edwards and Palsson, 2000; Reed and Palsson, 2003). Several *Arabidopsis* metabolic models based on FBA are available online (Poolman et al., 2009; Dal'Molin et al., 2010; Radrich et al., 2010).

Apart from FBA simulation, fluxomic changes can also be measured experimentally. To examine the response of sphingolipids to SA and BTH, we needed to determine and compare the turnover rates of sphingolipids. One of the major methods to measure turnover uses a time-course of stable isotopic incorporation into target metabolites, which are detected by mass spectrometry or nuclear magnetic resonance (Schwender, 2008; Hasunuma et al., 2010). The isotopic accumulation curve indicates the turnover of target metabolites.

Since metabolic changes substantially affect the crosstalk between SA and sphingolipids, in this study we constructed a metabolic model to simulate SA-related changes in the sphingolipid pathway. We constructed an *Arabidopsis* whole-cell FBA model including 23 pathways, 259 reactions, and 172 metabolites. Based on their relative enrichment and responsiveness to SA stimulation, our model includes 40 sphingolipid species, including LCBs, ceramides, hydroxyceramide, and glucosylceramides. Due to the lack of flux data on plant sphingolipid metabolism, we used ^{15}N-labeled metabolic turnover analysis to measure sphingolipid flux in untreated plants and calibrate the FBA model. After the calibration, we also supplied the model with additional expression profiles from plants treated with SA and BTH. The FBA model was calculated *in silico* for prediction and comparison of the optimal flux distribution and flux variability in SA- and BTH-treated and untreated conditions. We then used metabolic turnover analysis with ^{15}N-labeled samples to measure the flux changes directly. Both the computational model and the experiments showed consistent and significant changes in the sphingolipid pathway in response to SA and BTH. Our data gives us a systemic view of the effect of SA on sphingolipid homeostasis.

Materials and Methods

Plant Materials

Wild type *Arabidopsis thaliana* ecotype Columbia seedlings were grown vertically on 1/2x Murashige and Skoog (MS) medium for 10 days after 2-day vernalization. The culture dishes were incubated at 22°C under a 16 h light/8 h dark cycle. For labeling the plant seedlings in liquid medium, the culture dishes were incubated at 22°C with 24 h light.

Labeling and Treatments

The different sphingolipids have many carbon atoms in different positions; therefore, labeling the only nitrogen in the serine-based head group provides an easier approach for LC-MS/MS measurements. We used ^{15}N serine (Cambridge Isotope Laboratories, Inc. MA, USA) in the labeling experiment. Ten-day-old seedlings were transferred to N-deficient 1/2x MS liquid medium (Yoshimoto et al., 2004) in 12-well culture plates. 5 mM ^{15}N-labeled serine was supplied to compensate for the shortage of nitrogen (Hirner et al., 2006) and used as the only source of isotope. For SA and BTH treatments, 100 μM SA or 100 μM BTH was supplied in the labeling medium. The seedlings were treated or not treated for 0, 1, 3, 5, 7, 9, and 24 h for ^{15}N-labeled metabolic turnover analysis before sphingolipid extraction.

Experimental Measurement of Turnover Rate

Since serine has only one nitrogen atom and each sphingolipid has only one serine, the fraction of each labeled sphingolipid species can be measured as:

$$^{15}\text{N fraction\%} = {}^{15}\text{N}^{*}100/\text{N}$$

where ^{15}N is the concentration of ^{15}N-labeled molecules of a specific sphingolipid species, and N is the total concentration of that sphingolipid species, whether labeled or not.

The turnover rate of a sphingolipid species is calculated from the slope of the curve of the time-course of ^{15}N incorporation from the initial time that the fraction begins to increase to the time that the fraction stabilizes. Also, the isotopic incorporation rate r can be calculated as:

$$\text{r} = \frac{\text{d}^{15}\text{Nfraction}}{\text{dt}} * \text{N}$$

In the measurement, the natural enrichment of ^{15}N remains relatively constant between samples and treatments.

Sphingolipid Measurements

The plants cultured in labeling medium for the times described above were weighed and metabolically quenched by freezing in liquid nitrogen. Sphingolipid species were then extracted and measured by LC-MS/MS as described by Bi et al. (2014), with a slight modification to cope with isotopic-labeled sphingolipid species. Major sphingolipid species were subsequently analyzed with a Shimadzu 20A HPLC tandem AB SCIEX TripleTOF 5600^{+} mass spectrometer. The sphingolipid species were analyzed using the software Multiquant (AB SCIEX).

Metabolic Model Construction

The Arabidopsis whole-cell metabolic model was constructed with 23 pathways, 259 reactions, and 172 metabolites. Primary metabolic pathways refer to the KEGG (Kyoto Encyclopedia of Genes and Genomes http://www.genome.jp/kegg/ Kanehisa et al., 2008), the AraCyc database (Mueller et al., 2003), and the AraGEM model (Dal'Molin et al., 2010), with manual curation for sphingolipid metabolism, including major ceramide, hydroxyceramide, and glucosylceramide species (Table S1). We used biomass as the objective function and the stoichiometries of major components were assigned to their biomass fraction, which comprises major carbohydrates, amino acids, and lipids, according to experiments or data provided in the literature (Fiehn et al., 2000; Welti et al., 2002; Dal'Molin et al., 2010). For sphingolipid species, the objective function stoichiometries were set to the adjusted isotopic incorporation rate in labeling experiments.

Flux Balance Analysis (FBA)

Flux balance modeling uses a group of ordinary differential equations. The analysis requires a stoichiometric matrix (S) and a vector (v) built for each reaction, where s_{ij} in the S matrix is the stoichiometric number of the ith metabolite in the jth reaction and v_j is the rate of the jth reaction, which is subjected to upper and lower boundary constraints. To reach the *in silico* "quasi-steady state," the following condition must be fulfilled:

$$\text{S} \cdot v = 0$$

After solving the FBA equation with the constraints above (Edwards and Palsson, 2000; Edwards et al., 2001), a linear-programming optimization method (Edwards and Palsson, 2000) was applied to pick the most plausible (groups of) flux distributions among the solution space according to the objective setting.

We applied isotopic incorporation rate as the reference for stoichiometry in the objective function. Considering that the stoichiometries of other components are biomass-derived (from AraGEM, Dal'Molin et al., 2010), we used optimization to find the proper fold-change of all isotopic incorporation rates simultaneously (**Table 2**, the column showing untreated isotopic incorporation rate) of sphingolipids, as their stoichiometries, to make a new model that deviated the least from the optimized steady-state flux distribution from the AraGEM model. Then, we optimized the individual stoichiometry of every sphingolipid species from the results of the first step to get a set of final stoichiometries (**Table 1**).

In Silico SA and BTH Treatments

To incorporate the effect of exogenous SA and BTH on the wild-type plant into the model, we used published microarray data for SA- and BTH-treated *Arabidopsis* (for SA, van Leeuwen et al., 2007; for BTH, Wang et al., 2006). We assumed that the metabolic flux change followed the same trend as the respective gene expression levels. Therefore, we picked genes that changed more than 1.5-fold in SA-treated plants and more than 2-fold in BTH-treated plants (Table S2). Then, the adjusted model was recalculated for optimal flux distribution.

Flux Variability Analysis (FVA)

The stoichiometry model is a self-balancing model in that any flux distributions that fulfill the constraints are involved in its solution space. Through the sampling of the solution space or sensitivity analysis, each reaction is tested for its possible upper flux limit and lower flux limit under constraints (Mahadevan and Schilling, 2003). The calculated range of each flux is an important indicator of the role of the corresponding reaction in the robustness of the whole network. To make a physiologically relevant estimation, we sampled the flux space that achieved at least 80% of the optimal objective rate (in our model, the biomass production) in untreated or treated conditions.

Simulation Environment

The model of *Arabidopsis* was built in SBML (Systems Biology Makeup Language) (Hucka et al., 2003) in XML format. SBML Toolbox 2.0.2 (Keating et al., 2006; Schmidt and Jirstrand, 2006) and COBRA Toolbox 2.0.5 (Schellenberger et al., 2011) in MATLAB 2012a (Mathworks Inc.; Natick, MA) were used for model construction and calculation. Linear programming was performed with GLPK (GNU Linear Programming Kit, http://www.gnu.org/software/glpk/). The rank-test and multiple

TABLE 1 | Overview of sphingolipid species in the FBA model.

Symbol	Sphingolipid species	Pool size (nmol· g $^{-1}$)	Stoichiometry in objective function
d18:0 LCB	Long-chain base	0.2107728	0.050201
d18:1 LCB	Long-chain base	0.0404768	0.017119
t18:0 LCB	Long-chain base	0.280481	0.044619
t18:1 LCB	Long-chain base	0.1117734	8.05E-05
t18:1 c16:0	Long-chain ceramide	0.171892	0.14095
t18:0 c16:0	Long-chain ceramide	0.0097841	0.006289
d18:1 c16:0	Long-chain ceramide	0.0129473	0.017411
d18:0 c16:0	Long-chain ceramide	0.0404391	0.040446
t18:0 c24:0	Very-long-chain ceramide	2.1899963	0.47712
t18:1 c24:0	Very-long-chain ceramide	3.766825	0.775466
t18:0 c24:1	Very-long-chain ceramide	0.587771	0.119545
t18:1 c24:1	Very-long-chain ceramide	1.2656188	0.344293
t18:0 c26:0	Very-long-chain ceramide	0.7455185	0.129493
t18:1 c26:0	Very-long-chain ceramide	3.6843313	0.671015
t18:0 c26:1	Very-long-chain ceramide	0.0407943	0.005744
t18:1 c26:1	Very-long-chain ceramide	0.8207395	0.208064
t18:1 h160	Long-chain hydroxyceramide	0.8007893	0.154383
t18:0 h160	Long-chain hydroxyceramide	0.0852554	0.012748
d18:1 h16:0	Long-chain hydroxyceramide	0.0439154	0.020931
d18:0 h16:0	Long-chain hydroxyceramide	0.0365444	0.019623
t18:0 h24:0	Very-long-chain hydroxyceramide	1.2986488	0.01712
t18:1 h24:0	Very-long-chain hydroxyceramide	10.114958	1.148618
t18:0 h24:1	Very-long-chain hydroxyceramide	1.0769261	0.124845
t18:1 h24:1	Very-long-chain hydroxyceramide	0.0211909	1.53E-05
t18:0 h26:0	Very-long-chain hydroxyceramide	0.4134975	0.003149
t18:1 h26:0	Very-long-chain hydroxyceramide	2.2138763	0.218833
t18:0 h26:1	Very-long-chain hydroxyceramide	0.1257489	9.05E-05
t18:1 h26:1	Very-long-chain hydroxyceramide	1.268245	0.27478
t18:1 h16:0	Long-chain glucosylceramide	0.9171223	0.03589
t18:0 h16:0	Long-chain glucosylceramide	1.25E-06	9.00E-10
d18:1 h16:0	Long-chain glucosylceramide	2.908355	0.177984
d18:0 h16:0	Long-chain glucosylceramide	0.0239498	0.001506
t18:0 h24:0	Very-long-chain glucosylceramide	0.1940488	0.00014
t18:1 h24:0	Very-long-chain glucosylceramide	1.8239438	0.055296
t18:0 h24:1	Very-long-chain glucosylceramide	1.25E-06	9.00E-10
t18:1 h24:1	Very-long-chain glucosylceramide	2.1610275	0.057862
t18:0 h26:0	Very-long-chain glucosylceramide	1.25E-06	9.00E-10
t18:1 h26:0	Very-long-chain glucosylceramide	1.0588451	0.032563
t18:0 h26:1	Very-long-chain glucosylceramide	1.25E-06	9.00E-10
t18:1 h26:1	Very-long-chain glucosylceramide	0.7133198	0.016164

covariance analysis were performed using IBM SPSS Statistics 19 (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.).

Results

Model Construction for Plant Sphingolipid Metabolism

We used computational modeling and experiments to explore the changes in plant sphingolipid metabolism in response to SA. Although sphingolipids function as important components in plant development and stress responses, their metabolism remains obscure, with only a few network parameters that have been measured. FBA is well-suited to the simulation of a metabolic fluxome with poorly understood dynamics (Varma and Palsson, 1994), as optimization by FBA requires only the stoichiometric relationship in each reaction and the objective function. In our model, we obtained the numbers of molecules of reactants and products of known reactions from public databases (see Materials and Methods). For sphingolipid pathways (Table

S1), we inferred the reactions that have not been determined from their atomic composition or similar reactions. Considering that metabolic balances are mainly affected by a few metabolites that are either in a hub of the network or have high turnover, we picked the sphingolipid species that are relatively abundant or central to the known network (**Table 1**). Since inositolphosphorylceramide and its derivatives are difficult to measure in plants, we excluded those species from our model.

^{15}N-Labeled Metabolic Turnover Analysis of Sphingolipids

To inform the objective function and to validate the model's prediction, we used the *in vivo* fluxomic method of ^{15}N-labeled metabolic turnover analysis to directly measure the turnover rate of plant sphingolipids. In previous work, ^{13}C was mostly used to examine the fluxome of central pathways such as glucose metabolism or photosynthesis (Hasunuma et al., 2010; Noack et al., 2010; Nöh and Wiechert, 2011), where limited numbers of labeled fragments are detected by mass spectrometry. However, the simplest sphingolipid has at least 18 carbon atoms, and their combined transitions, modifications, and fragmentation would generate large numbers of labeled fragments; therefore mass spectrometry quantification of ^{13}C-labeled sphingolipid would be extremely difficult. To circumvent this difficulty, we used ^{15}N, which will label only the single nitrogen atom in the head of each sphingolipid. To distinguish between artificial and natural ^{15}N, we measured the composition of natural ^{15}N sphingolipid in unlabeled samples, finding different levels of natural ^{15}N in each sphingolipid species. This fraction is constant between measurements and treatments in each species, and thus cannot affect the comparison of isotopic incorporation rates between experiments.

We transiently labeled 10-day-old seedlings in a time course. The isotopic incorporation curves (see representative species shown in **Figure 1**) reveal that the labeled serine is absorbed and incorporated into sphingolipid in the first hour of labeling, and the sphingolipid then undergoes turnover at a uniform rate. For LCB (**Figure 1D**), ceramide (**Figure 1A**), and hydroxyceramide species (**Figure 1B**), the isotopic incorporation curves gradually flatten and finally reach a plateau of the isotopic fraction between 9 and 24 h. A noticeable, small drop occurs around the 5th hour of incorporation in LCB (**Figure 1D**). The incorporation of ^{15}N in these simple sphingolipids is fast, and the final balanced isotopic fraction can reach 40–65% (**Figures 1A,B,D**). By contrast, for the glucosylceramides the labeled fraction rose constantly between 9 and 24 h (**Figure 1C**), and the glucosylceramides had a lower rate of incorporation than the ceramides or hydroxyceramides. Combined with the concentration of sphingolipids, we calculated the isotopic incorporation rate as shown in **Table 2**.

Flux Balance Analysis (FBA) of the Flux Distribution in Untreated Plants

The objective function in the FBA model guides the flux determination by simulating a transient flux distribution. However, at each time point, biomass is the complex result of development throughout the organism's life, and hence cannot provide relevant information for setting the objective function in our model of the *Arabidopsis* seedling. Instead, we built and adjusted the objective function stoichiometries of the sphingolipid pathway from the isotopic incorporation rates in the labeling experiments (**Table 1**). Then, we performed flux balance optimization. **Figure 2** shows the simulated flux distributions of sphingolipid species in untreated plants.

The simulation data in **Figure 2** show that LCBs, very-long-chain ceramides, and hydroxyceramides compose the highest fraction of total flux. Combined with the rapid isotopic incorporation and high fraction of stabilized isotopic final levels of LCB, ceramides, and hydroxyceramides (**Figure 1**), the results demonstrate that LCBs, the sphingolipids that have the smallest pool size, also have the highest turnover among plant sphingolipids. Very-long-chain ceramides and hydroxyceramides are important not only for their hub position connecting glucosylceramides and sphingosine, but also because they carry a huge flux throughput in sphingolipid turnover and thus help maintain sphingolipid homeostasis. Both the simulation and experimental results indicate that these sphingolipid species are probably more responsive to disturbance, and thus are frequently used by pathogens to manipulate or interfere with host sphingolipid metabolism (Markham et al., 2011; Bi et al., 2014).

Although the glucosylceramides have much larger pool sizes (**Table 1**) than the ceramides, hydroxyceramides, or LCBs, they have smaller metabolic fluxes than their precursors (**Figure 2**). These results are validated by the slow but lasting incorporation of isotope into glucosylceramide pools (**Figure 1C**). The relatively slow turnover is in accordance with the function of glucosylceramides as membrane structural components, indicating a slow but continuous accumulation in the cell membrane during plant development. The accordance of simulation and experimental results also supports our choice of objective function stoichiometry setting, as the scale of simulated and measured sphingolipid metabolic flux distribution (**Figure 2** and **Table 2**) is nearly unrelated to the distribution of sphingolipid biomass (**Table 1**).

In Silico SA and BTH Treatments

The FBA model hypothesizes the quasi-steady state condition of the target network, and we assume that the sphingolipid pathway will reach at least a transient metabolic balance after SA treatment. Thus, we employed the previous model simulating the resting state to predict the effects of SA treatment. We first used data from microarray analysis of SA- and BTH-treated plants to simulate the effect of these treatments on sphingolipid flux. Sphingolipid-related genes were selected (see Method) from two microarrays (Table S2). *LAG 1 HOMOLOG 2* (*LOH2*), which encodes a ceramide synthase (Brandwagt et al., 2000; Ternes et al., 2011), showed the highest up-regulation after both SA and BTH treatments, and other genes *SPHINGOID BASE HYDROXYLASE 2* (*SBH2*), *FATTY ACID/SPHINGOLIPID DESATURASE* (*SLD*), *FATTY ACID HYDROXYLASE 2* (*FAH2*), *SPHINGOSINE-1-PHOSPHATE LYASE* (*AtDPL1*) also had different expression levels in the two treatments. The reactions regulated by the genes with altered transcript levels were then picked for incorporation into the model. The flux boundaries of these reactions were

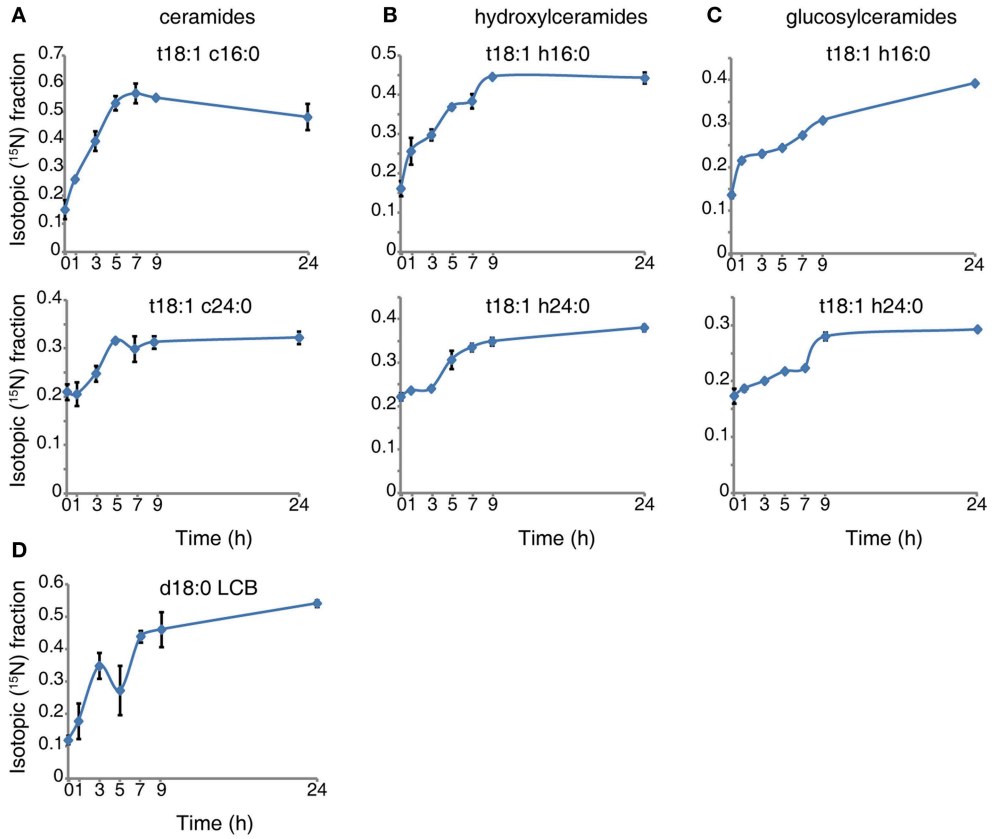


FIGURE 1 | ^{15}N incorporation curves for sphingolipid species. Ten-day-old wild-type seedlings were transferred to 5 mM ^{15}N- serine labeled N-deficient 1/2x MS liquid medium for the indicated times. Sphingolipids were then extracted and measured as described in Methods. The ^{15}N fraction incorporation curve was calculated based on the formula shown in Methods. Error bars represent the means ± SE from triplicate biological repeats. The measured sphingolipid species were: ceramide **(A)**, hydroxyceramide **(B)**, gluocosylceramide **(C)** and LCB **(D)**. LCB and fatty acid in ceramide species represent, LCB; d/t (di/trihydroxy) 18 (18 carbon chain), 1 (one desaturation) followed by fatty acid; c/h/g (non-hydroxyl/hydroxyl/glucosy and hydroxyl) 24 (24 carbon chain), 0 (no desaturations).

altered based on the gene expression, and the adjusted model was recalculated for flux balance analysis.

Compared with the model simulating the resting state, *in silico* SA and BTH treatments resulted in a nearly three-fold increase of predicted flux in long-chain ceramide species (**Figure 2**), as expected from the up-regulation of *LOH2* in the microarray data. In particular, simulated SA and BTH treatment both produced a significant rise in predicted metabolism of trihydroxy glucosylceramides. This increase was not specific to fatty acid species, which showed an increase in both trihydroxy long-chain and very-long-chain glucosylceramides (**Figure 2**). These results are consistent with the data from ^{15}N-labeled metabolic turnover analysis (**Table 2**). Interestingly, the microarray data showed no significant changes in genes that directly catalyze the pathways in glucosylceramide metabolism, nor any related to glucosylceramide, in response to SA or BTH treatment (Table S2). Considering the down-regulation of *SBH2* under BTH treatment, we believe that the increase of glucosylceramide metabolism may mainly be induced by the upstream up-regulation of *LOH2*. Since the increase of the turnover rate was not linked to metabolite concentration, the predicted changes of glucosylceramides are almost negligible by typical quantitative LC-MS/MS measurement, but the increase in lipid renewal may have indispensible functions in the sensitivity of membrane-based cell signaling.

In this simulation, although some genes change differently in response to SA and BTH treatment (Table S2), SA and BTH have similar effects on sphingolipid metabolism. Our model also proposes a possible mechanism by which BTH affects the network under flux balance constraint without mimicking all the gene expression changes of its counterpart.

^{15}N-Labeled Metabolic Turnover Measurement of the Effect of SA and BTH

To confirm the predictions of the model, we directly measured the *in vivo* flux change in response to SA and BTH treatments. For SA and BTH treatments, the isotope incorporation rate significantly increased for certain sphingolipid species such as C16 and C26 ceramides (**Table 2**). These results are consistent with our FBA model (**Figure 2**). After SA and BTH treatments, turnover increased for seven out of twenty-two and ten out of twenty-two major sphingolipids, respectively. Also, turnover

TABLE 2 | Isotopic incorporation rate for major sphingolipids, with or without 100 μM SA or 100 μM BTH treatments.

Symbol	Sphingolipid species	Isotope incorporation rate (nmol· g^{-1} ·h^{-1}) untreated	Isotope incorporation rate (nmol· g^{-1} ·h^{-1}) SA-treated	Isotope incorporation rate (nmol· g^{-1} ·h^{-1}) BTH-treated
d18:0 LCB	Long-chain base	0.062022	0.055779	0.038494#
d18:1 LCB	Long-chain base	0.005016	**0.059469***	**0.031829***
t18:0 LCB	Long-chain base	0.030297	0.049577	0.023784
t18:1 LCB	Long-chain base	1.43E-02	**8.94E-06#**	**5.44E-04#**
t18:1 c16:0	Long-chain ceramide	0.100845	0.241159*	0.221878*
d18:0 c16:0	Long-chain ceramide	0.04256	0.066754*	0.0477
t18:0 c24:0	Very-long-chain ceramide	0.386836	0.495358	0.505011*
t18:1 c24:0	Very-long-chain ceramide	0.418402	0.60068*	0.538219
t18:0 c24:1	Very-long-chain ceramide	0.217738	**0.144568#**	**0.176221**
t18:1 c24:1	Very-long-chain ceramide	0.485274	0.500902	0.547493
t18:0 c26:0	Very-long-chain ceramide	0.049354	0.048909	0.031827
t18:1 c26:0	Very-long-chain ceramide	0.136971	0.179349	0.184011*
t18:1 c26:1	Very-long-chain ceramide	3.44E-02	5.44E-02*	6.98E-02*
t18:1 h16:0	Long-chain hydroxyceramide	0.268339	0.253601	0.177361#
t18:1 h24:0	Very-long-chain hydroxyceramide	1.25246	1.139387	0.965043
t18:0 h24:1	Very-long-chain hydroxyceramide	0.092809	0.13231	**0.167954***
t18:1 h26:0	Very-long-chain hydroxyceramide	0.157256	**0.200213***	0.183134
t18:1 h26:1	Very-long-chain hydroxyceramide	1.86E-01	1.06E-01*	1.29E-01
d18:1 h16:0	Long-chain glucosylceramide	0.142007	0.126636	**0.199323***
t18:1 h24:0	Very-long-chain glucosylceramide	0.076921	0.13433*	0.265554*
t18:1 h24:1	Very-long-chain glucosylceramide	0.073858	0.076487	**0.15701***
t18:1 h26:0	Very-long-chain glucosylceramide	0.040668	0.053585	**0.060641***

** and # indicate significant up and down, respectively ($P < 0.05$, FDR < 0.05 in multiple covariance analysis) of incorporation rate compared to untreated plants. The bold numbers are in disagreement with simulation data shown in **Figure 2**.*

decreased for two out of twenty-two and three out of twenty-two major sphingolipids after SA and BTH treatments, respectively. We found that the few inconsistencies between *in silico* predictions (**Figure 2**) and experimental data (**Table 2**) mainly came from LCB and glucosylceramides. Given the low *in vivo* level of LCB and the high variability of LCB measurement, the inconsistency of LCB turnover could result from experimental error. Interestingly, we found discrepancies between the effect of BTH and SA on glucosylceramide turnover. For example, the isotope incorporation rate significantly increased for glucosylceramides after BTH treatments (**Table 2**), indicating that it may underlie different mechanisms in the responses to BTH and SA.

Flux Variability Analysis

To examine the change in network rigidity in response to SA and BTH treatments, we estimated the accessible flux ranges of sphingolipid species *in silico*. To make a physiologically relevant estimation, we sampled the flux space that achieved at least 80% of the optimal objective rate (in our model, the biomass production) under untreated or treated conditions. We sorted the flux range into three types (Oberhardt et al., 2010): rigid flux (flux range near zero but with non-zero flux value), bounded flexible flux, and infinitely flexible flux (boundary spans from 0 or -1000 to 1000 in the model). In the fluxome of treated and untreated plants, LCB fluxes were infinitely flexible (showing a high capacity to tolerate disturbance), ceramide and glycosylceramide fluxes showed bounded flexibility, and hydroxyceramide fluxes were rigid (**Table 3**). The limited flux variability of most sphingolipids is consistent with stoichiometric modeling result in *S. cerevisiae* (Ozbayraktar and Ulgen, 2011). Similar to the isotopic incorporation experiments, we found disturbances of flux variability in ceramide and glucosylceramide metabolic fluxes after SA and BTH treatments, indicating that plant cells have the freedom to adjust their sphingolipid flux homeostasis during defense processes.

Discussion

Our FBA model and isotope labeling experiments systematically explored the alterations in the sphingolipid pathway that occur in response to SA and BTH. Traditional metabolic responses can cause significant changes in the concentrations of certain metabolites. However, the systematic responses caused by plant activators and phytohormones cannot be achieved by only doubling the concentration of certain nodes; these responses also affect the dynamic properties of the whole network. To detect the underlying changes of network parameters caused by the modulation, both up and down, of certain nodes, one of the most direct measurements is the fluxome. FBA analysis has been applied in microbial metabolic engineering and

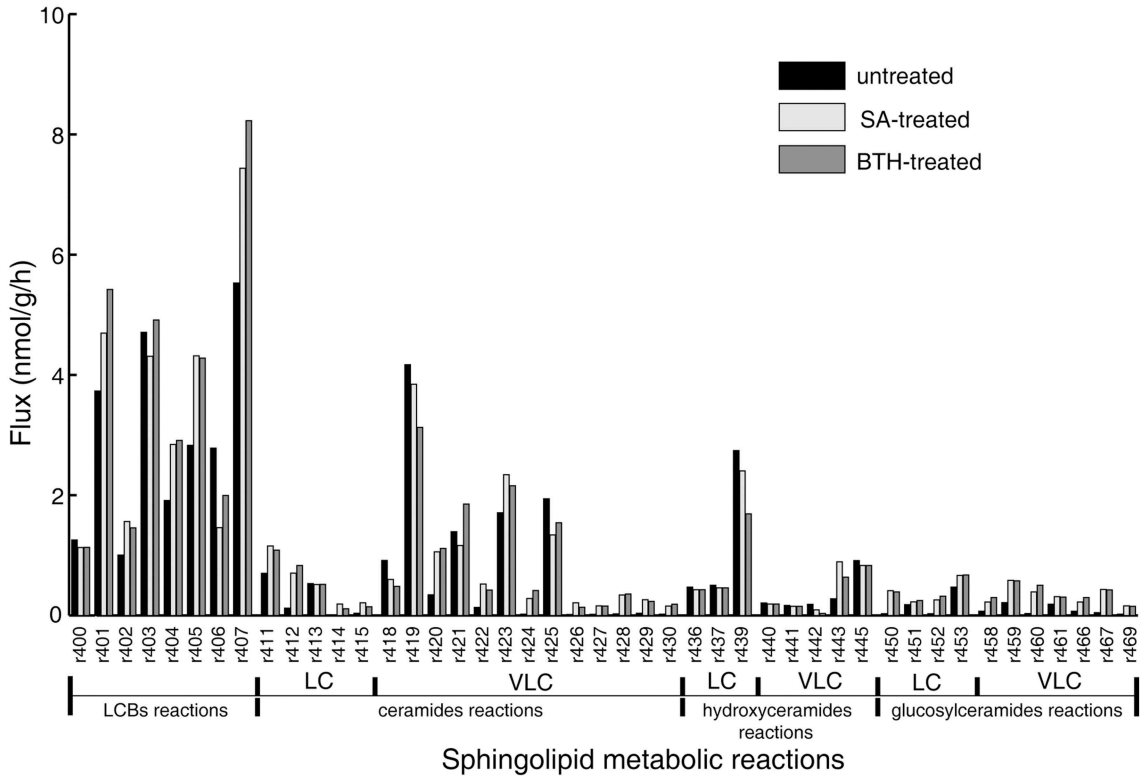


FIGURE 2 | Simulated flux distribution of selected sphingolipid species. The untreated plants (black) and *in silico* SA (light gray) and BTH-treated plants (gray) were taken from the flux balance model. The effects of exogenous SA and BTH were simulated by changing the target flux bound proportional to its related gene expression alteration identified by published microarray data (Wang et al., 2006; van Leeuwen et al., 2007). LC, long-chain (≤C18); VLC: very-long-chain (>C18).

modeling of other systems. However, construction of the model for sphingolipid metabolism presented difficulties related to the unique features of sphingolipid pathways. Although sphingolipid species are among the most reactive components in plant development and stress responses, they reside in the periphery of the network of plant metabolism, having loose metabolic connections with other subnetworks. Their lack of connection and remote position make the flux in the self-balanced function more susceptible to the objective settings, rather than being affected by artificial constraints and neighboring subnetworks.

Indeed, studies of sphingolipids in *S. cerevisiae* (Ozbayraktar and Ulgen, 2011) found that the sphingolipid pathways are also remote from central metabolism, but these models are backed by experimental data on enzyme kinetic parameters or known fluxes. Experimental exploration of plant sphingolipid pathways has been hindered by the vast diversity, low abundance, and lack of sensitive and replicable measurements of sphingolipids. In addition, the enzymes linking metabolites often are embedded in the layers of membranes, making the isolation and estimation of their kinetic properties difficult. Until now, a limited set of experiments has revealed only a rough sketch of plant sphingolipid metabolism. Considering that, we used the experimentally measured isotopic incorporation rate to set the stoichiometry of each sphingolipid species in the objective function, and we found that the resulting flux distribution of each species was in accordance with the isotopic incorporation pattern, demonstrating that isotopic incorporation data produce a better fit than biomass fraction in objective stoichiometry determination, as the maximization of biomass is often considered as the aim of plant metabolism regardless of any inconsistency between biomass contents and the generation rate of each component.

In our experiments, isotopic transient labeling provided a direct measurement of *in vivo* flux. We note that none of the sphingolipid species reached 100% labeled. Similar phenomena were also observed in other experiments (Delwiche and Sharkey, 1993; Hasunuma et al., 2010). Considering the internal serine sources and anaplerotic reactions of complex existing sphingolipids, the pattern indicates a balance of labeled and unlabeled sphingolipids in the metabolic pool. Since the only exogenous source of nitrogen is labeled, we can also speculate that sphingolipid synthesis uses external and internal sources of nitrogen, based on the isotopic incorporation curve.

There are various models linking plant sphingolipid pathways with hormones and their synergistic roles in plant development and stress responses. In these models, the possible sphingolipid inducers of defense responses include LCBs (Saucedo-García et al., 2011) and ceramides (Markham et al., 2011; Bi et al., 2014),

TABLE 3 | Simulated flux variability of sphingolipid-related reactions in untreated and SA-treated plants.

Reaction ID	Reaction property	Flux range of untreated plant (nmol/g/h)	Flux category	Flux range of *in silico* SA-treated plant (nmol/g/h)	Flux category	Flux range of *in silico* BTH-treated plant (nmol/g/h)	Flux category
r400	LCB synthesis	0.6816942	BF	0.933054	BF	0.7323672	BF
r401	LCB hydroxylation	996.1271	IF	990.0517	IF	940.56259	IF
r402	LCB desaturation	891.39928	IF	633.8894	IF	588.71726	IF
r403	LCB desaturation	744.41581	IF	858.1732	IF	898.85107	IF
r404	LCB hydroxylation	555.71359	IF	740.9661	IF	602.70542	IF
r405	LCB degradation	965.6077	IF	962.8005	IF	757.88787	IF
r406	LCB degradation	669.54014	IF	678.1669	IF	572.0321	IF
r407	LCB degradation	961.18543	IF	981.1921	IF	985.3995	IF
r408	LCB degradation	0.6124747	BF	0.886632	BF	0.6845704	BF
r409	LCB degradation	0.6124747	BF	0.886632	BF	0.6845704	BF
r410	Long-chain ceramide synthesis	2.7399016	BF	1.488821	BF	2.5018621	BF
r411	Long-chain ceramide synthesis	1.3103871	BF	1.71169	BF	1.9698516	BF
r412	Long-chain ceramide synthesis	1.7084488	BF	1.822309	BF	2.0970869	BF
r413	Long-chain ceramide synthesis	3.3413012	BF	2.752579	BF	2.0658496	BF
r414	Long-chain ceramide degradation	2.739888	BF	1.488661	BF	2.501841	BF
r415	Long-chain ceramide degradation	1.3095469	BF	1.70886	BF	1.9655798	BF
r416	Long-chain ceramide degradation	1.7077694	BF	1.821901	BF	2.097133	BF
r417	Long-chain ceramide degradation	3.3422485	BF	2.75117	BF	2.0645697	BF
r418	Very-long-chain ceramide synthesis	4.3578539	BF	7.715646	BF	4.3136348	BF
r419	Very-long-chain ceramide synthesis	8.7817641	BF	5.694421	BF	6.7077636	BF
r420	Very-long-chain ceramide synthesis	3.5295194	BF	2.408687	BF	2.9528709	BF
r421	Very-long-chain ceramide synthesis	4.2127446	BF	3.453244	BF	5.0346985	BF
r422	Very-long-chain ceramide synthesis	4.608139	BF	3.854737	BF	3.4107203	BF
r423	Very-long-chain ceramide synthesis	5.6709963	BF	7.263345	BF	6.1313472	BF
r424	Very-long-chain ceramide synthesis	3.5244325	BF	3.770128	BF	4.3695179	BF
r425	Very-long-chain ceramide synthesis	4.6239162	BF	3.720505	BF	3.7953091	BF
r426	Very-long-chain ceramide degradation	2.1905924	BF	4.899526	BF	2.6481642	BF
r427	Very-long-chain ceramide degradation	1.9294142	BF	1.400288	BF	2.3380799	BF
r428	Very-long-chain ceramide degradation	2.7824852	BF	2.919854	BF	2.5284588	BF
r429	Very-long-chain ceramide degradation	6.2532973	BF	3.182878	BF	2.3868717	BF
r430	Very-long-chain ceramide degradation	2.7924997	BF	3.175204	BF	3.2417941	BF
r431	Very-long-chain ceramide degradation	1.654875	BF	3.178278	BF	2.1331369	BF
r432	Very-long-chain ceramide degradation	3.7449578	BF	3.378223	BF	4.2554344	BF
r433	Very-long-chain ceramide degradation	2.7185461	BF	4.234617	BF	4.8273498	BF
r434	Ceramide LCB-hydroxylation	3.2847564	BF	3.234191	BF	4.0981726	BF
r435	Ceramide LCB-hydroxylation	2.3948574	BF	3.112839	BF	2.9876163	BF
r436	Long-chain ceramide alpha-hydroxylation	0.0002983	RF	0.000267	RF	0.0002828	RF
r437	Long-chain ceramide alpha-hydroxylation	0.0065064	RF	0.005832	RF	0.0061698	RF
r438	Long-chain ceramide alpha-hydroxylation	0.0006397	RF	0.000573	RF	0.0006066	RF
r439	Long-chain ceramide alpha-hydroxylation	0.0069564	RF	0.006235	RF	0.0065965	RF
r440	Very-long-chain ceramide alpha-hydroxylation	4.7098229	BF	3.792224	BF	3.5955032	BF
r441	Very-long-chain ceramide alpha-hydroxylation	9.5915915	BF	5.22986	BF	6.284673	BF
r442	Very-long-chain ceramide alpha-hydroxylation	0.0028754	RF	0.002577	RF	0.0027266	RF
r443	Very-long-chain ceramide alpha-hydroxylation	0.0023156	RF	0.002075	RF	0.0021958	RF
r444	Very-long-chain ceramide alpha-hydroxylation	4.7663799	BF	4.426039	BF	5.1332466	BF
r445	Very-long-chain ceramide alpha-hydroxylation	6.2567661	BF	5.436772	BF	6.3043047	BF
r446	Very-long-chain ceramide alpha-hydroxylation	2.916E-06	RF	2.61E-06	RF	2.765E-06	RF
r447	Very-long-chain ceramide alpha-hydroxylation	0.0126184	BF	0.01131	BF	0.0119656	BF
r448	Long-chain hydroxylceramide glucosylation	2.4344115	BF	2.286215	BF	4.6218412	BF

(Continued)

TABLE 3 | Continued

Reaction ID	Reaction property	Flux range of untreated plant (nmol/g/h)	Flux category	Flux range of *in silico* SA-treated plant (nmol/g/h)	Flux category	Flux range of *in silico* BTH-treated plant (nmol/g/h)	Flux category
r449	Long-chain hydroxylceramide glucosylation	1.6341334	BF	2.627672	BF	1.7824886	BF
r450	Long-chain hydroxylceramide glucosylation	1.592099	BF	1.690888	BF	2.2631503	BF
r451	Long-chain hydroxylceramide glucosylation	1.9261375	BF	1.513117	BF	2.5673956	BF
r452	Long-chain glucosylceramide degradation	2.4344115	BF	2.286215	BF	4.6218412	BF
r453	Long-chain glucosylceramide degradation	1.634039	BF	2.627359	BF	1.7825829	BF
r454	Long-chain glucosylceramide degradation	1.5920983	BF	1.690883	BF	2.2631602	BF
r455	Long-chain glucosylceramide degradation	1.9267482	BF	1.513729	BF	2.568496	BF
r456	Very-long-chain hydroxylceramide glucosylation	3.118642	BF	2.280832	BF	1.7163731	BF
r457	Very-long-chain hydroxylceramide glucosylation	1.9581782	BF	3.500058	BF	2.1010147	BF
r458	Very-long-chain hydroxylceramide glucosylation	1.8737974	BF	2.168017	BF	1.6308077	BF
r459	Very-long-chain hydroxylceramide glucosylation	1.865647	BF	2.35413	BF	2.1378746	BF
r460	Very-long-chain hydroxylceramide glucosylation	2.2127127	BF	1.990514	BF	3.1107668	BF
r461	Very-long-chain hydroxylceramide glucosylation	1.9563111	BF	2.108021	BF	1.9282944	BF
r462	Very-long-chain hydroxylceramide glucosylation	2.773781	BF	2.214492	BF	2.2287123	BF
r463	Very-long-chain hydroxylceramide glucosylation	2.3197591	BF	2.983733	BF	4.8624845	BF
r464	Very-long-chain glucosylceramide degradation	3.1186404	BF	2.280831	BF	1.7163742	BF
r465	Very-long-chain glucosylceramide degradation	1.9582823	BF	3.49983	BF	2.1013099	BF
r466	Very-long-chain glucosylceramide degradation	1.8737974	BF	2.168017	BF	1.6308077	BF
r467	Very-long-chain glucosylceramide degradation	1.8649995	BF	2.353971	BF	2.1378732	BF
r468	Very-long-chain glucosylceramide degradation	2.2127127	BF	1.990514	BF	3.1107668	BF
r469	Very-long-chain glucosylceramide degradation	1.9565562	BF	2.107267	BF	1.9284395	BF
r470	Very-long-chain glucosylceramide degradation	2.773781	BF	2.214492	BF	2.2287123	BF
r471	Very-long-chain glucosylceramide degradation	2.3197341	BF	2.983678	BF	4.862662	BF

We used the criteria described by Oberhardt et al. (2010) to classify different reaction fluxes based on their flux ranges. RF represents Rigid Flux; IF represents Infinitely Flexible flux; BF represents Bounded Flexible flux.

with SA both up- and downstream of sphingolipid-mediated PCD (Saucedo-García et al., 2011; Bi et al., 2014). As mutants affecting sphingolipids often accumulate SA, the effect of SA on ceramide species may include positive feedback on the imbalance of sphingolipids. Our results are in accordance with the observed frequent variation in the concentration of LCB and sometimes ceramide, and the reduced variation in the concentrations of hydroxyceramide and glucosylceramide in wild-type *Arabidopsis*. Functionally speaking, since LCB and ceramides are fundamental to sphingolipid metabolism and show high flexibility in their flux, they can be more responsive to stimuli such as SA or BTH without disrupting the total fluxomic balance of sphingolipid metabolism.

In a living cell, the synthesis and degradation of all substances occurs through metabolism. However, current research tends to separate metabolites and functional molecules. The most exciting aspect of plant sphingolipids is that they are metabolites and functional molecules. Our current model only deals with their metabolic properties in a self-balanced manner. It will be interesting to incorporate the signaling network that involves sphingolipids to build an integrated model that can consider the direct effect of metabolism on cell signaling.

Conclusion

In this study, we established a sphingolipid FBA model and used ^{15}N-labeled isotopic transient labeling to systematically explore the effects of SA and BTH on sphingolipid metabolic pathways. The results show that increases in ceramide and glucosylceramide flux occur in response to exogenous SA and BTH and that alteration of their flux variability also occurs. Our results also give us insights that help explain the mechanism of crosstalk between SA and sphingolipids, and their roles in the plant defense response.

Acknowledgments

We thank members in the Yao and Ren laboratories for assistance with this work. This work was supported by the National Key Basic Science 973 Program (2012CB114006), National Natural Science Foundation of China (31170247), and the National Basic Scientific Training Funds (J1310025).

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2015.00186/abstract

References

Allen, D. K., Libourel, I. G. L., and Shachar-Hill, Y. (2009). Metabolic flux analysis in plants: coping with complexity. *Plant Cell Environ.* 32, 1241–1257. doi: 10.1111/j.1365-3040.2009.01992.x

Bi, F.-C., Liu, Z., Wu, J.-X., Liang, H., Xi, X.-L., Fang, C., et al. (2014). Loss of ceramide kinase in *Arabidopsis* impairs defenses and promotes ceramide accumulation and mitochondrial H_2O_2 bursts. *Plant Cell* 26, 3449–3467. doi: 10.1105/tpc.114.127050

Brandwagt, B. F., Mesbah, L. A., Takken, F. L., Laurent, P. L., Kneppers, T. J., Hille, J., et al. (2000). A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumonisin B1. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4961–4966. doi: 10.1073/pnas.97.9.4961

Brodersen, P., Petersen, M., Pike, H. M., Olszak, B., Skov, S., Oedum, N., et al. (2002). Knockout of *Arabidopsis* ACCELERATED CELL-DEATH11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes Dev.* 16, 490–502. doi: 10.1101/gad.218202

Chen, M., Cahoon, E. B., Saucedo-García, M., Plasencia, J., and Gavilanes-Ruíz, M. (2009). "Plant sphingolipids: structure, synthesis and function," in *Lipids in Photosynthesis: Essential and Regulatory Functions*, eds H. Wada and N. Murata (Dordrecht: Springer), 77–115.

Dal'Molin, C. G. D., Quek, L., Palfreyman, R. W., Brumbley, S. M., and Nielsen, L. K. (2010). AraGEM, a genome-scale reconstruction of the primary metabolic network in *Arabidopsis*. *Plant Physiol.* 152, 579–589. doi: 10.1104/pp.109.148817

Delwiche, C. F., and Sharkey, T. D. (1993). Rapid appearance of ^{13}C in biogenic isoprene when $^{13}CO2$ is fed to intact leaves. *Plant Cell Environ.* 16, 587–591. doi: 10.1111/j.1365-3040.1993.tb00907.x

Durner, J., and Klessig, D. F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 92, 11312–11316. doi: 10.1073/pnas.92.24.11312

Durner, J., and Klessig, D. F. (1996). Salicylic acid is a modulator of tobacco and mammalian catalases. *J. Biol. Chem.* 271, 28492–28501. doi: 10.1074/jbc.271.45.28492

Edwards, J. S., Ibarra, R. U., and Palsson, B. O. (2001). *In silico* predictions of *Escherichia coli* metabolic capabilities are consistent with experimental data. *Nat. Biotechnol.* 19, 125–130. doi: 10.1038/84379

Edwards, J. S., and Palsson, B. O. (2000). The *Escherichia coli* MG1655 *in silico* metabolic genotype: its definition, characteristics, and capabilities. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5528–5533. doi: 10.1073/pnas.97.10.5528

Fiehn, O., Kopka, J., Dörmann, P., Altmann, T., Trethewey, R. N., and Willmitzer, L. (2000). Metabolite profiling for plant functional genomics. *Nat. Biotechnol.* 18, 1157–1161. doi: 10.1038/81137

Greenberg, J. T., Silverman, F. P., and Liang, H. (2000). Uncoupling salicylic acid dependent cell death and defense-related responses from disease resistance in the *Arabidopsis* mutant *acd5*. *Genetics* 156, 341–350.

Hannun, Y. A., and Obeid, L. M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat. Rev.* 9, 139–150. doi: 10.1038/nrm2329

Hasunuma, T., Harada, K., Miyazawa, S., Kondo, A., Fukusaki, E., and Miyake, C. (2010). Metabolic turnover analysis by a combination of *in vivo* ^{13}C-labelling from $^{13}CO2$ and metabolic profiling with CE-MS/MS reveals rate-limiting steps of the C3 photosynthetic pathway in *Nicotiana tabacum* leaves. *J. Exp. Bot.* 61, 1041–1051. doi: 10.1093/jxb/erp374

Hirner, A., Ladwig, F., Stransky, H., Okumoto, S., Keinath, M., Harms, A., et al. (2006). Arabidopsis LHT1 is a high-affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *Plant Cell* 18, 1931–1946. doi: 10.1105/tpc.106.041012

Hucka, M., Finney, A., Sauro, H. M., Bolouri, H., Doyle, J. C., Kitano, H., et al. (2003). The systems biology markup language (SBML): a medium for representation and exchange of biochemical network models. *Bioinformatics* 19, 524–531. doi: 10.1093/bioinformatics/btg015

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., et al. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36, D480–D484. doi: 10.1093/nar/gkm882

Keating, S. M., Bornstein, B. J., Finney, A., and Hucka, M. (2006). SBMLToolbox: an SBML toolbox for MATLAB users. *Bioinformatics* 22, 1275–1277. doi: 10.1093/bioinformatics/btl111

König, S., Feussner, K., Schwarz, M., Kaever, A., Iven, T., Landesfeind, M., et al. (2012). *Arabidopsis* mutants of sphingolipid fatty acid a-hydroxylases accumulate ceramides and salicylates. *New Phytol.* 196, 1086–1097. doi: 10.1111/j.1469-8137.2012.04351.x

Kruger, N. J., Masakapalli, S. K., and Ratcliffe, R. G. (2012). Strategies for investigating the plant metabolic network with steady-state metabolic flux analysis: lessons from an *Arabidopsis* cell culture and other systems. *J. Exp. Bot.* 63, 2309–2323. doi: 10.1093/jxb/err382

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., et al. (1996). Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of systemic acquired resistance signal transduction pathway. *Plant J.* 10, 71–82. doi: 10.1046/j.1365-313X.1996.10010071.x

Liang, H., Yao, N., Song, J. T., Luo, S., Lu, H., and Greenberg, J. T. (2003). Ceramides modulate programmed cell death in plants. *Genes Dev.* 17, 2636–2641. doi: 10.1101/gad.1140503

Libourel, I. G. L., and Shachar-Hill, Y. (2008). Metabolic flux analysis in plants: from intelligent design to rational engineering. *Annu. Rev. Plant Biol.* 59, 625–650. doi: 10.1146/annurev.arplant.58.032806.103822

Lonien, J., and Schwender, J. (2009). Analysis of metabolic flux phenotypes for two *Arabidopsis* mutants with severe impairment in seed storage lipid synthesis. *Plant Physiol.* 151, 1617–1634. doi: 10.1104/pp.109.144121

Mahadevan, R., and Schilling, C. H. (2003). The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. *Metab. Eng.* 5, 264–276. doi: 10.1016/j.ymben.2003.09.002

Markham, J. E., Lynch, D. V., Napier, J. A., Dunn, T. M., and Cahoon, E. B. (2013). Plant sphingolipids: function follows form. *Curr. Opin. Plant Biol.* 16, 350–357. doi: 10.1016/j.pbi.2013.02.009

Markham, J. E., Molino, D., Gissot, L., Bellec, Y., Hématy, K., Marion, J., et al. (2011). Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in *Arabidopsis*. *Plant Cell* 23, 2362–2378. doi: 10.1105/tpc.110.080473

Masakapalli, S. K., Le Lay, P., Huddleston, J. E., Pollock, N. L., Kruger, N. J., and Ratcliffe, R. G. (2010). Subcellular flux analysis of central metabolism in a heterotrophic *Arabidopsis* cell suspension using steady-state stable isotope labeling. *Plant Physiol.* 152, 602–619. doi: 10.1104/pp.109.151316

Meng, P. H., Raynaud, C., Tcherkez, G., Blanchet, S., Massoud, K., Domenichini, S., et al. (2009). Crosstalks between Myo-Inositol Metabolism, Programmed Cell Death and Basal Immunity in *Arabidopsis*. *PLoS ONE* 4:e7364. doi: 10.1371/journal.pone.0007364

Miura, K., and Tada, Y. (2014). Regulation of water, salinity, and cold stress responses by salicylic acid. *Front. Plant Sci.* 5:4. doi: 10.3389/fpls.2014.00004

Moore, A. L., Albury, M. S., Crichton, P. G., and Affourtit, C. (2002). Function of the alternative oxidase: is it still a scavenger? *Trends Plant Sci.* 7, 478–481. doi: 10.1016/S1360-1385(02)02366-X

Mueller, L. A., Zhang, P., and Rhee, S. Y. (2003). AraCyc: a biochemical pathway database for *Arabidopsis*. *Plant Physiol.* 132, 453–460. doi: 10.1104/pp.102.017236

Noack, S., Nöh, K., Moch, M., Oldiges, M., and Wiechert, W. (2010). Stationary versus non-stationary 13C-MFA: a comparison using a consistent dataset. *J. Biotechnol.* 154, 179–190. doi: 10.1016/j.jbiotec.2010.07.008

Nöh, K., and Wiechert, W. (2011). The benefits of being transient: isotope-based metabolic flux analysis at the short time scale. *Appl. Microbiol. Biotechnol.* 91, 1247–1265. doi: 10.1007/s00253-011-3390-4

Oberhardt, M. A., Goldberg, J. B., Hogardt, M., and Papin, J. A. (2010). Metabolic network analysis of *Pseudomonas aeruginosa* during chronic cystic fibrosis lung infection. *J. Bacteriol.* 192, 5534–5548. doi: 10.1128/JB.00900-10

Ozbayraktar, F. B. K., and Ulgen, K. O. (2011). Stoichiometric network reconstruction and analysis of yeast sphingolipid metabolism incorporating different states of hydroxylation. *Biosystems* 104, 63–75. doi: 10.1016/j.biosystems.2011.01.001

Pata, M. O., Hannun, Y. A., and Ng, C. K. (2009). Plant sphingolipids: decoding the enigma of the Sphinx. *New Phytol.* 185, 611–630. doi: 10.1111/j.1469-8137.2009.03123.x

Poolman, M. G., Miguet, L., Sweetlove, L. J., and Fell, D. A. (2009). A genome-scale metabolic model of *Arabidopsis* and some of its properties. *Plant Physiol.* 151, 1570–1581. doi: 10.1104/pp.109.141267

Radrich, K., Tsuruoka, Y., Dobson, P., Gevorgyan, A., Swainston, N., Baart, G., et al. (2010). Integration of metabolic databases for the reconstruction of genome-scale metabolic networks. *BMC Syst. Biol.* 4:114. doi: 10.1186/1752-0509-4-114

Reed, J. L., and Palsson, B. O. (2003). Thirteen years of building constraint based *in silico* models of *Escherichia coli*. *J. Bacteriol.* 185, 2692–2699. doi: 10.1128/JB.185.9.2692-2699.2003

Saucedo-García, M., Guevara-García, A., Gonzalez-Solís, A., Cruz-García, F., Vazquez-Santana, S., Markham, J. E., et al. (2011). MPK6, sphinganine and the LCB2a gene from serine palmitoyltransferase are required in the signaling pathway that mediates cell death induced by long chain bases in *Arabidopsis*. *New Phytol.* 191, 943–957. doi: 10.1111/j.1469-8137.2011.03727.x

Schellenberger, J., Que, R., Fleming, R. M., Thiele, I., Orth, J. D., Feist, A. M., et al. (2011). Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0. *Nat. Protoc.* 6, 1290–1307. doi: 10.1038/nprot.2011.308

Schmidt, H., and Jirstrand, M. (2006). Systems Biology Toolbox for MATLAB: a computational platform for research in systems biology. *Bioinformatics* 22, 514–515. doi: 10.1093/bioinformatics/bti799

Schwender, J. (2008). Metabolic flux analysis as a tool in metabolic engineering of plants. *Curr. Opin. Biotechnol.* 19, 131–137. doi: 10.1016/j.copbio.2008.02.006

Seaver, S. M. D., Henry, C. S., and Hanson, A. D. (2012). Frontiers in metabolic reconstruction and modeling of plant genomes. *J. Exp. Bot.* 63, 2247–2258. doi: 10.1093/jxb/err371

Shimono, M., Sugano, S., Nakayama, A., Jiang, C. J., Ono, K., Toki, S., et al. (2007). Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance. *Plant Cell* 19, 2064–2076. doi: 10.1105/tpc.106.046250

Slaymaker, D. H., Navarre, D. A., Clark, D., del Pozo, O., Martin, G. B., and Klessig, D. F. (2002). The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant capacity and plays a role in the hypersensitive response. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11640–11645. doi: 10.1073/pnas.182427699

Smith, A. M., and Stitt, M. (2007). Coordination of carbon supply and plant growth. *Plant Cell Environ.* 30, 1126–1149. doi: 10.1111/j.1365-3040.2007.01708.x

Spoel, S. H., and Dong, X. N. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Stitt, M., Sulpice, R., and Keurentjes, J. (2010). Metabolic networks: how to identify key components in the regulation of metabolism and growth. *Plant Physiol.* 152, 428–444. doi: 10.1104/pp.109.150821

Sweetlove, L. J., and Ratcliffe, R. G. (2011). Flux-balance modeling of plant metabolism. *Front. Plant Sci.* 2:38. doi: 10.3389/fpls.2011.00038

Ternes, P., Feussner, K., Werner, S., Lerche, J., Iven, T., Heilmann, I., et al. (2011). Disruption of the ceramide synthase LOH1 causes spontaneous cell death in *Arabidopsis thaliana*. *New Phytol.* 192, 841–854. doi: 10.1111/j.1469-8137.2011.03852.x

Thiele, I., and Palsson, B. O. (2010). A protocol for generating a high-quality genome-scale metabolic reconstruction. *Nat. Protoc.* 5, 93–121. doi: 10.1038/nprot.2009.203

van Leeuwen, H., Kliebenstein, D. J., West, M. A., Kim, K., van Poecke, R., Katagiri, F., et al. (2007). Natural variation among *Arabidopsis thaliana* accessions for transcriptome response to exogenous salicylic acid. *Plant Cell* 19, 2099–2110. doi: 10.1105/tpc.107.050641

Varma, A., and Palsson, B. O. (1994). Metabolic flux balancing: basic concepts, scientific and practical use. *Nat. Biotechnol.* 12, 994–998. doi: 10.1038/nbt1094-994

Vicente, M. R., and Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *J. Exp. Bot.* 62, 3321–3338. doi: 10.1093/jxb/err031

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Wang, D., Amornsiripanitch, N., and Dong, X. N. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2, 1042–1050. doi: 10.1371/journal.ppat.0020123

Wang, W. M., Yang, X. H., Tangchaiburana, S., Ndeh, R., Markham, J. E., Tsegaye, Y., et al. (2008). An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*. *Plant Cell.* 20, 3163–3179. doi: 10.1105/tpc.108.060053

Welti, R., Li, W., Li, M., Sang, Y., Biesiada, H., Zhou, H., et al. (2002). Profiling membrane lipids in plant stress responses. *J. Bio. Chem.* 277, 31194–32002. doi: 10.1074/jbc.M205375200

Yan, S. P., and Dong, X. N. (2014). Perception of the plant immune signal salicylic acid. *Curr. Opin. Plant Biol.* 20, 64–68. doi: 10.1016/j.pbi.2014.04.006

Yoshimoto, K., Hanaoka, H., Sato, S., Kato, T., Tabata, S., Noda, T., et al. (2004). Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. *Plant Cell* 16, 2967–2983. doi: 10.1105/tpc.104.025395

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 28 May 2015
doi: 10.3389/fpls.2015.00387

Lipids in salicylic acid-mediated defense in plants: focusing on the roles of phosphatidic acid and phosphatidylinositol 4-phosphate

Qiong Zhang[1] *and Shunyuan Xiao*[1,2]*

[1] *Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD, USA,* [2] *Department of Plant Sciences and Landscape Architecture, University of Maryland, Rockville, MD, USA*

Plants have evolved effective defense strategies to protect themselves from various pathogens. Salicylic acid (SA) is an essential signaling molecule that mediates pathogen-triggered signals perceived by different immune receptors to induce downstream defense responses. While many proteins play essential roles in regulating SA signaling, increasing evidence also supports important roles for signaling phospholipids in this process. In this review, we collate the experimental evidence in support of the regulatory roles of two phospholipids, phosphatidic acid (PA), and phosphatidylinositol 4-phosphate (PI4P), and their metabolizing enzymes in plant defense, and examine the possible mechanistic interaction between phospholipid signaling and SA-dependent immunity with a particular focus on the immunity-stimulated biphasic PA production that is reminiscent of and perhaps mechanistically connected to the biphasic reactive oxygen species (ROS) generation and SA accumulation during defense activation.

Keywords: plant defense signaling, lipid signaling, salicylic acid, phosphatidic acid, phosphatidylinositol 4-phosphate, phospholipase D, phospholipase C, biphasic generation of ROS

OPEN ACCESS

Edited by:
Jean Toby Greenberg, The University of Chicago, USA

Reviewed by:
Xuemin Wang, University of Missouri-St Louis & Donald Danforth Plant Science Center, USA
Mats X. Andersson, University of Gothenburg, Sweden

***Correspondence:**
Shunyuan Xiao, Institute for Bioscience and Biotechnology Research, University of Maryland, 9600 Gudelsky Dr., Rockville, MD 20850, USA
xiao@umd.edu

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *12 March 2015*
Accepted: *14 May 2015*
Published: *28 May 2015*

Citation:
Zhang Q and Xiao S (2015) Lipids in salicylic acid-mediated defense in plants: focusing on the roles of phosphatidic acid and phosphatidylinositol 4-phosphate. Front. Plant Sci. 6:387. doi: 10.3389/fpls.2015.00387

1. Introduction

Plants have evolved multilayered preformed and inducible defense mechanisms to fight against various pathogens. In most cases, plant defense responses are induced upon recognition of non-adapted and adapted pathogens by a two-branched innate immune system (Jones and Dangl, 2006). For the first branch, defense is triggered upon recognition of conserved pathogen- or microbial- associated molecular patterns (PAMPs or MAMPs) by plant cell surface-localized pattern recognition receptors (PRRs) thus it is referred to as PAMP/MAMP-triggered immunity (PTI/MTI). For the second branch, plants employ cell-surface receptor-like proteins or intracellular nucleotide binding site leucine-rich-repeats (NB-LRR) proteins (genetically defined as R proteins) to recognize effectors that are secreted by pathogens to suppress PTI and promote pathogenesis, thereby inducing defense responses termed effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI and ETI are believed to be evolutionarily inter-related and mechanistically interconnected, as both involve activation of an overlapping array of downstream defense responses including *PR* gene expression, reactive oxygen species (ROS) production and callose deposition via conserved interwoven signaling pathways that are regulated by salicylic acid (SA), jasmonic acid (JA), and ethylene (C_2H_4) (Bari and Jones, 2009; Pieterse et al., 2012), despite clear branch-specific differences in crosstalk directionality and outcome strength.

SA, the best-studied small phenolic phytohormone, plays a major role in mediating defense against biotrophic and hemi-biothrophic pathogens that rely on living host cells for establishing infection (Vlot et al., 2009). Cellular SA accumulation constitutes an early signaling event during PTI and ETI and is essential for induction of defense responses. This step requires components including EDS1, and its homologous & interacting partners PAD4 and SAG101 (positive regulators of SA signaling) (Wagner et al., 2013), as well as SID2 (required for 90% stress-induced SA biosynthesis) (Wildermuth et al., 2001) and EDS5 (required for SA transport from the chloroplast to the cytoplasm) (Serrano et al., 2013). Elevation of SA level is perceived by SA receptors NPR3 and NPR4, which leads to degradation of NPR1, the master regulator of SA, in the infected cell, resulting in effector-triggered cell death; whereas NPR1 accumulates in neighboring cells to promote cell survival and SA-mediated resistance (Fu and Dong, 2013; Yan and Dong, 2014). In addition, SA signaling engages a feedback circuit to amplify defense responses (Wiermer et al., 2005), which is negatively regulated by EDR1, a MAPKKK (Frye et al., 2001; Xiao et al., 2005).

While protein components are essential for plant immunity and have been extensively studied, important roles for signaling lipids and their metabolizing enzymes in plant immunity have also been observed but relevant studies on the latter lag far behind. Even less is known about the possible mechanistic connection between lipid signaling and SA-dependent defense responses. In this mini-review, we will examine recent literatures on the "lipid-SA" connection with a focus on discussing how two phospholipids, i.e., phosphatidic acid (PA) and phosphatidylinositol 4-phosphate (PI4P), and the related enzymes [phospholipase D (PLD), phospholipase C (PLC), diacylglycerol kinase (DGK), and phosphatidylinositol-4-kinases (PI4Ks)] are implicated in SA signaling during PTI and ETI. For more detailed information on the biochemistry of these phospholipids and their metabolizing enzymes, and their roles in plant stress responses, we recommend several excellent reviews (Wang, 2004, 2014; Bargmann and Munnik, 2006; Arisz et al., 2009; Li et al., 2009; Munnik and Nielsen, 2011; Testerink and Munnik, 2011).

2. Role of PA in Defense: Both Positive and Negative

Being the simplest phospholipid class, PA has rather versatile functions: it is not only a central intermediate in glycerolipid biosynthesis but also a signaling molecule involved in regulating cellular processes such as lipid metabolism, signal transduction, cytoskeletal rearrangements, and vesicular trafficking. The concentration of PA is normally very low in plant tissues and can be induced rapidly by various stimuli. This signal-induced PA is mainly produced via two distinct enzymatic pathways. The first route is accomplished in a two-step enzymatic process that involves generation of diacylglycerol (DAG) from inositol phospholipids by PLC, followed by production of PA through phosphorylation of DAG by DGK. The other route engages PLD to produce PA through direct hydrolysis of phospholipids such as phosphatidylcholine and phosphatidylethanolamine by removing their head groups (Testerink and Munnik, 2011). In the presence of primary alcohols such as n-butanol, PLD prefers alcohols over water molecules to produce phosphatidyl alcohols instead of PA through a reaction called transphosphatidylation (Yang et al., 1967). This unique property allows researchers to easily monitor PLD activity to study the role of PLD-derived PA under different conditions, and distinguish PA produced by PLD from that produced by PLC-DGK (Arisz et al., 2009). Conceivably, PA derived from the above-mentioned two pathways may possess structural diversity (fatty acyl chain length and degree of saturation) as well as distinct spatiotemporal characteristics at the tissue, cell or subcellular level. Hence, a multifaceted role of PA in cellular signaling is anticipated and can be attributed largely to the properties of the specific enzymes that produce different pools of signaling PA with spatiotemporal specificity.

A potential role of PA in plant defense was inferred by the observations that transcription of plant *PLC*, *DGK* or *PLD* genes and/or their protein enzymatic activities were induced to higher levels upon pathogen infection or elicitor treatment in rice (*Oryza sativa*) (Young et al., 1996; Yamaguchi et al., 2003, 2005), tomato (*Solanum lycopersicum*) (van der Luit et al., 2000), tobacco (*Nicotiana tabacum*) (Suzuki et al., 2007), and Arabidopsis (*Arabidopsis thaliana*) plants (de Torres Zabela et al., 2002). Subsequent genetic or biochemical studies provided more definitive evidence to support differential or even opposing roles of PA in regulation of plant defense response under different pathocontexts (Supplemental Table 1). In tomato suspension-cultured cells expressing the *Cf-4* resistance gene, treatment of the cognate pathogen effector Avr4 rapidly induced accumulation of PA, via the PLC-DGK route (De Jong et al., 2004). Further studies showed that silencing of the tomato *SlPLC4* gene impaired Cf-4/Avr4-induced HR and resulted in increased susceptibility of *Cf-4* plants to *Cladosporium fulvum* expressing *Avr4* (Vossen et al., 2010). Interestingly, silencing of *SlPLC6* in tomato did not affect Cf-4/Avr4-induced HR, but compromised resistance mediated by *R* genes like *Cf-4*, *Ve1* or *Pto/Prf*. These observations demonstrate that PLC-DGK-derived PA probably acts as a positive regulator of ETI. In Arabidopsis, two recent studies have established a positive role for PLD-derived PA in basal defense and non-host resistance. These studies showed that abrogation of PLD-derived PA by n-butanol in Arabidopsis compromised both basal (cell-wall-based) resistance to non-adapted powdery mildew pathogens and RPM1(an NB-LRR)/AvrRpm1(the cognate effector)-triggered immunity (Pinosa et al., 2013; Johansson et al., 2014). Genetic analysis of Arabidopsis mutants identified *At*PLDδ, one of the 12 *At*PLDs, to be the only isoform that contributes to penetration resistance against non-adapted powdery mildew (Pinosa et al., 2013), yet no single PLD isoform was found to be responsible for RPM1/AvrRpm1-triggered immunity, highlighting functional redundancy among different *At*PLD isoforms (Johansson et al., 2014).

Interestingly, while so far there is no evidence for PLC-DGK-derived PA in negative regulation of plant defense, genetic depletion of specific PLD isoforms in tomato, rice,

and Arabidopsis resulted in elevated defense responses. These genes include *SlPLDβ1* (its silencing resulted in priming for a subset of defense responses in tomato cells treated with elicitors) (Bargmann et al., 2006), *OsPLDβ1* (its silencing in rice resulted in enhanced resistance to multiple pathogens) (Yamaguchi et al., 2009), and *AtPLDβ1* (its expression was suppressed by SA and genetic depletion led to elevated levels of SA, ROS, and enhanced resistance to virulent *P. syringae*) (Zhao et al., 2013).

Apparently, further studies are required to gain more mechanistic insight into how PA derived from different PLDs might oppositely regulate defense responses in plants. In the following section, we carefully examined the temporal kinetics of PA generation and manifestation of defense response in searching for possible intrinsic causal relationships between PA and SA signaling.

3. A Biphasic Connection between PA and SA Signaling

Several earlier studies showed that both pathogen- or elicitor-induced production of ROS, SA, and C_2H_4 exhibited a biphasic pattern (Alvarez et al., 1998; Mur et al., 2000, 2003, 2008, 2009). Interestingly, as seen from the data summarized in Supplemental Table 1 a biphasic PA production upon PAMP/effector treatment has also been either inferred from increased PLC and/or PLD gene/enzyme activities or direct detection. Since (i) PA production appeared to occur earlier than ROS generation (Sang et al., 2001; De Jong et al., 2004; Park et al., 2004), (ii) PA was indeed shown to induce ROS production by activating the NADPH oxidase RbohD (Zhang et al., 2009; Tetiana et al., 2013) which is the main NADPH oxidase responsible for H_2O_2 generation during PTI (Kadota et al., 2014; Li et al., 2014), and (iii) ROS generation could lead to SA level elevation (Lamb and Dixon, 1997; Chamnongpol et al., 1998; Mur et al., 2009), we propose that PA likely functions as an important initial signal in the biphasic defense signaling waves.

3.1. A Potential Biphasic PA Production During PTI?

In tomato suspension-cultured cells, formation of PA (by *Sl*PLC) was detected within a few minutes after application of elicitors N,N,N,N-tetraacetylchitotetraose, xylanase, and flg22, which coincided with H_2O_2 production (van der Luit et al., 2000; Bargmann et al., 2006). Whether treatment of these PAMPs triggered the second wave of PA and ROS generation was not known in these circumstances since the measurement was restricted to the first 2 h post-elicitation which may preclude the second wave of PA and ROS production at later time points. Notably, using rice suspension-cultured cells, Yamaguchi and colleagues did detect a biphasic induction of ROS that coincided with (and probably was preceded by) *Os*PLC and/or *Os*PLD activation, in which case the first peak at 20 min was associated with the activation of both *Os*PLC and *Os*PLD whereas the second peak at 120 min was associated mostly with the activation of *Os*PLD, after application of a PAMP-like elicitor N-acetylchitooligosaccharide (Yamaguchi et al., 2005). Exogenous application of PA could induce ROS generation by itself, suggesting that ROS production was induced by enzymatic activities of *Os*PLC and/or *Os*PLD. Thus, although no direct quantification of PA was conducted in this study, a biphasic PA production (as a result of *Os*PLD activation) before the biphasic ROS production was anticipated. It seems clear that PAMPs could trigger the first phase of PA production, but whether or not they can also induce the second phase remains to be determined.

3.2. A Biphasic PA Production During ETI

Using transgenic tobacco cells expressing the tomato *Cf-4*-resistance gene as a model system, it was found that within 2 min after challenge with the fungal effector Avr4, a largely *Sl*PLC-DGK-dependent PA production was detected, followed by an oxidative burst a few minutes later (De Jong et al., 2004). Since no measurement for PA or ROS was done beyond 30 min, occurrence of the second wave of PA and ROS production, though anticipated, was not determined. However, because silencing of *SlPLC4* impaired Cf-4-dependent resistance and silencing of *SlPLC6* compromised several R-mediated resistance (Vossen et al., 2010), one can infer that the initial PA production is essential for ROS generation, HR and defense during ETI. It is worth pointing out that Cf-4 and Avr4, which are genetically defined as R and Avr, respectively, may arguably qualify for a PRR and a PAMP, respectively (Thomma et al., 2011). If so, Cf-4/Avr4 interaction-induced PA production before ROS generation would also render support to a biphasic PA production as an early signaling step of PTI.

Supporting this notion, Andersson and colleagues found that the first detectable wave of PA accumulation (via the PLC-DGK route) started in ∼60 min which was followed by a second wave of PA production (via PLD route) occurred around 3∼4 h after application of dexamethasone to induce expression of *AvrRpm1* or *AvrRpt2* as transgenes in Arabidopsis plants containing the cognate receptor (Andersson et al., 2006). Given that PTI and ETI signaling mechanisms are believed to be interconnected, it should not be a surprise that an early signaling step conserved for PTI and ETI is channeled through PA production. Recent findings that PAD4 functions upstream of SA in defense signaling during both PTI and ETI (Tsuda et al., 2009; Kim et al., 2014), and that SA can further up-regulate expression and signaling of PRRs (Zhang et al., 2014) provide likely mechanistic connection between PTI and ETI concerning PA production.

3.3. Possible Biphasic PA-ROS-SA Signaling Amplification?

While a robust biphasic production of ROS or SA was described in Arabidopsis (Shapiro and Gutsche, 2003), potato (Yoshioka et al., 2001), and tobacco plants (Lamb and Dixon, 1997; Mur et al., 2000) during PTI/ETI, a clear biphasic PA production in a similar time window from elicitation to manifestation of immune response was also observed in Arabidopsis (Andersson et al., 2006), and rice cells (Yamaguchi et al., 2005). Unfortunately, there were no time-course studies in which levels of PA, ROS, and SA were measured using the same pathosystem, making it impossible to directly assess the timing of these signaling events because of the differences in the experimental systems reported (plant species, cell types, and pathogens/elicitors).

However, given that ROS production and SA accumulation are tightly linked and form a self-amplifying feedback circuit during defense signaling (Mur et al., 2009; Vlot et al., 2009), we can envision that PAMP/effector-triggered PA production constitutes an important early signaling step that results in the first wave of ROS production, which in turn triggers SA biosynthesis, forming the first signaling phase that potentiates the second phase. Such signaling waves may also involve other signaling molecules such as calcium fluxes (Grant et al., 2000) and C_2H_4 (Mur et al., 2008).

Based on the evidence from multiple studies described above and summarized in Supplemental Table 1 we propose a model to illustrate biphasic PA-ROS-SA signaling during plant defense activation (**Figure 1**). The main points of the model are as follows: (i) The first wave is rapid and transient, and is attributable to PTI and/or ETI; whereas the second wave occurs in plant cells undergoing ETI or ectopically strengthened PTI (i.e., suspension-cultured cells treated with high-concentration of PAMPs). (ii) The signaling order is probably from PA (mainly from the PLC-DGK route) to ROS (De Jong et al., 2004; Park et al., 2004), and from ROS to SA (Lamb and Dixon, 1997; Chamnongpol et al., 1998; Mur et al., 2009) in the first wave based on time sequence and some known mechanistic connections. (iii) Elevated SA in the first wave above a threshold level plays an essential role in potentiation (priming) of the second wave of PA (mainly produced by PLDs), ROS and SA production through multi-layered positive feedback amplification circuits where EDS1/PAD4/SAG101 may be essential components required for SA signaling and PTI-ETI connection (Kim et al., 2014; Zhang et al., 2014). Conceivably, the spatiotemporal kinetics and amplitude of the biphasic defense signal amplification may vary under different pathocontexts, which may at least partially account for the discrepancies in the results from different studies (Supplemental Table 1). Nevertheless, the biphasic PA-ROS-SA signal amplification, together with production of other signaling molecules such as nitric oxide and C_2H_4 (Mur et al., 2008, 2009), likely orchestrates the eventual development of HR and other defense responses in many cases.

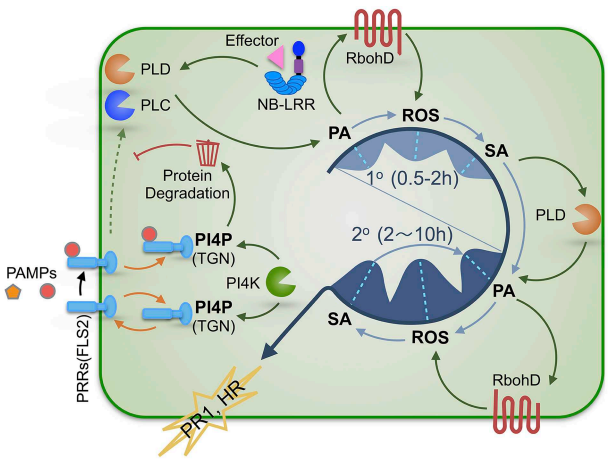


FIGURE 1 | A schematic illustration of the regulatory roles of PA and PI4P in SA-dependent plant defense signaling. Plants activate immune responses upon recognition of PAMPs or effectors by PRRs or NB-LRR immune receptors, respectively. Bioactive PA and PI4P play distinct roles in regulating defense signaling. PA production via immunity-activated PLC and/or PLD is required for SA-dependent defense activation and exhibits a biphasic pattern (1° & 2°) that precedes the kinetically similar biphasic ROS generation and SA accumulation. Therefore, we propose that these three signaling molecules are sequentially interconnected with PA most likely being the initial signal of the PA-ROS-SA signaling module. The first wave (1°) of PA-ROS-SA signal amplification (occurring during PTI and ETI) may potentiate the second wave (2°) of PA-ROS-SA signal amplification (occurring mostly during ETI or strengthened PTI), constituting a tunable signaling module for defense in plant cells. PI4P derived from PI4KIIIβ1 and PI4KIIIβ2 functions to maintain the homeostasis of PRRs via facilitating its recycling and/or degradation, thereby preventing inappropriate activation of PTI in the absence of pathogens and allowing measured PTI signaling upon pathogen attack. PAMPs, pathogen-associated molecular patterns; PTI, PAMP-triggered immunity; ETI, effector-triggered immunity; PRR, pattern recognition receptor; NB-LRR, nucleotide binding site leucine-rich-repeats; PA, phosphatidic acid; PI4P, phosphatidylinositol 4-phosphate; TGN, trans-Golgi network; PLC, phospholipase C; PLD, phospholipase D.

4. PI4P Chimes in to Put a Brake on and Fine-Tune PTI

One critical question one may ask is why plant defense signaling is biphasic but not monophasic or incremental. A logical explanation is that there must be concomitant or instantaneous negative regulation on it. Indeed, PAMP-elicited or EDS1-dependent defense signaling has been demonstrated to be tightly regulated by a number of negative regulators. These include the E3-ubiquitin ligase PUB13 (Lu et al., 2011; Zhou et al., 2014), the Ca^{2+}/calmodulin-binding transcription factor SR1 (Du et al., 2009) and a MAPKKK EDR1 (Frye et al., 2001; Xiao et al., 2005). Interestingly, recent studies showed that PI4KIIIβ1, PI4KIIIβ2, and their product PI4P negatively regulated SA signaling via modulating homeostasis of FLS2, a PRR that recognizes flagellin (a PAMP from bacteria) (Antignani et al., 2015), providing a possible braking mechanism for PTI.

PI4Ks catalyze the phosphorylation of phosphatidylinositol at the 4th -OH position of its inositol head group to produce PI4P, the precursor of $PI(4,5)P_2$. PI4Ks are divided into two major types, II and III, according to their sizes and sensitivities to pharmacological treatments. Based on sequence and structure similarities, type III PI4Ks are further grouped into two subfamilies, α and β (Mueller-Roeber and Pical, 2002).

In an earlier report, PI4KIIIβ1 and PI4KIIIβ2 were shown to be negative regulators of SA signaling in Arabidopsis, as the *pi4kIII*β1β2 double mutant plants accumulated high levels of SA and ROS, constitutively expressed the *PR-1* gene and showed enhanced resistance to *P. syringae* (Šašek et al., 2014; Antignani et al., 2015). Interestingly, PI4KIIIβ1 and PI4KIIIβ2 were reported to interact with a small GTPase RabA4B in the Arabidopsis root tip to regulate polarized expansion of root hair cells (Preuss et al., 2006). Recently, Antignani and colleagues showed that both RabA4B and PI4P interacted with PUB13 and the authors proposed that PI4KIIIβ1 and PI4KIIIβ2 were recruited by RabA4b to assist in the enrichment of PI4P at the trans-Golgi network (TGN) for (i) proper sorting of FLS2 via

recycling it back to the plasma membrane, or (ii) promoting FLS2 turnover by recruiting PUB13 to FLS2 (Antignani et al., 2015). Thus, PI4KIIIβ1 and PI4KIIIβ2, and more relevantly PI4P, function to negatively regulate SA signaling by maintaining FLS2 homeostasis. Meanwhile, PI4P can be converted to $PI(4,5)P_2$ which can activate PLDβ (Zheng et al., 2002), a genetically defined negative regulator of SA signaling in Arabidopsis (Zhao et al., 2013). Thus, PI4P may also exert its negative role via stimulating PLDβ indirectly. Intriguingly, another study showed that PI4Ks could be activated within 2 min upon SA treatment in Arabidopsis suspension-cultured cells, preceding the activation of PLD (45 min after SA treatment) (Krinke et al., 2007). Whether PI4KIIIβ1 and PI4KIIIβ2 were among the activated PI4Ks is not known. Regardless, PAMP-elicitation may lead to recruitment of PI4Ks to the TGN and subsequent local enrichment of PI4P, which may assist in recruiting PUB13 and facilitating its role in targeted degradation of PRRs, thereby down-regulating PTI signaling, resulting in a measured initial wave of PA-ROS-SA production for potentiating the second defense signaling wave (**Figure 1**).

5. Conclusions and Perspectives

Increasing evidence from biochemical and genetic studies suggests that PA from different sources may play distinct roles in plant immune responses, while PI4P may negatively regulate PTI signaling. Interestingly, as demonstrated by or inferred from multiple studies, immunity-stimulated PA production exhibits a biphasic pattern that is reminiscent of the biphasic ROS generation and SA accumulation. Hence, it appears likely that a major role of PA in plant immunity is to initiate and orchestrate biphasic amplification of ROS- and SA-dependent signaling leading to downstream defense responses. However, because of the intrinsic complexity of such regulatory mechanisms, the diverse experimental systems used, the genetic redundancies, and the difficulty in measuring the (sub)cellular levels of target lipid molecules, results from many individual studies are either descriptive in nature or fragmental. Future studies will be directed to defining the roles of signaling phospholipids and their metabolizing phospholipases in plant immunity by (i) using higher-order genetic mutants to circumvent functional redundancy, (ii) using novel tools and technologies to investigate the spatiotemporal dynamics of target molecules at the subcellular level, and (iii) studying of multiple defense signaling molecules in the same pathosystem.

Acknowledgments

Funding for this work was provided by a scholarship from the Ministry of Education of the People's Republic of China to QZ and by the National Science Foundation Grant IOS-1146589 to SX.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00387/abstract

References

Alvarez, M. E., Pennell, R. I., Meijer, P.-J., Ishikawa, A., Dixon, R. A., and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92, 773–784.

Andersson, M. X., Kourtchenko, O., Dangl, J. L., Mackey, D., and Ellerström, M. (2006). Phospholipase-dependent signalling during the AvrRpm1-and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*. *Plant J.* 47, 947–959. doi: 10.1111/j.1365-313X.2006.02844.x

Antignani, V., Klocko, A. L., Bak, G., Chandrasekaran, S. D., Dunivin, T., and Nielsen, E. (2015). Recruitment of PLANT U-BOX13 and the PI4Kβ1/β2 phosphatidylinositol-4 kinases by the small GTPase RabA4B plays important roles during salicylic acid-mediated plant defense signaling in Arabidopsis. *Plant Cell* 27, 243–261. doi: 10.1105/tpc.114.134262

Arisz, S. A., Testerink, C., and Munnik, T. (2009). Plant PA signaling via diacylglycerol kinase. *Biochim. Biophys. Acta* 1791, 869–875. doi: 10.1016/j.bbalip.2009.04.006

Bargmann, B. O., Laxalt, A. M., Riet, B. T., Schouten, E., Van Leeuwen, W., Dekker, H. L., et al. (2006). LePLDβ1 activation and relocalization in suspension-cultured tomato cells treated with xylanase. *Plant J.* 45, 358–368. doi: 10.1111/j.1365-313X.2005.02631.x

Bargmann, B. O., and Munnik, T. (2006). The role of phospholipase D in plant stress responses. *Curr. Opin. Plant Biol.* 9, 515–522. doi: 10.1016/j.pbi.2006.07.011

Bari, R., and Jones, J. D. (2009). Role of plant hormones in plant defence responses. *Plant Mol. Biol.* 69, 473–488. doi: 10.1007/s11103-008-9435-0

Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H., Van Montagu, M., et al. (1998). Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5818–5823. doi: 10.1073/pnas.95.10.5818

De Jong, C. F., Laxalt, A. M., Bargmann, B. O., De Wit, P. J., Joosten, M. H., and Munnik, T. (2004). Phosphatidic acid accumulation is an early response in the Cf-4/Avr4 interaction. *Plant J.* 39, 1–12. doi: 10.1111/j.1365-313X.2004.02110.x

de Torres Zabela, M., Fernandez-Delmond, I., Niittyla, T., Sanchez, P., and Grant, M. (2002). Differential expression of genes encoding *Arabidopsis* phospholipases after challenge with virulent or avirulent *Pseudomonas* isolates. *Mol. Plant Microbe Interact.* 15, 808–816. doi: 10.1094/MPMI.2002.15.8.808

Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A., et al. (2009). Ca^{2+}/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* 457, 1154–1158. doi: 10.1038/nature07612

Frye, C. A., Tang, D., and Innes, R. W. (2001). Negative regulation of defense responses in plants by a conserved MAPKK kinase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 373–378. doi: 10.1073/pnas.98.1.373

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., and Mansfield, J. (2000). The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *Plant J.* 23, 441–450. doi: 10.1046/j.1365-313x.2000.00804.x

Johansson, O. N., Fahlberg, P., Karimi, E., Nilsson, A. K., Ellerström, M., and Andersson, M. X. (2014). Redundancy among phospholipase d isoforms in resistance triggered by recognition of the *Pseudomonas syringae* effector avrrpm1 in *Arabidopsis thaliana*. *Front. Plant Sci.* 5:639. doi: 10.3389/fpls.2014.00639

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kadota, Y., Sklenar, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., et al. (2014). Direct regulation of the NADPH oxidase RBOHD by the

PRR-associated kinase BIK1 during plant immunity. *Mol. Cell* 54, 43–55. doi: 10.1016/j.molcel.2014.02.021

Kim, Y., Tsuda, K., Igarashi, D., Hillmer, R. A., Sakakibara, H., Myers, C. L., et al. (2014). Mechanisms underlying robustness and tunability in a plant immune signaling network. *Cell Host Microbe* 15, 84–94. doi: 10.1016/j.chom.2013.12.002

Krinke, O., Ruelland, E., Valentová, O., Vergnolle, C., Renou, J.-P., Taconnat, L., et al. (2007). Phosphatidylinositol 4-kinase activation is an early response to salicylic acid in Arabidopsis suspension cells. *Plant Physiol.* 144, 1347–1359. doi: 10.1104/pp.107.100842

Lamb, C., and Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Biol.* 48, 251–275.

Li, L., Li, M., Yu, L., Zhou, Z., Liang, X., Liu, Z., et al. (2014). The FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RbohD to control plant immunity. *Cell Host Microbe* 15, 329–338. doi: 10.1016/j.chom.2014.02.009

Li, M., Hong, Y., and Wang, X. (2009). Phospholipase D- and phosphatidic acid-mediated signaling in plants. *Biochim. Biophys. Acta* 1791, 927–935. doi: 10.1016/j.bbalip.2009.02.017

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., et al. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* 332, 1439–1442. doi: 10.1126/science.1204903

Mueller-Roeber, B., and Pical, C. (2002). Inositol phospholipid metabolism in Arabidopsis. Characterized and putative isoforms of inositol phospholipid kinase and phosphoinositide-specific phospholipase C. *Plant Physiol.* 130, 22–46. doi: 10.1104/pp.004770

Munnik, T., and Nielsen, E. (2011). Green light for polyphosphoinositide signals in plants. *Curr. Opin. Plant Biol.* 14, 489–497. doi: 10.1016/j.pbi.2011.06.007

Mur, L., Santosa, I., Laarhoven, L., Harren, F., and Smith, A. (2003). A new partner in the danse macabre: the role of nitric oxide in the hypersensitive response. *Bulg. J. Plant Physiol.* 110, 123.

Mur, L. A., Brown, I. R., Darby, R. M., Bestwick, C. S., Bi, Y.-M., Mansfield, J. W., et al. (2000). A loss of resistance to avirulent bacterial pathogens in tobacco is associated with the attenuation of a salicylic acid-potentiated oxidative burst. *Plant J.* 23, 609–621. doi: 10.1046/j.1365-313x.2000.00825.x

Mur, L. A., Laarhoven, L. J., Harren, F. J., Hall, M. A., and Smith, A. R. (2008). Nitric oxide interacts with salicylate to regulate biphasic ethylene production during the hypersensitive response. *Plant Physiol.* 148, 1537–1546. doi: 10.1104/pp.108.124404

Mur, L. A., Lloyd, A. J., Cristescu, S. M., Harren, F. J., Hall, M., and Smith, A. (2009). Biphasic ethylene production during the hypersensitive response in Arabidopsis: a window into defence priming mechanisms? *Plant Signal. Behav.* 4, 610–613. doi: 10.4161/psb.4.7.8904

Park, J., Gu, Y., Lee, Y., Yang, Z., and Lee, Y. (2004). Phosphatidic acid induces leaf cell death in Arabidopsis by activating the Rho-related small G protein GTPase-mediated pathway of reactive oxygen species generation. *Plant Physiol.* 134, 129–136. doi: 10.1104/pp.103.031393

Pieterse, C. M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Pinosa, F., Buhot, N., Kwaaitaal, M., Fahlberg, P., Thordal-Christensen, H., Ellerström, M., et al. (2013). *Arabidopsis* phospholipase Dδ Is involved in basal defense and nonhost resistance to powdery mildew fungi. *Plant Physiol.* 163, 896–906. doi: 10.1104/pp.113.223503

Preuss, M. L., Schmitz, A. J., Thole, J. M., Bonner, H. K., Otegui, M. S., and Nielsen, E. (2006). A role for the RabA4b effector protein PI-4Kβ1 in polarized expansion of root hair cells in *Arabidopsis thaliana*. *J. Cell Biol.* 172, 991–998. doi: 10.1083/jcb.200508116

Sang, Y., Cui, D., and Wang, X. (2001). Phospholipase D and phosphatidic acid-mediated generation of superoxide in *Arabidopsis*. *Plant Physiol.* 126, 1449–1458. doi: 10.1104/pp.126.4.1449

Šašek, V., Janda, M., Delage, E., Puyaubert, J., Guivarc'h, A., López Maseda, E., et al. (2014). Constitutive salicylic acid accumulation in pi4kIIIβ1β2 Arabidopsis plants stunts rosette but not root growth. *New Phytol.* 203, 805–816. doi: 10.1111/nph.12822

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., et al. (2013). Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiol.* 162, 1815–1821. doi: 10.1104/pp.113.218156

Shapiro, A. D., and Gutsche, A. T. (2003). Capillary electrophoresis-based profiling and quantitation of total salicylic acid and related phenolics for analysis of early signaling in *Arabidopsis* disease resistance. *Anal. Biochem.* 320, 223–233. doi: 10.1016/S0003-2697(03)00405-6

Suzuki, K., Yano, A., Nishiuchi, T., Nakano, T., Kodama, H., Yamaguchi, K., et al. (2007). Comprehensive analysis of early response genes to two different microbial elicitors in tobacco cells. *Plant Sci.* 173, 291–301. doi: 10.1016/j.plantsci.2007.06.002

Testerink, C., and Munnik, T. (2011). Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. *J. Exp. Bot.* 62, 2349–2361. doi: 10.1093/jxb/err079

Tetiana, K., Oksana, I., and Sergii, K. (2013). Involvement of phospholipase D and NADPH-oxidase in salicylic acid signaling cascade. *Plant Physiol. Biochem.* 66, 127–133. doi: 10.1016/j.plaphy.2013.02.006

Thomma, B. P., Nürnberger, T., and Joosten, M. H. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *Plant Cell* 23, 4–15. doi: 10.1105/tpc.110.082602

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. (2009). Network properties of robust immunity in plants. *PLoS Genet.* 5:e1000772. doi: 10.1371/journal.pgen.1000772

van der Luit, A. H., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T., et al. (2000). Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Physiol.* 123, 1507–1516. doi: 10.1104/pp.123.4.1507

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Vossen, J. H., Abd-El-Haliem, A., Fradin, E. F., Van Den Berg, G., Ekengren, S. K., Meijer, H. J., et al. (2010). Identification of tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) family members and the role of PLC4 and PLC6 in HR and disease resistance. *Plant J.* 62, 224–239. doi: 10.1111/j.1365-313X.2010.04136.x

Wagner, S., Stuttmann, J., Rietz, S., Guerois, R., Brunstein, E., Bautor, J., et al. (2013). Structural basis for signaling by exclusive EDS1 heteromeric complexes with SAG101 or PAD4 in plant innate immunity. *Cell Host Microbe* 14, 619–630. doi: 10.1016/j.chom.2013.11.006

Wang, X. (2004). Lipid signaling. *Curr. Opin. Plant Biol.* 7, 329–336. doi: 10.1016/j.pbi.2004.03.012

Wang, X. (2014). *Phospholipases in Plant Signaling*. Heidelberg: Springer. doi: 10.1007/978-3-642-42011-5

Wiermer, M., Feys, B. J., and Parker, J. E. (2005). Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* 8, 383–389. doi: 10.1016/j.pbi.2005.05.010

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Xiao, S., Calis, O., Patrick, E., Zhang, G., Charoenwattana, P., Muskett, P., et al. (2005). The atypical resistance gene, *RPW8*, recruits components of basal defence for powdery mildew resistance in *Arabidopsis*. *Plant J.* 42, 95–110. doi: 10.1111/j.1365-313X.2005.02356.x

Yamaguchi, T., Kuroda, M., Yamakawa, H., Ashizawa, T., Hirayae, K., Kurimoto, L., et al. (2009). Suppression of a phospholipase D gene, OsPLDβ1, activates defense responses and increases disease resistance in rice. *Plant Physiol.* 150, 308–319. doi: 10.1104/pp.108.131979

Yamaguchi, T., Minami, E., and Shibuya, N. (2003). Activation of phospholipases by n-acetylchitooligosaccharide elicitor in suspension-cultured rice cells mediates reactive oxygen generation. *Physiol. Plant.* 118, 361–370. doi: 10.1034/j.1399-3054.2003.00131.x

Yamaguchi, T., Minami, E., Ueki, J., and Shibuya, N. (2005). Elicitor-induced activation of phospholipases plays an important role for the induction of defense responses in suspension-cultured rice cells. *Plant Cell Physiol.* 46, 579–587. doi: 10.1093/pcp/pci065

Yan, S., and Dong, X. (2014). Perception of the plant immune signal salicylic acid. *Curr. Opin. Plant Biol.* 20, 64–68. doi: 10.1016/j.pbi.2014.04.006

Yang, S. F., Freer, S., and Benson, A. (1967). Transphosphatidylation by phospholipase D. *J. Biol. Chem.* 242, 477–484.

Yoshioka, H., Sugie, K., Park, H.-J., Maeda, H., Tsuda, N., Kawakita, K., et al. (2001). Induction of plant gp91 *phox* homolog by fungal cell wall, arachidonic acid, and salicylic acid in potato. *Mol. Plant Microbe Interact.* 14, 725–736. doi: 10.1094/MPMI.2001.14.6.725

Young, S. A., Wang, X., and Leach, J. E. (1996). Changes in the plasma membrane distribution of rice phospholipase D during resistant interactions with *Xanthomonas oryzae* pv *oryzae*. *Plant Cell* 8, 1079–1090.

Zhang, Y., Zhu, H., Zhang, Q., Li, M., Yan, M., Wang, R., et al. (2009). Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in *Arabidopsis*. *Plant Cell* 21, 2357–2377. doi: 10.1105/tpc.108.062992

Zhang, Z., Shrestha, J., Tateda, C., and Greenberg, J. T. (2014). Salicylic acid signaling controls the maturation and localization of the Arabidopsis defense protein ACCELERATED CELL DEATH6. *Mol. Plant* 7, 1365–1383. doi: 10.1093/mp/ssu072

Zhao, J., Devaiah, S. P., Wang, C., Li, M., Welti, R., and Wang, X. (2013). *Arabidopsis* phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens. *New Phytol.* 199, 228–240. doi: 10.1111/nph.12256

Zheng, L., Shan, J., Krishnamoorthi, R., and Wang, X. (2002). Activation of plant phospholipased by phosphatidylinositol 4,5-bisphosphate: characterization of binding site and mode of action. *Biochemistry* 41, 4546–4553. doi: 10.1021/bi0158775

Zhou, J., He, P., and Shan, L. (2014). "Ubiquitination of plant immune receptors," in *Plant Endosomes*, ed M. S. Otegui (New York, NY: Springer), 219–231. doi: 10.1007/978-1-4939-1420-3_17

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 18 February 2015
doi: 10.3389/fpls.2015.00059

Phospholipase D affects translocation of NPR1 to the nucleus in *Arabidopsis thaliana*

Martin Janda[1,2], Vladimír Šašek[2], Hana Chmelařová[3], Jan Andrejch[1], Miroslava Nováková[1,2], Jana Hajšlová[3], Lenka Burketová[2] and Olga Valentová[1]*

[1] *Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague, Prague, Czech Republic*
[2] *Laboratory of Pathological Plant Physiology, Institute of Experimental Botany AS CR, Prague, Czech Republic*
[3] *Department of Food Analysis and Nutrition, University of Chemistry and Technology Prague, Prague, Czech Republic*

Edited by:
Loreto Holuigue, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Günther F. E. Scherer, Leibniz Universität Hannover, Germany
Mats X. Andersson, University of Gothenburg, Sweden

***Correspondence:**
Olga Valentová, CSc., Laboratory of Plant Biochemistry, Department of Biochemistry and Microbiology, University of Chemistry and Technology Prague, Technická 5, Prague 6 – Dejvice, 16628, Czech Republic
e-mail: olga.valentova@vscht.cz

Phytohormone salicylic acid (SA) is a crucial component of plant-induced defense against biotrophic pathogens. Although the key players of the SA pathway are known, there are still gaps in the understanding of the molecular mechanism and the regulation of particular steps. In our previous research, we showed in *Arabidopsis* suspension cells that *n*-butanol, which specifically modulates phospholipase D activity, significantly suppresses the transcription of the pathogenesis related (*PR-1*) gene, which is generally accepted as the SA pathway marker. In the presented study, we have investigated the site of *n*-butanol action in the SA pathway. We were able to show in *Arabidopsis* plants treated with SA that *n*-butanol inhibits the transcription of defense genes (*PR-1, WRKY38*). Fluorescence microscopy of *Arabidopsis thaliana* mutants expressing *35S::NPR1-GFP* (nonexpressor pathogenesis related 1) revealed significantly decreased nuclear localization of NPR1 in the presence of *n*-butanol. On the other hand, *n*-butanol did not decrease the nuclear localization of NPR1 in *35S::npr1C82A-GFP* and *35S::npr1C216A-GFP* mutants constitutively expressing NPR1 monomers. Mass spectrometric analysis of plant extracts showed that *n*-butanol significantly changes the metabolic fingerprinting while *t*-butanol had no effect. We found groups of the plant metabolites, influenced differently by SA and *n*-butanol treatment. Thus, we proposed several metabolites as markers for *n*-butanol action.

Keywords: *n*-butanol, NPR1, salicylic acid, phospholipase D, signaling, *PR-1*, metabolome

INTRODUCTION

The resistance of plants to pathogens relies on a sophisticated immune system comprising an orchestra of defense mechanisms. The efficiency is highly dependent on the speed of the process starting with pathogen recognition and resulting in the expression of appropriate defense proteins.

Salicylic acid (SA) is a crucial phytohormone involved in the defense response mostly to biotrophs (Glazebrook, 2005; Tsuda et al., 2008; Tsuda and Katagiri, 2010), but several reports on the defense against necrotrophs also exist (Novakova et al., 2014). The key enzyme in SA biosynthesis is isochorismate synthase (ICS; EC 5.4.4.2) that catalyses the conversion of chorismate into isochorismate. ICS is encoded by two genes in *Arabidopis thaliana*. This pathway has been shown to be the dominant SA biosynthetic pathway in response to attack by pathogenic bacteria, contributing to approximately 90% of total SA, with most ICS activity attributed to ICS1 and ICS2, which ICS2 plays only a marginal role (Wildermuth et al., 2001). SA is catabolized in infected and senescing plants by the recently found enzyme salicylic acid-3-hydroxylase (S3H), which catalyzes conversion of SA to 2,3-dihydroxybenzoic acid (2,3-DHBA; gentisic acid) and thus regulates the level of SA in plants (Zhang et al., 2013). The SA mode of action has been intensively studied for more than 20 years (Vlot et al., 2009). The crucial component of the SA pathway is a nonexpressor of pathogenesis related 1 (NPR1) protein (Cao et al., 1994). It was shown that NPR1 influences transcription of ∼90% of the SA dependent defense genes (Wang et al., 2005; Blanco et al., 2009). In cytosol, NPR1 occurs as an oligomer. Increased amounts of SA cause the monomerization of the NPR1 oligomer due to the change of the redox state in the plant cell (Mou et al., 2003). Thereafter, the NPR1 monomer is translocated to the nucleus where the NPR1 monomers bind to the TGA transcription factors followed by their direct binding to the as-1 (activation sequence 1) cis-regulatory element that is present in the promoters of *PR* (pathogenesis related) genes, thus activating their expression (Jakoby et al., 2002). The *PR-1* gene is generally accepted as the marker for SA signaling. The monomeric NPR1, in the nucleus, is continuously degraded by proteasome, a process which plays a dual function in the induction of transcription of the SA related genes (e.g., *PR-1*) (Wang et al., 2005; Spoel et al., 2009). Proteasome degradation lowers the amount of NPR1 in

Abbreviations: NPR1, nonexpressor of pathogenesis related 1; PA, phosphatidic acid; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidyl inositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PR, patogenesis related; PLD, phospholipase D; SA, salicylic acid.

the nucleus, but considering that newly formed NPR1 is needed for the induction of *PR-1* transcription, the proteasome plays a key role in the regulation of NPR1 turnover (Spoel et al., 2009). Recently, a crucial step forward was made in the understanding of SA awareness; the long sought after SA receptor was probably found. Xinnian Dong's group showed that NPR3 and NPR4 (two orthologs of NPR1) have a binding affinity to SA. Interestingly, the binding affinity of NPR4 is much higher than that of NPR3, but this property is crucial for the correct regulation of NPR1 degradation and SA awareness (Fu et al., 2012).

Currently, it seems more obvious that the SA pathway is connected with the phospholipid signaling system (Janda et al., 2013), but the details are unknown. One of the key players of the phospholipid signaling in plants is phosphatidic acid (PA), produced by the action of phospholipase C and DAG kinase or directly by phospholipase D (PLD) (EC 3.1.4.4). PLD activity is specifically modulated by *n*-butanol due to the unique transphosphatidylation reaction catalyzed by this enzyme (Yang et al., 1967; Munnik et al., 1995). In the presence of low concentrations of primary alcohols, the phosphatidate moiety is preferentially transferred to the alcohol hydroxyl group rather than to the water molecule and the products of this reaction—phosphatidylalcohols are metabolically stable (Liscovitch et al., 2000). PLD occurs in *A. thaliana* in 12 isoforms with distinct biochemical and structural properties (Pleskot et al., 2012). Activation or increased expression of PLD isoforms after infection was shown in rice (Young et al., 1996; Lee et al., 1997; McGee et al., 2003) and *A. thaliana* (De Torres Zabela et al., 2002). The treatment with SA increased the PA level or PLD activity in *A. thaliana, Brassica napus* and soybean (Profotova et al., 2006; Kalachova et al., 2012; Rainteau et al., 2012). Zhao et al. (2013) investigated the role of AtPLDβ1 in defense responses to bacterial pathogens. PLDβ1-deficient plants were less susceptible to *Pseudomonas syringae* and the transcription of SA responsive genes rose in infected plants compared to the wild-type infected plants (Zhao et al., 2013). Krinke et al. (2009) described that in *A. thaliana* suspension cells, *n*-butanol blocked the *PR-1* transcription in the presence of SA. However, the mechanism of PLD/PA involvement in SA signaling remains unclear.

This work provides evidence that *n*-butanol, the most effective primary alcohol modulating the activity of PLD, is involved in the regulation of *PR-1* transcription in the seedlings of *A. thaliana*. We show also that its action proceeds or participates in the process of NPR1 transfer to the nucleus. The non-targeted metabolomic fingerprinting provides evidence that *n*-butanol has a substantial impact on metabolome whereas *t*-butanol remains ineffective.

MATERIALS AND METHODS

PLANT MATERIAL

Seedlings of *A. thaliana* ecotype Col-0 (WT), and transgenic plants *35S::NPR1-GFP*, *35S::npr1C82A-GFP, 35S::npr1C216A-GFP* (Kinkema et al., 2000; Mou et al., 2003) were grown in 24-well plates in 400 μL of MS liquid medium (Clay et al., 2009) for 10 days in a cycle of 10 h days (120 $\mu E\ m^{-2}\ s^{-1}$, 22°C) and 14 h nights (22°C) at 70% relative humidity. MS liquid medium in the wells was changed on the 7th day.

CHEMICAL TREATMENTS

The plants were treated directly in the wells of plates by changing the growing medium for the chemical-containing medium. 10-day-old seedlings were treated for 6 h with 50 μM and 250 μM salicylic acid sodium salt (Sigma; NaSA), 0.1 and 1% *n*-butanol (Sigma) or *t*-butanol (Penta).

GENE TRANSCRIPTION ANALYSIS

The whole seedlings from three wells were immediately frozen in liquid nitrogen. The tissue was homogenized in tubes with 1 g of 1.3 mm silica beads using a FastPrep-24 instrument (MP Biomedicals,CA, USA). RNA isolation and reverse transcription were performed as previously described (Sasek et al., 2012). An equivalent of 6.25 ng of RNA was loaded into a 10 μl reaction with qPCR mastermix EvaLine—E1LC (GeneOn, Ludwigshafen am Rhein, Germany). The reactions were performed in polycarbonate capillaries (Genaxxon, Ulm, Germany) and a LightCycler 1.5 (Roche). The following PCR program was used for PCR assays: 95°C for 10 min; 45 cycles: 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s; finished with a melting curve analysis. Threshold cycles and melting curves were calculated using LightCycler Software 4.1 (Roche). Alternatively, the LightCycler® 480 SYBR Green I master kit was used. The reactions were performed in the LightCycler® 480 Multiwell Plate 96 white. The following PCR program was used for PCR assays: 95°C for 10 min; 45 cycles: 95°C for 20 s, 55°C for 20 s, and 72°C for 10 s; finished with a melting curve analysis. The threshold cycles and melting curves were calculated using LightCycler Software 4.2 (Roche). The relative transcription was calculated with the efficiency correction and normalization (Czechowski et al., 2005). The primers were designed using PerlPrimer v1.1.17 (Marshall, 2004). The list of *A. thaliana* genes and corresponding accession numbers and primers follows: *SAND*, AT2G28390, FP: 5′CTG TCT TCT CAT CTC TTG TC 3′, RP: 5′ TCT TGC AAT ATG GTT CCT G 3′, *PR-1*, AT2G14610, FP: 5′ AGT TGT TTG GAG AAA GTC AG 3′, RP: 5′ GTT CAC ATA ATT CCC ACG A, *S3H*, AT4G10500, FP: 5′GGA TGA TAA ATG GGT CGC T 3′, RP: 5′TGT TTA CTA CGG CTC TAT GG 3′; *WRKY38*, AT5G22570, FP: 5′GCC CCT CCA AGA AAA GAA AG 3′, RP: 5′ CCT CCA AAG ATA CCC GTC GT 3′, *ICS1* AT1G74710 FP: 5′GCA AGA ATC ATG TTC CTA CC 3′, RP: 5′AAT TAT CCT GCT GTT ACG AG 3′.

CONFOCAL MICROSCOPY

The slide with seedlings was positioned onto an inverted platform (with a cover slip at the bottom) of the confocal laser scanning Zeiss LSM 5 DUO microscope. The GFP fluorescence was excited by the 488 nm line of a laser, the DAPI fluorescence was excited by the 405 nm line. The epidermal cells were viewed using an Zeiss Plan-Apochromat 20x/0,8 objective. The emitted light was captured using the HFT405/488 beam splitter and a 505–550 nm or 420–480 nm band-pass filter, respectively. Image analysis was performed using the software APS Asess 2.0.

METABOLOMIC SCREENING

The extraction procedure was modified according to Vaclavik et al. (2013). Whole seedlings from three independent wells were immediately frozen using liquid nitrogen. Six independent

samples for one type of treatment were prepared for one biological replicate. 150–250 mg of plant tissue was homogenized in tubes with 1 g of 1.3 mm silica beads using a FastPrep-24 instrument (MP Biomedicals,CA, USA). After the addition of 700 μL of methanol (p.a.; PENTA), the plant tissue was homogenized again. The silica beads were washed once with 700 μL methanol and both extracts were combined. The samples were kept on ice during the extraction. Prior to instrumental analysis, the samples were stored in a dark and dry environment at −70°C. The UHPLC–Q-TOF-MS analyses were performed using an Acquity Ultra-Performance LC system coupled to a Synapt G2 high definition mass spectrometer (Waters, USA). The LC separation was performed by an Acquity UPLC® HSS T3 column (100 × 1.8 mm, 1.7 μm particle size; Waters, USA). The gradient elution was used with the mobile phases consisting of (A) 0.1% formic acid in Milli-Q water and (B) 0.1% formic acid in methanol.

The Synapt G2 HD instrument was operated in the negative electrospray ionization (ESI) mode. The parameter settings used during the measurements were as follows: capillary voltage (−700 V), cone voltage (−25 V), source temperature (120°C), and desolvation temperature (350°C). Nitrogen was used as both desolvation and cone gas at a flow rate of 800 and 10 L/h, respectively. Both full MS and MS/MS fragmentation mass spectra were acquired at a rate of two spectra per second in the range *m/z* 50–1000. In order to diminish any possible time dependent changes in the UHPLC-MS chromatographic fingerprints, the sequence of the samples was randomized and one sample was chosen as a quality control sample, which was injected after every set of 20 samples. The MassLynx 4.1 software (Waters, USA) was used for data acquisition and the MarkerLynx software (Waters, USA) was used for data mining and processing. The software SIMCA (v. 13.0, Umetrics, Sweden) was then used for data processing based on Principal Components Analysis (PCA).

DATA EVALUATION

Values are expressed as means ± standard error (SE). For statistical analysis, Student's *t*-test or One-Way ANOVA followed by Fisher's Least-Significant-Difference (LSD) were used as appropriate, with a value $P < 0.05$ considered significant for mean differences using STATGRAPHICS® Centurion XVII software.

RESULTS

n-BUTANOL ALTERS SALICYLIC ACID RELATED GENES TRANSCRIPTION

The increased levels of SA or exogenous treatment with this phytohormone activates the signaling pathway resulting in the transcription of defense related genes (e.g., pathogenesis related). The generally accepted marker of SA signaling is the *PR-1* gene. In order to examine possible role of PLD/PA in this process, we co-treated 10-day-old seedlings of *A. thaliana* with both SA

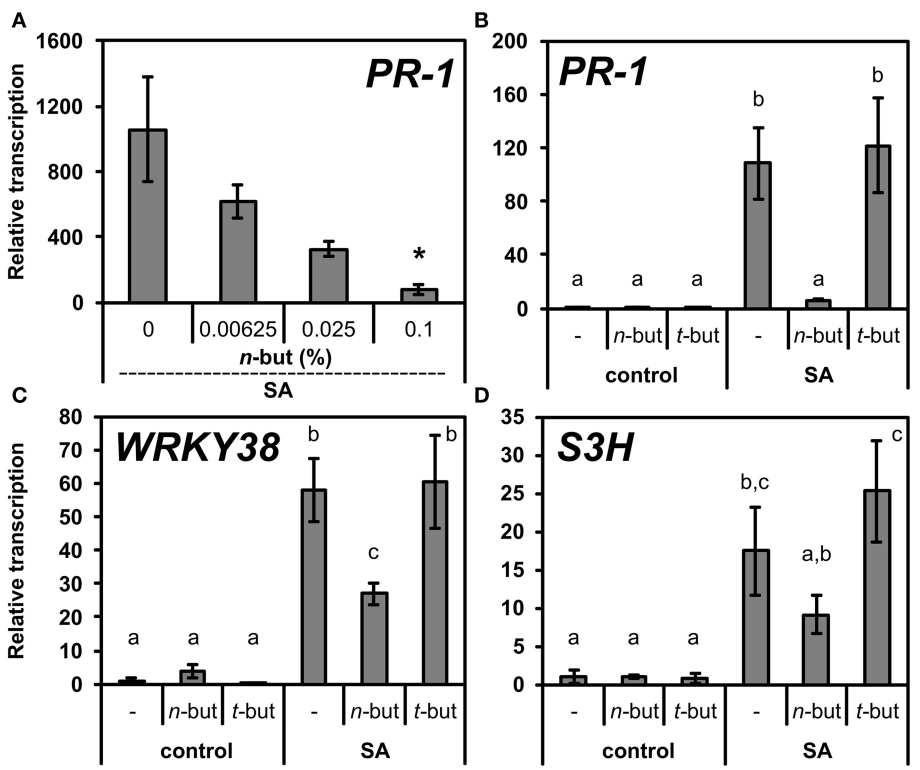


FIGURE 1 | Effect of *n*-butanol on SA related genes transcription. (A) Ten-day-old *A. thaliana* seedlings were treated for 6 h with 50 μM NaSA (SA) and 0.00625, 0.025, 0.1% *n*-butanol. **(B–D)** 10-day-old *A. thaliana* seedlings were treated for 6 h with 0.1% *n*-butanol and 0.1% *t*-butanol or with 50 μM NaSA together with the above mentioned alcohols. Pure MS was used as a control. Error bars represent SE from three biological repeats. Asterisks indicate statistically significant differences compared to NaSA-treated plants without *n*-butanol (*$P < 0.05$, Student's *t*-test) for **(A)**. Different letters indicate significant differences ($P < 0.05$) and were calculated with One-Way ANOVA and Fisher's LSD test. Transcription was normalized to a reference gene *SAND*.

and increasing concentrations of *n*-butanol. The SA induced *PR-1* transcription was decreased in the presence of *n*-butanol in a strongly dose-dependent manner (**Figure 1A**). Contrarily, the *t*-butanol showed no effect on the *PR-1* transcription (**Figure 1B**).

We also examined the effect of both alcohols on the transcription of other SA related genes, *WRKY38* and *S3H*, both encoding proteins with different functions. While the PR-1 protein is responsible for a direct antimicrobial effect as the end product of SA pathway, WRKY38 is a transcription factor which negatively regulates *PR-1* transcription, but it is NPR1 dependent (Kim et al., 2008). S3H is an enzyme responsible for the conversion of SA to a less biologically active compound, gentisic acid (Zhang et al., 2013). *WRKY38* and *S3H* transcriptions were not as significantly blocked as *PR-1* transcription. The relative transcription of *WRKY38* decreased only two times and even less in the case of *S3H* (**Figures 1B–D**). Also, the dose dependence of the *n*-butanol effect on the transcription of these two genes was far less apparent (Supplemental Figure S1).

n-BUTANOL AFFECTS NPR1 ACCUMULATION IN NUCLEUS

We further intended to take a closer look at the site of *n*-butanol action in the SA signaling pathway. To decipher, we used *35S::NPR1-GFP A. thaliana* transgenic plants. It was confirmed earlier that the treatment of these mutants with 2,6-dichloroisonicotinic acid (INA), a functional analog of SA, causes monomerization of NPR1, which is afterwards accumulated in the plant cell nucleus (Mou et al., 2003). We treated 10-day-old *35S::NPR1-GFP A. thaliana* seedlings with 250 μM NaSA and observed a significant increase of fluorescence in the nuclei (**Figures 2A,B**), the same effect was described for INA treatment. The accumulation of *35S::NPR1-GFP* in the nuclei in the presence of NaSA decreased after addition of 1% *n*-butanol (**Figures 2A,B**). When *t*-butanol was applied as a negative control, no effect on the *35S::NPR1-GFP* accumulation in the nuclei was observed (**Figures 2A,B**). *n*-butanol alone decreased the basal accumulation of NPR1 in the nuclei in the control plants, while no effect was observed for *t*-butanol. The localization of NPR1-GFP in the nuclei was verified by DAPI staining (Supplemental Figure S2). All these results correlate with the aforementioned *PR-1* transcription analysis (**Figure 1B**). Consequently, we wanted to examine whether the decreased amount of NPR1 in the nucleus caused by *n*-butanol is due to the higher actvity of proteasomes in NPR1 degradation (Spoel et al., 2009). For this experiment, we used *35S::npr1C82A-GFP* and *35S::npr1C216A-GFP* seedlings expressing constitutively monomerized NPR1, which is overaccumulated in the nucleus (Mou et al., 2003). The treatment of these mutants with 0.1% and 1% *n*-butanol did not decrease the accumulation of NPR1 in the nuclei (**Figure 3**). This experiment also provides evidence that *n*-butanol does not influence fluorescence intensity.

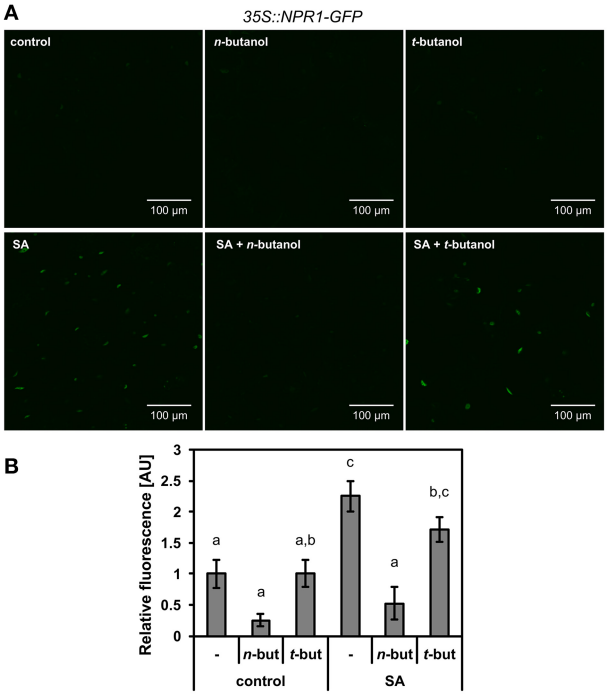


FIGURE 2 | Effect of SA and *n*-butanol on the localization of NPR1. Ten-day-old seedlings of *A. thaliana 35S::NPR1-GFP* mutants were treated for 6 h with fresh MS medium (control), 1% *n*-butanol (*n*-but), 1% *t*-butanol (*t*-but), 250 μM NaSA (SA), 250 μM NaSA (SA) and 1% *n*-butanol or 1% *t*-butanol. **(A)** Representative micrographs of *35S::NPR1-GFP A. thaliana* seedlings 6 h after treatment, **(B)** Image analysis of relative fluorescence using APS Assess 2.0 software. The values represent means ± SE from 16 images (8 seedlings). Different letters indicate significant differences ($P < 0.05$) and were calculated with One-Way ANOVA and Fisher's LSD test. The experiment was performed in three biological replicates with similar results.

n-BUTANOL INDUCES *ICS1* TRANSCRIPTION

Zhang et al. (2010) showed that nuclear localization of NPR1 is required for SA accumulation, *ICS1* transcription and SA tolerance. When NPR1 was retained in the cytoplasm, plants accumulated higher levels of *ICS1* transcripts compared to the wild type. Based on this, we measured the transcription of *ICS1* upon the addition of the *n*-butanol treatment and as expected, *n*-butanol induced *ICS1* transcription in a dose-dependent manner (**Figure 4A**), while *t*-butanol had no effect (**Figure 4B**). These results support our suggestion that *n*-butanol inhibits the translocation of NPR1 to the nucleus. Accordingly, we also found that the *ICS1* transcription in *35S::npr1C82A-GFP* and *35S::npr1C216-GFP* mutants were significantly decreased but *n*-butanol treatment partially reverted this effect (**Figure 4C**).

n-BUTANOL CAUSES CHANGES IN *A. THALIANA* METABOLOME

As the accumulation of SA leads to the massive reprogramming of the plant transcriptome, it was obviously accompanied by significant changes in the whole metabolome. We investigated these changes in plants treated with SA and *n*-butanol to test if we could reveal compounds involved in the SA/phospholipid signaling pathway.

The principle component analysis (PCA) represents a highly useful and widely employed tool for the interpretation of complex data sets generated by several modern instruments including mass spectrometry. In our study, PCA was employed to explore alterations in the metabolomes of differently treated *A. thaliana*

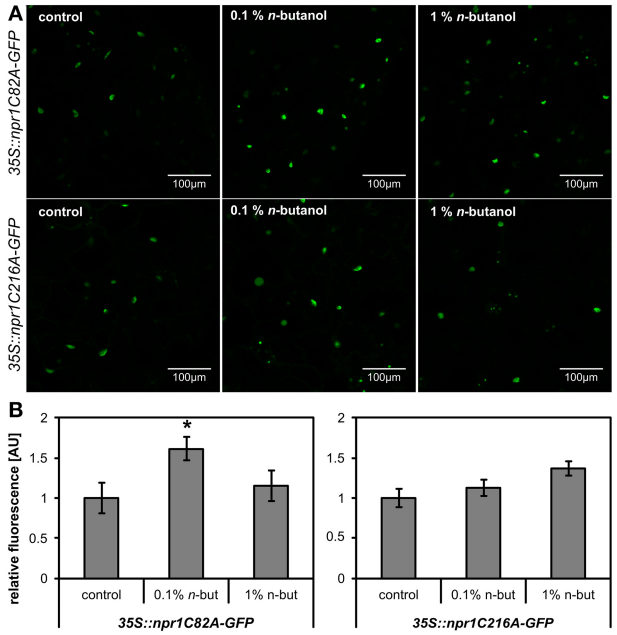


FIGURE 3 | Effects of *n*-butanol on the accumulation of NPR1 in the nuclei of *Arabidopsis thaliana* mutants constitutively expressing monomers of NPR1. Ten-day-old seedlings of *35S::npr1C82A-GFP* and *35S::npr1C216A-GFP A. thaliana* mutants were treated 6 h with fresh MS medium (control), 0.1% *n*-butanol and 1% *n*-butanol (*n*-but). **(A)** Representative images of *35S::npr1C82A-GFP* and *35S::npr1C216A-GFP A. thaliana* seedlings 6 h after treatment, **(B)** Image analysis of relative fluorescence using APS Assess 2.0 software. The values represent means ± SE from 12 images (6 seedlings). Asterisks indicate statistically significant differences compared to the control, non-treated plants (* $P <$ 0.05, Student's *t*-test). The experiment was performed in two biological replicates with similar results.

samples measured by LC-MS. As shown in **Figure 5**, there was a significant difference in the metabolomic fingerprints of samples treated with *n*-butanol (right side of the PCA plot) and untreated ones (left side of the PCA plot). The samples treated with SA were clearly differentiated (bottom part of the PCA plot) from the untreated samples (top part of the PCA plot). The list of the most distinct markers (ions recovered from the LC-MS records) is summarized in **Table 1**. Obviously, SA and its metabolite SA hexoside are typical markers for the samples treated with SA. Unfortunately, the identification of markers present in *n*-butanol treated plants was mostly unsuccessful, mainly due to their high *m/z* values resulting in many possible elemental formulas and also due to the limited information about the changes induced by *n*-butanol in the metabolism. Important observations were that the *t*-butanol treated samples did not differentiate from the untreated samples and also that *t*-butanol had no effect on the SA treated samples (**Figure 5**).

We identified 114 metabolites, from which 61 were statistically ($P = 0.05$; two tailed Student's *t*-test) changed at least in one of the used treatments (**Figure 6**). Based on the response to treatment, we were able to divide the metabolites into six groups (**Figure 6**) according to whether they were induced or suppressed by SA, *n*-butanol or both chemicals together.

DISSCUSSION

Plant response to biotic stress mediated by the phytohormone SA is a fundamental process. It was shown that NPR1 protein is a crucial component of the SA signaling (Cao et al., 1994). The structural changes and localization of this protein in plant cells is responsible for the plant defense signaling (Kinkema et al., 2000). Whereas an oligomer form occurs in cytosol, the monomer, which is formed when SA level increases, is translocated to the nucleus (Mou et al., 2003), where it binds to the TGA transcription factors and induces a transcription of the most of SA related genes (Zhang et al., 1999; Wang et al., 2005). NPR1 nuclear localization is responsible for regulation of plant tolerance to SA, a negative regulation of *ICS1* transcription and leads to SA accumulation (Zhang et al., 2010). Thus, NPR1 is really a master regulator of the SA signaling pathway although the NPR1 independent pathway also exists (Janda and Ruelland, 2014). Nevertheless, there are still gaps in the knowledge of the regulation of SA signaling needing to be filled in.

n-BUTANOL AND NPR1 DEPENDENT SA SIGNALING PATHWAY

In our study, we have shown that *n*-butanol is a molecule with a high impact on the SA signaling pathway in *A. thaliana* seedlings. *n*-butanol has been for a long time accepted by the "PLD community" as a modulator of PLD activity due to its preference for primary alcohols as substrates (Yang et al., 1967; Munnik et al., 1995). Potocky et al. (2014) recently provided excellent evidence that *n*-butanol alters the concentration of PA on the pollen tube's plasma membrane *in vivo*. *n*-butanol was used to establish the PLD/PA signaling connection with G proteins, ABA triggered germination, primary root elongation, hypocotyl length, cotyledon expansion, inhibition of pollen tube germination and growth, proline accumulation, actin cytoskeleton rearangement and microtubule reorganization (Munnik et al., 1995; Ritchie and Gilroy, 1998; Dhonukshe et al., 2003; Gardiner et al., 2003; Potocky et al., 2003; Thiery et al., 2004; Motes et al., 2005; Pleskot et al., 2010, 2014). We would like to mention that it is necessary to keep in mind the possibility that the effect of *n*-butanol is not so specific as was mentioned by Hirase et al. (2006), who observed that *n*-butanol induced the depolymerization of microtubules. Although the use of *t*-butanol, as a control, can serve as convincing proof.

In our study, the treatment of *A. thaliana* seedlings with *n*-butanol rapidly decreased *PR-1* transcription in the presence of SA and this effect is clearly dose dependent (**Figures 1A,B**). The effect on the transcription of *WRKY38* and *S3H* was much less pronounced (**Figures 1C,D**) but in the case of *WRKY38* the decrease was significant (more than two times) and so the transcription pattern seems similar to *PR-1*. It is not surprising, as the transcription of *WRKY38* is also NPR1 dependent. Our results are in agreement with the results obtained by Krinke et al. (2009) in *A. thaliana* suspension cells. *n*-butanol did not have a significant effect on *S3H* transcription. S3H is responsible for a conversion of SA, therefore its transcription should be induced immediately by higher levels of SA and the signaling events downstream to SA can have only a minor effect on *S3H* transcription (**Figure 1D**). In fact, the connection between the S3H effect and NPR1 has not yet been described in detail.

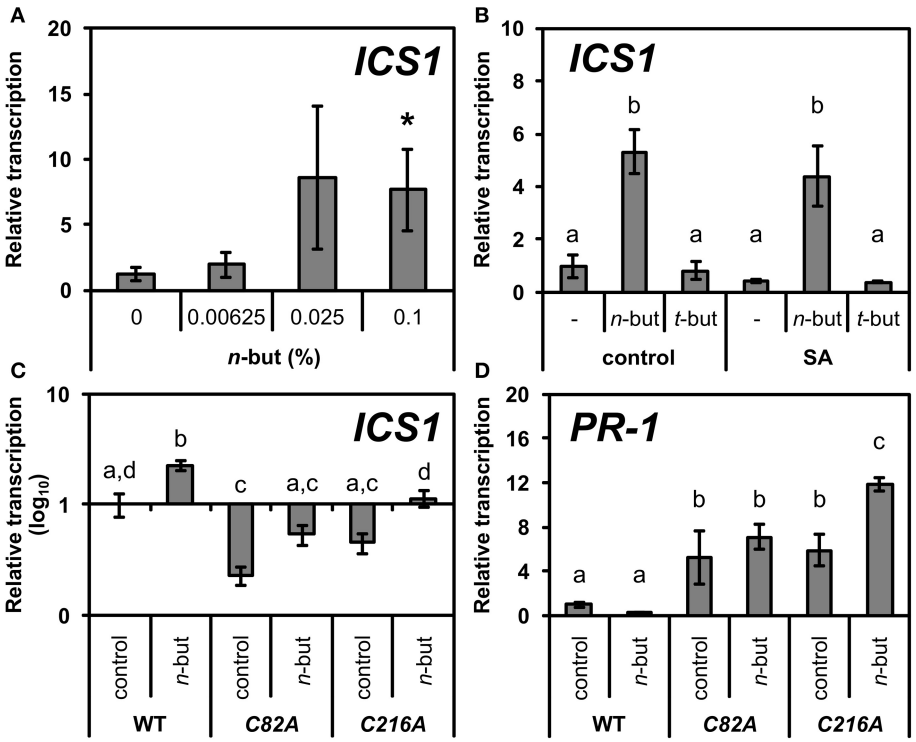


FIGURE 4 | Transcription of *ICS1* and *PR-1* in wild type and mutants of *Arabidopsis thaliana* constitutively expressing monomeric NPR1 in the presence of *n*-butanol. (A) Ten-day-old *A. thaliana* seedlings (wt) were treated for 6 h with 50 μM NaSA (SA) and 0.00625, 0.025, 0.1% *n*-butanol. **(B)** Ten-day-old *A. thaliana* seedlings (wt) were treated for 6 h with 0.1% *n*-butanol and 0.1% *t*-butanol or with 50 μM NaSA (SA) together with the above mentioned alcohols. **(C,D)** Ten-day-old seedlings of *35S::npr1C82A-GFP (C82A)* and *35S::npr1C216A-GFP (C216A) A. thaliana* mutants were treated for 6 h with fresh MS medium (control) or MS with 0.1% *n*-butanol. Error bars represent SE from three independent repeats. Asterisks indicate statistically significant differences compared to control, non-treated plants (*$P < 0.05$, Student's *t*-test) for **(A)**. Different letters indicate significant differences ($P < 0.05$) and were calculated with One-Way ANOVA and Fisher's LSD test for **(B–D)**. The *ICS1* and *PR-1* transcription was normalized to a reference gene *SAND*.

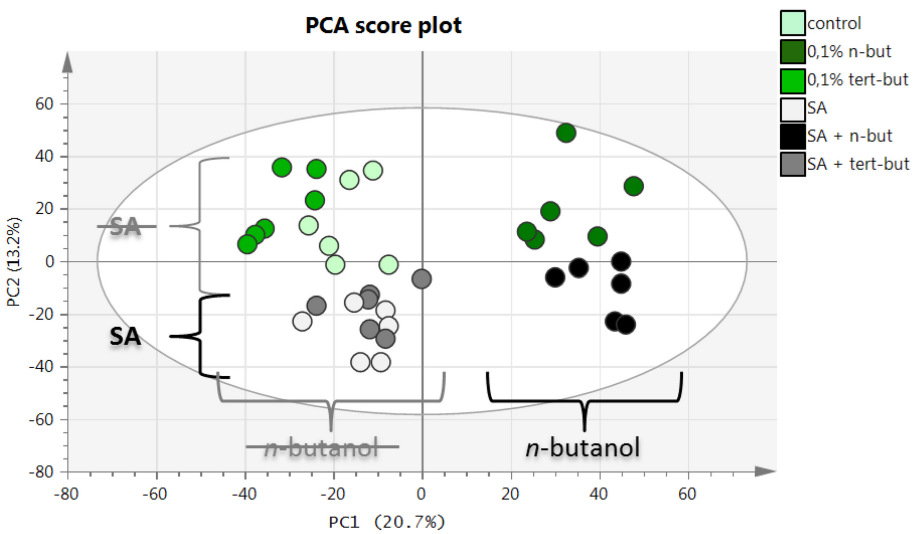


FIGURE 5 | PCA score plot for LC-ESI(-)-MS data of *n*-butanol and salicylic acid metabolome in *A. thaliana*. Ten-day-old *A. thaliana* seedlings were treated for 6 h with 0.1% *n*-butanol or 0.1% *t*-butanol or with 50 μM NaSA (SA) together with the aformentioned alcohols. Fresh MS medium was used as a control. This experiment was done in three biological repeats with similar results.

Table 1 | The most distinct metabolites (markers) of *A. thaliana* seedlings.

m/z	RT (min)	Ion elemental Formula	Tentative identification	Ion	Mass error (ppm)	Marker of treatment
137.0240	3.31	$C_7H_5O_3$	Salicylic acid	[M-H]-	5.0	SA
202.0714	1.61	$C_8H_{12}NO_5$	N-Butyryl-L-aspartic acid	[M-H]-	2.0	n-but
235.1180	2.63	$C_{10}H_{19}O_6$	Butyl—hexoside	[M-H]-	1.6	n-but
295.1028	1.79	$C_{11}H_{19}O_9$	?	?	1.5	n-but
299.0768	2.04	$C_{13}H_{15}O_8$	Salicylic acid—hexoside	[M-H]-	2.2	SA
536.1651	0.69	?	?	?	?	n-but
643.2446	2.63	?	?	?	?	n-but

A deeper insight into the mode of action of *n*-butanol in SA signaling provided the experiment with *35S::NPR1-GFP A. thaliana* mutants. We observed that effect of *n*-butanol is closely connected with the NPR1 localization in plant cells. In the *35S::NPR1-GFP* plants, *n*-butanol blocks NPR1 accumulation in the nucleus in the presence of SA (**Figure 2**). This finding well corresponds with the suppressive effect of *n*-butanol on the transcription of *PR-1*. Zhang et al. (2010) showed that nuclear localization of NPR1 negatively regulates the transcription of the *ICS1* gene. We observed that *n*-butanol induces the transcription of *ICS1* (**Figure 4**), which supports the idea that *n*-butanol blocks translocation of monomeric NPR1 to the nucleus. It was reported that the proteasome degrades NPR1 monomers in the nucleus (Spoel et al., 2009). Based on that fact we used *35S::npr1C82A-GFP* and *35S::npr1C216A-GFP* mutants which constitutively express a higher amount of NPR1 monomers and also exhibit a higher accumulation of NPR1 in the nucleus (Mou et al., 2003). In these mutants, we investigated the effect of *n*-butanol. As *n*-butanol treatment revealed no effect on the nuclear localization of NPR1 in the *35S::npr1C82A-GFP* and *35S::npr1C216A-GFP* mutants (**Figure 3**), we can assume that *n*-butanol acts in the cytosol in the SA pathway before or during NPR1 translocation to the nucleus. *n*-butanol could either affect the transmission of the monomer from the cytosol to the nucleus e.g., by direct effect of *n*-butanol on the nucleopores or by active transport which can be mediated by PA. This mechanism was recently shown in the nuclear localization of the MYB transcription factor (Yao et al., 2013). Nevertheless, we cannot exclude the possibility that *n*-butanol acts upstream to NPR1 monomerization (**Figure 8**).

PHOSPHOLIPIDS IN SA SIGNALING

Based on the above mentioned observations, we would like to highlight the possible connection between the phospholipid signaling system and the SA pathway. SA treatment increased the PA level or the PLD activity in *A. thaliana, B. napus.* and soybean (Profotova et al., 2006; Kalachova et al., 2012, 2013; Rainteau et al., 2012). Krinke et al. (2009) showed in *A. thaliana* suspension cells that SA treatment led to a rapid increase of the PA level *in vivo*. We observed that the exogenous PA is capable of preventing the disruption of the actin cytoskeleton caused by SA (Matouskova et al., 2014). PLD and PA are not the only members of large phospholipid family involved in SA signaling. Interestingly, it was shown that SA treatment activates type-III phosphatidylinositol-4-kinase (PI4K), which is responsible for the formation of phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol-4,5-bisphosphate (PIP_2) in *A. thaliana* suspension cells (Krinke et al., 2007). Recently, we showed that double knock-out mutation of two isoforms PI4Kβ1β2 triggers SA signaling, suggesting them to be negative regulators of SA signaling (Sasek et al., 2014). For more information about the connection between hormones and phospholipid signaling see the review (Janda et al., 2013).

The interest of importance is to find out the particular isoform(s) of PLD responsible for the effect of *n*-butanol. Based on this, we performed the *in silico* experiment to find the possible PLD isoforms involved in SA signaling (**Figure 7**). We investigated the transcription of all PLD isoforms in the publicly available database, Genevestigator (Hruz et al., 2008), in response to SA, BTH (benzothiadiazole; a functional analog of SA), EF-Tu, flg22, pep2 (well described PAMPs) triggering SA signaling. All studies, which we included to this analysis, were performed on the *A. thaliana* ecotype Col-0. The screening showed that the promising candidates could be PIP_2-dependent isoforms from the PLDβ, PLDγ and PLDζ families (**Figure 7**). We can speculate that they could be connected with the above mentioned PI4K activity, producing precursor for PIP_2 biosynthesis. The evidence that PLD isoforms exhibit redundant effect upon *Pseudomonas syrigae* infection was provided recently by Johansson et al. (2014).

METABOLOMIC SCREENING

The aim of this part of our study was to find the metabolic compounds, which are affected by *n*-butanol and involved in the SA pathway. For this purpose, we used the mass spectrometry-based metabolomic fingerprinting described by Vaclavik et al. (2013). A very important output from the screening is the evidence that *t*-butanol treatment, used in our study (but also by other researchers studying PLD function) as a negative control to *n*-butanol, is really biologically "inactive." This is based on the fact that the PCA analysis of the samples determined no differences between control samples vs. *t*-butanol and the SA treated samples vs. *t*-butanol (i.e., their metabolomes were similar). On the other hand, the samples treated with *n*-butanol clustered very well (**Figure 5**). We were able to identify several characteristic metabolites for the samples treated with *n*-butanol. We were also able to predict the molecular formulas and we proposed their tentative identification (**Table 1**). It is not surprising that a higher amount of SA and SA-hexoside was found in the samples treated by SA. In fact, we were able to identify only a few metabolites affected by SA, probably due to the relatively short time of treatment. It was shown that BTH causes significant alterations in metabolome 24 h after treatment, while after 4 h the changes were much less

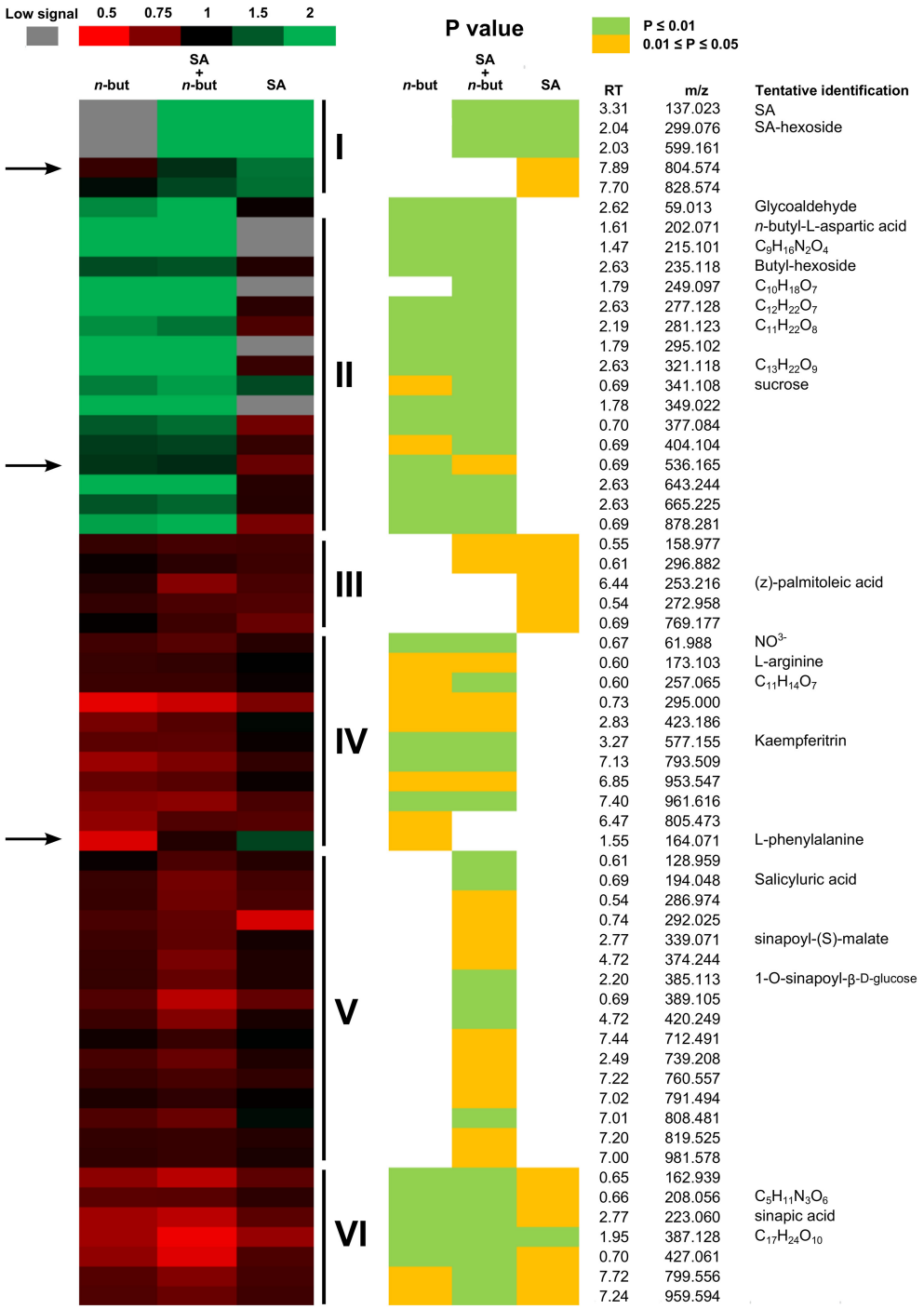


FIGURE 6 | Heat map of metabolites. Ten-day-old *Arabidopsis thaliana* seedlings were treated for 6 h with 0.1% *n*-butanol or with 50 μM NaSA (SA) and both chemicals together. Fresh MS was used as a control. The Heat map values represent a ratio between the treated and control samples (treatment/control). The green color indicates increased values, red indicates decreased values and black indicates zero; see the color scale. The gray color indicates metabolites with a low signal in particular treated samples. *P*-value is represented by yellow $0.01 < P < 0.05$; bright green $P < 0.01$ (Student's *t*-test). The arrows indicate putative metabolites with a similar pattern of response to treatment as the *PR-1* gene transcription. Group I represents metabolites induced by SA. Group II represents metabolites induced by *n*-butanol. Group III represents metabolites suppressed by SA. Group IV represents metabolites suppressed by *n*-butanol. Group V represents metabolites suppressed only when SA and *n*-butanol were applied together. Group VI represents metabolites suppressed by all treatments. This experiment was repeated in three biological repeats with similar results. RT, retention time; m/z, mass to charge ratio.

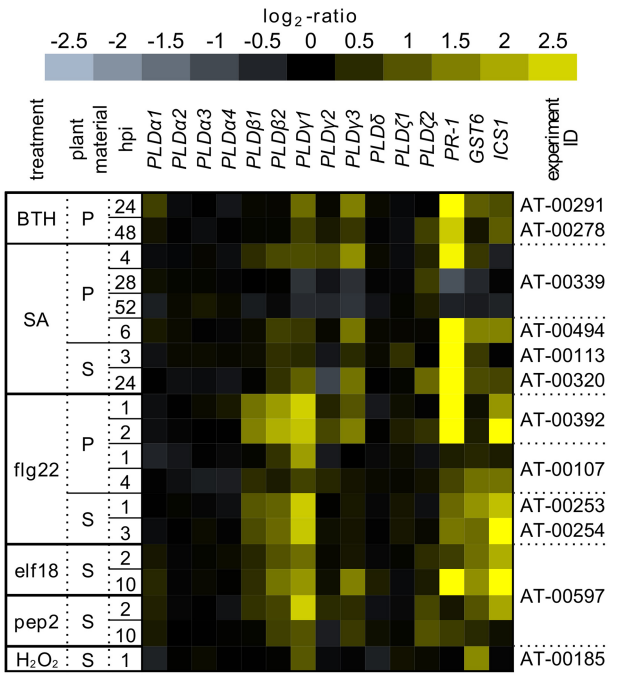


FIGURE 7 | The transcription pattern of PLDs in *A. thaliana* under stress conditions. The transcriptomic data collected from the public database Genevestigator after treatment by BTH, SA, flg22, elf18, pep2, and H_2O_2. All experiments were performed on the *A. thaliana* ecotype Col-0 in the different developmental stages treated with different concentrations of compounds and in different time points. The experiment ID provides the identification for the particular experiment in Genevestigator. For comparison, the transcription of *PR-1*, *GST6,* and *ICS1* genes were added.

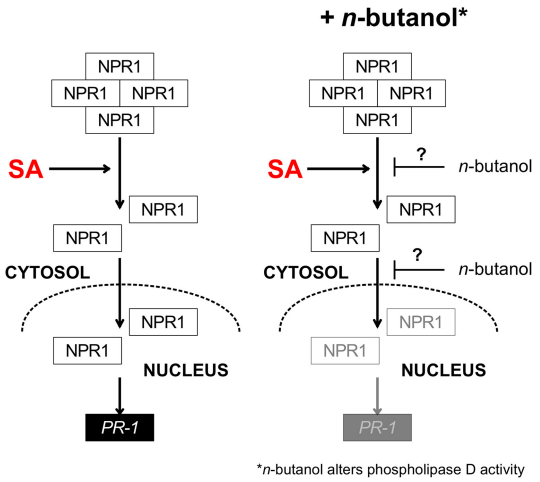


FIGURE 8 | Scheme summarizing the effect of *n*-butanol on the SA pathway in *A. thaliana*. In the presence of salicylic acid NPR1 monomerize, translocates into the nucleus and induces the *PR-1* gene transcription. In the presence of *n*-butanol the accumulation of NPR1 in the nucleus is decreased and the transcription of *PR-1* is supressed. The proposed site of *n*-butanol effect is shown by blunt-end arrows with question mark. SA—salicylic acid, NPR1—nonexpressor of pathogenesis related1, *PR-1*—pathogenesis related 1.

significant (Hien Dao et al., 2009). We chose the 6 h time-point to get the information in the same time frame as we used for the transcriptional study (**Figure 1**). Interestingly, *n*-butanol had a higher impact on the *A. thaliana* metabolome compared to the SA treatment. Seventeen metabolites were affected by SA and 34 by *n*-butanol (**Figure 6**).

We were able to predict structure for several metabolites that changed upon treatment. The heat map representing the changes of 61 metabolites supplemented with RT-m/z and the putative names of several predicted compounds is shown in **Figure 6**. Interestingly, the behavior of phenylalanine, a precursor of SA biosynthesis, exhibits a similar pattern as *PR-1* transcription upon treatment. Another two compounds exhibit patterns similar to *PR-1* (RT_m/z 7.89_804.5 and 0.69_536.1; **Figure 6**). These compounds could be interesting targets of further research.

CONCLUSION

The observations were summarized in the scheme presented in **Figure 8**. *n*-butanol affects *PR-1* transcription and NPR1 accumulation in the nucleus in the presence of SA. We propose that our current study should be a new puzzle fitting in the previous idea that PA produced by PLD is involved in the SA signaling pathway as *n*-butanol alters PLD activity. We found 61 metabolites whose levels were changed upon the treatment with *n*-butanol and SA. We showed that the *n*-butanol treatment has a higher impact on the metabolome than treatment with SA. We provided the metabolomic evidence that *t*-butanol can be really used as a negative control in studies using *n*-butanol.

AUTHOR CONTRIBUTIONS

Martin Janda created the conception and design, performed and analyzed the experiments and also composed the manuscript. Vladimír Šašek designed, performed and analyzed the experiments. Jan Andrejch performed and analyzed the confocal microscopy experiments. Hana Chmelařová performed and analyzed the experiments (metabolomic screening). Miroslava Nováková performed and analyzed the experiments (confocal microscopy) and critically revised the manuscript. Jana Hajšlová analyzed the data (metabolomic screening) and critically revised the manuscript. Lenka Burketová critically revised the manuscript. Olga Valentová created the conception, critically revised the manuscript and also composed the manuscript. All authors concurred in the final version of the manuscript.

ACKNOWLEDGMENTS

We would like to thank Myrta Pařízková for her excellent technical support and also to Bc. Lucie Lamparová for her support. This work was supported by the Czech Science Foundation grants nos. 501/11/1654 and from specific university research (MSMT No 21/2015). Martin Janda would like to thank associate professor Zuzana Novotná for her supervising during master study. Martin Janda also want to thank to Dr. Kenichi Tsuda from Max Planck Institute of Plant Breeding Research and Lucie Trdá PhD. for providing advices during research. Special thanks belong to professor Xinnian Dong from Duke University, who kindly provided us the

seeds of *35S::NPR1-GFP*, *35S::npr1C82A-GFP*, *35S::npr1C216A-GFP*. The authors express their thanks to the developers of open source software used in the preparation of this study, particularly Gimp and Inkscape.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2015.00059/abstract

REFERENCES

Blanco, F., Salinas, P., Cecchini, N. M., Jordana, X., Van Hummelen, P., Alvarez, M. E., et al. (2009). Early genomic responses to salicylic acid in *Arabidopsis*. *Plant Mol. Biol.* 70, 79–102. doi: 10.1007/s11103-009-9458-1

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. N. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired-resistance. *Plant Cell* 6, 1583–1592.

Clay, N. K., Adio, A. M., Denoux, C., Jander, G., and Ausubel, F. M. (2009). Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science* 323, 95–101. doi: 10.1126/science.1164627

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., and Scheible, W.-R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* 139, 5–17. doi: 10.1104/pp.105.063743

De Torres Zabela, M., Fernandez-Delmond, I., Niittyla, T., Sanchez, P., and Grant, M. (2002). Differential expression of genes encoding *Arabidopsis* phospholipases after challenge with virulent or avirulent *Pseudomonas* isolates. *Mol. Plant Microbe Interact.* 15, 808–816. doi: 10.1094/MPMI.2002.15.8.808

Dhonukshe, P., Laxalt, A. M., Goedhart, J., Gadella, T. W., and Munnik, T. (2003). Phospholipase d activation correlates with microtubule reorganization in living plant cells. *Plant Cell* 15, 2666–2679. doi: 10.1105/tpc.014977

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gardiner, J., Collings, D. A., Harper, J. D., and Marc, J. (2003). The effects of the phospholipase D-antagonist 1-butanol on seedling development and microtubule organisation in Arabidopsis. *Plant Cell Physiol.* 44, 687–696. doi: 10.1093/pcp/pcg095

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Hien Dao, T. T., Puig, R. C., Kim, H. K., Erkelens, C., Lefeber, A. W., Linthorst, H. J., et al. (2009). Effect of benzothiadiazole on the metabolome of *Arabidopsis thaliana*. *Plant Physiol. Biochem.* 47, 146–152. doi: 10.1016/j.plaphy.2008.10.001

Hirase, A., Hamada, T., Itoh, T. J., Shimmen, T., and Sonobe, S. (2006). *n*-Butanol induces depolymerization of microtubules *in vivo* and *in vitro*. *Plant Cell Physiol.* 47, 1004–1009. doi: 10.1093/pcp/pcj055

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., et al. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics* 2008:420747. doi: 10.1155/2008/420747

Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T., et al. (2002). bZIP transcription factors in Arabidopsis. *Trends Plant Sci.* 7, 106–111. doi: 10.1016/S1360-1385(01)02223-3

Janda, M., Planchais, S., Djafi, N., Martinec, J., Burketova, L., Valentova, O., et al. (2013). Phosphoglycerolipids are master players in plant hormone signal transduction. *Plant Cell Rep.* 32, 839–851. doi: 10.1007/s00299-013-1399-0

Janda, M., and Ruelland, E. (2014). Magical mystery tour: salicylic acid signalling. *Environ. Exp. Bot.* doi: 10.1016/j.envexpbot.2014.07.003. (in press).

Johansson, O. N., Fahlberg, P., Karimi, E., Nilsson, A. K., Ellerström, M., and Andersson, M. X. (2014). Redundancy among phospholipase D isoforms in resistance triggered by recognition of the *Pseudomonas syringae* effector AvrRpm1 in *Arabidopsis thaliana*. *Front. Plant Sci.* 5:639. doi: 10.3389/fpls.2014.00639

Kalachova, T., Lakovenko, O., Kretinin, S., and Kravets, V. (2013). Involvement of phospholipase D and NADPH-oxidase in salicylic acid signaling cascade. *Plant Physiol. Biochem.* 66, 127–133. doi: 10.1016/j.plaphy.2013.02.006

Kalachova, T. A., Iakovenko, O. M., Kretinin, S. V., and Kravets, V. S. (2012). Effects of salicylic and jasmonic acid on Phospholipase D activity and the level of active oxygen species in soybean seedlings. *Biochemistry (Moscow)* 29, 169–176. doi: 10.1134/S1990747812030099

Kim, K. C., Lai, Z., Fan, B., and Chen, Z. (2008). Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. *Plant Cell* 20, 2357–2371. doi: 10.1105/tpc.107.055566

Kinkema, M., Fan, W. H., and Dong, X. N. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.1105/tpc.12.12.2339

Krinke, O., Ruelland, E., Valentova, O., Vergnolle, C., Renou, J. P., Taconnat, L., et al. (2007). Phosphatidylinositol 4-kinase activation is an early response to salicylic acid in arabidopsis suspension cells. *Plant Physiol.* 144, 1347–1359. doi: 10.1104/pp.107.100842

Krinke, O., Flemr, M., Vergnolle, C., Collin, S., Renou, J. P., Taconnat, L., et al. (2009). Phospholipase D activation is an early component of the salicylic acid signaling pathway in *Arabidopsis* cell suspensions. *Plant Physiol.* 150, 424–436. doi: 10.1104/pp.108.133595

Lee, S. M., Suh, S., Kim, S., Crain, R. C., Kwak, J. M., Nam, H. G., et al. (1997). Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. *Plant J.* 12, 547–556. doi: 10.1046/j.1365-313X.1997.00547.x

Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000). Phospholipase D: molecular and cell biology of a novel gene family. *Biochem. J.* 345(Pt 3), 401–415. doi: 10.1042/0264-6021:3450401

Marshall, O. J. (2004). PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* 20, 2471–2472. doi: 10.1093/bioinformatics/bth254

Matouskova, J., Janda, M., Fiser, R., Sasek, V., Kocourkova, D., Burketova, L., et al. (2014). Changes in actin dynamics are involved in salicylic acid signaling pathway. *Plant Sci.* 223, 36–44. doi: 10.1016/j.plantsci.2014.03.002

McGee, J. D., Roe, J. L., Sweat, T. A., Wang, X. M., Guikema, J. A., and Leach, J. E. (2003). Rice phospholipase D isoforms show differential cellular location and gene induction. *Plant Cell Physiol.* 44, 1013–1026. doi: 10.1093/pcp/pcg125

Motes, C. M., Pechter, P., Yoo, C. M., Wang, Y. S., Chapman, K. D., and Blancaflor, E. B. (2005). Differential effects of two phospholipase D inhibitors, 1-butanol and N-acylethanolamine, on *in vivo* cytoskeletal organization and Arabidopsis seedling growth. *Protoplasma* 226, 109–123. doi: 10.1007/s00709-005-0124-4

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Munnik, T., Arisz, S. A., De Vrije, T., and Musgrave, A. (1995). G Protein activation stimulates phospholipase D signaling in plants. *Plant Cell* 7, 2197–2210. doi: 10.1105/tpc.7.12.2197

Novakova, M., Sasek, V., Dobrev, P. I., Valentova, O., and Burketova, L. (2014). Plant hormones in defense response of *Brassica napus* to Sclerotinia sclerotiorum - reassessing the role of salicylic acid in the interaction with a necrotroph. *Plant Physiol. Biochem.* 80, 308–317. doi: 10.1016/j.plaphy.2014.04.019

Pleskot, R., Pejchar, P., Bezvoda, R., Lichtscheidl, I. K., Wolters-Arts, M., Marc, J., et al. (2012). Turnover of phosphatidic acid through distinct signaling pathways affects multiple aspects of pollen tube growth in tobacco. *Front. Plant Sci.* 3:54. doi: 10.3389/fpls.2012.00054

Pleskot, R., Pejchar, P., Staiger, C. J., and Potocky, M. (2014). When fat is not bad: the regulation of actin dynamics by phospholipid signaling molecules. *Front. Plant Sci.* 5:5. doi: 10.3389/fpls.2014.00005

Pleskot, R., Potocky, M., Pejchar, P., Linek, J., Bezvoda, R., Martinec, J., et al. (2010). Mutual regulation of plant phospholipase D and the actin cytoskeleton. *Plant J.* 62, 494–507. doi: 10.1111/j.1365-313X.2010.04168.x

Potocky, M., Elias, M., Profotova, B., Novotna, Z., Valentova, O., and Zarsky, V. (2003). Phosphatidic acid produced by phospholipase D is required for tobacco pollen tube growth. *Planta* 217, 122–130. doi: 10.1007/s00425-002-0965-4

Potocky, M., Pleskot, R., Pejchar, P., Vitale, N., Kost, B., and Zarsky, V. (2014). Live-cell imaging of phosphatidic acid dynamics in pollen tubes visualized by Spo20p-derived biosensor. *New Phytol.* 203, 483–494. doi: 10.1111/nph.12814

Profotova, B., Burketova, L., Novotna, Z., Martinec, J., and Valentova, O. (2006). Involvement of phospholipases C and D in early response to SAR and ISR inducers in *Brassica napus* plants. *Plant Physiol. Biochem.* 44, 143–151. doi: 10.1016/j.plaphy.2006.02.003

Rainteau, D., Humbert, L., Delage, E., Vergnolle, C., Cantrel, C., Maubert, M. A., et al. (2012). Acyl Chains of Phospholipase D transphosphatidylation products in arabidopsis cells: a study using multiple reaction monitoring mass spectrometry. *PLoS ONE* 7:e41985. doi: 10.1371/journal.pone.0041985

Ritchie, S., and Gilroy, S. (1998). Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. *Proc. Natl. Acad. Sci. U.S.A.* 95, 2697–2702. doi: 10.1073/pnas.95.5.2697

Sasek, V., Janda, M., Delage, E., Puyaubert, J., Guivarc'h, A., Lopez Maseda, E., et al. (2014). Constitutive salicylic acid accumulation in pi4kIIIbeta1beta2 Arabidopsis plants stunts rosette but not root growth. *New Phytol.* 203, 805–816. doi: 10.1111/nph.12822

Sasek, V., Novakova, M., Jindrichova, B., Boka, K., Valentova, O., and Burketova, L. (2012). Recognition of avirulence gene AvrLm1 from hemibiotrophic ascomycete Leptosphaeria maculans triggers salicylic acid and ethylene signaling in *Brassica napus*. *Mol. Plant Microbe Interact.* 25, 1238–1250. doi: 10.1094/MPMI-02-12-0033-R

Spoel, S. H., Mou, Z. L., Tada, Y., Spivey, N. W., Genschik, P., and Dong, X. N. A. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* 137, 860–872. doi: 10.1016/j.cell.2009.03.038

Thiery, L., Leprince, A. S., Lefebvre, D., Ghars, M. A., Debarbieux, E., and Savoure, A. (2004). Phospholipase D is a negative regulator of proline biosynthesis in *Arabidopsis thaliana*. *J. Biol. Chem.* 279, 14812–14818. doi: 10.1074/jbc.M308456200

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* 13, 459–465. doi: 10.1016/j.pbi.2010.04.006

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses (vol 53, pg 763, 2008). *Plant J.* 55, 1061–1061. doi: 10.1111/j.1365-313X.2008.03589.x

Vaclavik, L., Mishra, A., Mishra, K. B., and Hajslova, J. (2013). Mass spectrometry-based metabolomic fingerprinting for screening cold tolerance in *Arabidopsis thaliana* accessions. *Anal. Bioanal. Chem.* 405, 2671–2683. doi: 10.1007/s00216-012-6692-1

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. (2005). Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 308, 1036–1040. doi: 10.1126/science.1108791

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Yang, S. F., Freer, S., and Benson, A. A. (1967). Transphosphatidylation by Phospholipase D. *J. Biol. Chem.* 242, 477.

Yao, H., Wang, G., Guo, L., and Wang, X. (2013). Phosphatidic acid interacts with a MYB transcription factor and regulates its nuclear localization and function in Arabidopsis. *Plant Cell* 25, 5030–5042. doi: 10.1105/tpc.113.120162

Young, S. A., Wang, X. M., and Leach, J. E. (1996). Changes in the plasma membrane distribution of rice phospholipase D during resistant interactions with Xanthomonas oryzae pv oryzae. *Plant Cell* 8, 1079–1090.

Zhang, K., Halitschke, R., Yin, C., Liu, C. J., and Gan, S. S. (2013). Salicylic acid 3-hydroxylase regulates Arabidopsis leaf longevity by mediating salicylic acid catabolism. *Proc. Natl. Acad. Sci. U.S.A.* 110, 14807–14812. doi: 10.1073/pnas.1302702110

Zhang, X., Chen, S., and Mou, Z. (2010). Nuclear localization of NPR1 is required for regulation of salicylate tolerance, isochorismate synthase 1 expression and salicylate accumulation in Arabidopsis. *J. Plant Physiol.* 167, 144–148. doi: 10.1016/j.jplph.2009.08.002

Zhang, Y. L., Fan, W. H., Kinkema, M., Li, X., and Dong, X. N. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528. doi: 10.1073/pnas.96.11.6523

Zhao, J., Devaiah, S. P., Wang, C. X., Li, M. Y., Welti, R., and Wang, X. M. (2013). Arabidopsis phospholipase D1 modulates defense responses to bacterial and fungal pathogens. *New Phytol.* 199, 228–240. doi: 10.1111/Nph.12256

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 November 2014; accepted: 22 January 2015; published online: 18 February 2015.

Citation: Janda M, Šašek V, Chmelařová H, Andrejch J, Nováková M, Hajšlová J, Burketová L and Valentová O (2015) Phospholipase D affects translocation of NPR1 to the nucleus in Arabidopsis thaliana. Front. Plant Sci. ***6****:59. doi: 10.3389/fpls.2015.00059*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

frontiers in Plant Science

REVIEW
published: 19 May 2015
doi: 10.3389/fpls.2015.00349

Microbial effectors target multiple steps in the salicylic acid production and signaling pathway

*Shigeyuki Tanaka, Xiaowei Han and Regine Kahmann**

Department of Organismic Interactions, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

Microbes attempting to colonize plants are recognized through the plant immune surveillance system. This leads to a complex array of global as well as specific defense responses, which are often associated with plant cell death and subsequent arrest of the invader. The responses also entail complex changes in phytohormone signaling pathways. Among these, salicylic acid (SA) signaling is an important pathway because of its ability to trigger plant cell death. As biotrophic and hemibiotrophic pathogens need to invade living plant tissue to cause disease, they have evolved efficient strategies to downregulate SA signaling by virulence effectors, which can be proteins or secondary metabolites. Here we review the strategies prokaryotic pathogens have developed to target SA biosynthesis and signaling, and contrast this with recent insights into how plant pathogenic eukaryotic fungi and oomycetes accomplish the same goal.

Keywords: virulence effector, salicylic acid, bacterial plant pathogens, fungal plant pathogens, oomycete plant pathogens

OPEN ACCESS

Edited by:
Hua Lu,
University of Maryland, Baltimore County, USA

Reviewed by:
Roger W. Innes,
Indiana University, USA
Barbara N. Kunkel,
Washington University in St. Louis, USA

***Correspondence:**
Regine Kahmann,
Department of Organismic Interactions, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse 10, D-35043 Marburg, Germany
kahmann@mpi-marburg.mpg.de

Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *28 February 2015*
Accepted: *03 May 2015*
Published: *19 May 2015*

Citation:
Tanaka S, Han X and Kahmann R (2015) Microbial effectors target multiple steps in the salicylic acid production and signaling pathway. Front. Plant Sci. 6:349. doi: 10.3389/fpls.2015.00349

Introduction

The plant hormone salicylic acid (SA) has been extensively studied because of its influence on various plant developmental processes as well as its role on resistance to abiotic and biotic stresses (Vlot et al., 2009). In the context of biotic stress SA has been shown to be a crucial player in pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) as well as effector-triggered immunity (ETI; Jones and Dangl, 2006). PAMP-triggered immunity is a plant defense reaction in which pathogens are recognized through conserved molecular patterns like flg22, an epitope of bacterial flagellin, elf18, a component of bacterial elongation factor EF-Tu, bacterial peptidoglycans, and chitin, a typical component of the fungal cell wall. PAMPs are perceived by membrane localized pattern-recognition receptors (PRRs), many of which are receptor-like kinases (RLKs) that function together with co-receptors (Macho and Zipfel, 2015). Activation of these PRRs by PAMP ligand binding elicits plant defense responses that confer a certain level of protection against virulent pathogens. PAMP-induced defense responses include calcium spiking, the production of reactive oxygen species, callose deposition which interferes with pathogen spread, the production of antimicrobial compounds, accumulation of the plant hormone SA, and the synthesis of pathogenesis-related (PR) proteins, many of which exhibit toxicity directed against the pathogen (Newman et al., 2013). Plants can also recognize an invading pathogen through secreted protein effectors and mount a highly effective defense response that is associated with programmed cell death (hypersensitive response, HR) at the site of pathogen infection. This ETI is induced by direct or indirect recognition of pathogen effectors by plant resistance (R) proteins. Direct interactions between R proteins and effector proteins have been demonstrated only rarely (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). More common are indirect interactions which involve host targets that guard the R protein or act as decoy

to detect pathogen effectors via R proteins, respectively (see van der Hoorn and Kamoun, 2008 for details). Pathogen effectors triggering ETI were initially identified as the products of avirulence genes (Avr). However, with the advent of whole genome sequencing and elucidation of genome-wide effectomes, Avr proteins are now included in the large group of microbial effectors and are termed effectors triggering ETI in resistant plants. R proteins typically belong to the nucleotide-binding leucine-rich repeat (NB-LRR) class, a large family of intracellular receptors (Zipfel, 2014; Macho and Zipfel, 2015), that respond to the respective pathogen effectors translocated from the pathogen to the host. Gram-negative plant pathogenic bacteria possess type III secretion systems (T3SS) to inject bacterial type III effectors (T3Es) into host cells through a specialized syringe-like structure. T3Es of pathogenic bacteria can downregulate PAMP-triggered defense responses at many levels, i.e., by direct targeting the membrane bound PRRs or their co-receptors to affect their signaling function, by specifically interfering with expression of PRR proteins, by affecting the stability of PRRs or by inactivating downstream components like MAP kinases or interfering with vesicle trafficking, which is necessary to downregulate PTI responses like callose deposition. These processes have recently been reviewed comprehensively (Macho and Zipfel, 2015) and will not be covered here as they do not specifically address SA signaling.

Eukaryotic plant pathogenic microbes like oomycetes and fungi also transfer effectors to their hosts, and this has been functionally demonstrated for many Avr proteins by expressing the respective genes in resistant hosts and demonstrating the elicitation of cell death. While this provides a simple assay for the Avr function of effectors, it is much more difficult to determine the virulence function of effectors. In addition, the mechanisms how filamentous eukaryotic plant pathogens translocate effectors are still under debate (Rafiqi et al., 2012; Doehlemann et al., 2014; Lo Presti et al., 2015).

Salicylic acid acts as a crucial signaling molecule in pathways conferring local and systemic immunity against a large number of pathogens. SA was first shown to be the key plant hormone for triggering systemic acquired resistance (SAR), an induced defense elicited by an avirulent pathogen involving the entire plant and providing protection against a broad spectrum of pathogens (Durrant and Dong, 2004). The important role of SA as a signaling molecule during basal and induced responses to virulent pathogens has been demonstrated by the isolation of plant mutants exhibiting increased susceptibility to virulent as well as avirulent pathogens. This includes the SALICYLIC ACID INDUCTION-DEFICIENT 2 (*sid2*), the ENHANCED DISEASE SUSCEPTIBILITY 5 (*eds5*), and the NON-EXPRESSOR OF PR GENES (*npr1*) mutants of *Arabidopsis thaliana*. Compared to these plant mutants NahG expressing plants, in which endogenous SA is removed by expressing a bacterial SA hydoxylase, show even stronger disease susceptibility toward virulent as well as avirulent pathogens (Cao et al., 1994; Delaney et al., 1994, 1995; Glazebrook et al., 1996; Nawrath and Metraux, 1999; Glazebrook, 2005). Furthermore it was demonstrated that SA signaling is generally important for immunity against biotrophs, while jasmonic acid (JA) and ethylene (ET) signaling confer immunity against necrotrophs (Glazebrook, 2005).

Given the importance of SA signaling in basal and induced plant defense it is clear that virulent hemibiotrophic and biotrophic pathogens that rely on living plant tissue have to downregulate SA levels to establish themselves inside the plant and cause disease. In this review we will address the intricate ways such microbes have developed to target the SA pathway to promote disease at the level of biosynthesis, signal transduction, and by affecting the crosstalk between SA and JA pathways. We will contrast modes of molecular intervention in these processes by bacterial and eukaryotic plant pathogen effectors, and highlight specifically recent findings in filamentous fungi and oomycetes.

Effectors Interfering with SA Biosynthesis and Accumulation

In plants two distinct pathways exist for the biosynthesis of SA and both start out with chorismate, the end product of the shikimate pathway. The isochorismate pathway (IC) is operative in plastids (**Figure 1**). The IC pathway is the prime source for SA accumulation in non-challenged and pathogen-challenged plants (Dempsey et al., 2011; Seyfferth and Tsuda, 2014). Chorismate is converted to isochorismate by isochorismate synthase (ICS). *A. thaliana* has two *ICS* genes (*ICS1* and *ICS2*), the products of which are localized in chloroplasts. In *A. thaliana eds16* mutants and *sid2* mutants where ICS1 is defective, SA accumulation is 90% lower than in wild-type plants upon pathogen challenge (Dewdney et al., 2000). ICS2 participates only weakly in SA synthesis and its contribution is only detectable in *ics1 ics2* double-mutants (Garcion et al., 2008; Dempsey et al., 2011). Isochorismate is then converted to SA either through an isochorismate pyruvate lyase-like enzyme in the chloroplast (that has not been identified) or a chloroplast enzyme related to chorismate mutase but with a higher affinity for isochorismate (**Figure 1**; Dempsey et al., 2011). The transmembrane protein EDS5 from *A. thaliana* belongs to the MATE transporter family (Nawrath et al., 2002). EDS5 is chloroplast-localized (**Figure 1**) and presumed to play a role in exporting SA from the plastid to the cytosol (Nawrath et al., 2002; Ishihara et al., 2008). The *eds5* mutants accumulate very little SA, and display hypersusceptibility to pathogens (Nawrath and Metraux, 1999; Nawrath et al., 2002).

The second pathway for producing SA is the phenylalanine ammonia-lyase (PAL) pathway (**Figure 1**) in which phenylalanine is converted by PAL to *trans*-cinnamic acid, which then serves as a precursor for various routes of SA biosynthesis (Dempsey et al., 2011). Because of the minor role of this pathway in SA biosynthesis in defense signaling, we will not discuss this pathway here in detail. We will also not discuss SA modifications like glucosylation, conjugation to amino acids, or methylation (Dempsey et al., 2011), because so far these processes have not been shown to be targeted by pathogen effectors.

Turning now to effectors modulating SA biosynthesis and/or accumulation, it has been shown that the *Pseudomonas syringae* virulence effector HopI1 belongs to this group. HopI1 is targeted to plastids where it induces the remodeling of thylakoid structures. The C-terminal domain of HopI1 binds to HSP70 resulting in the formation of large complexes in association with HSP70 and the

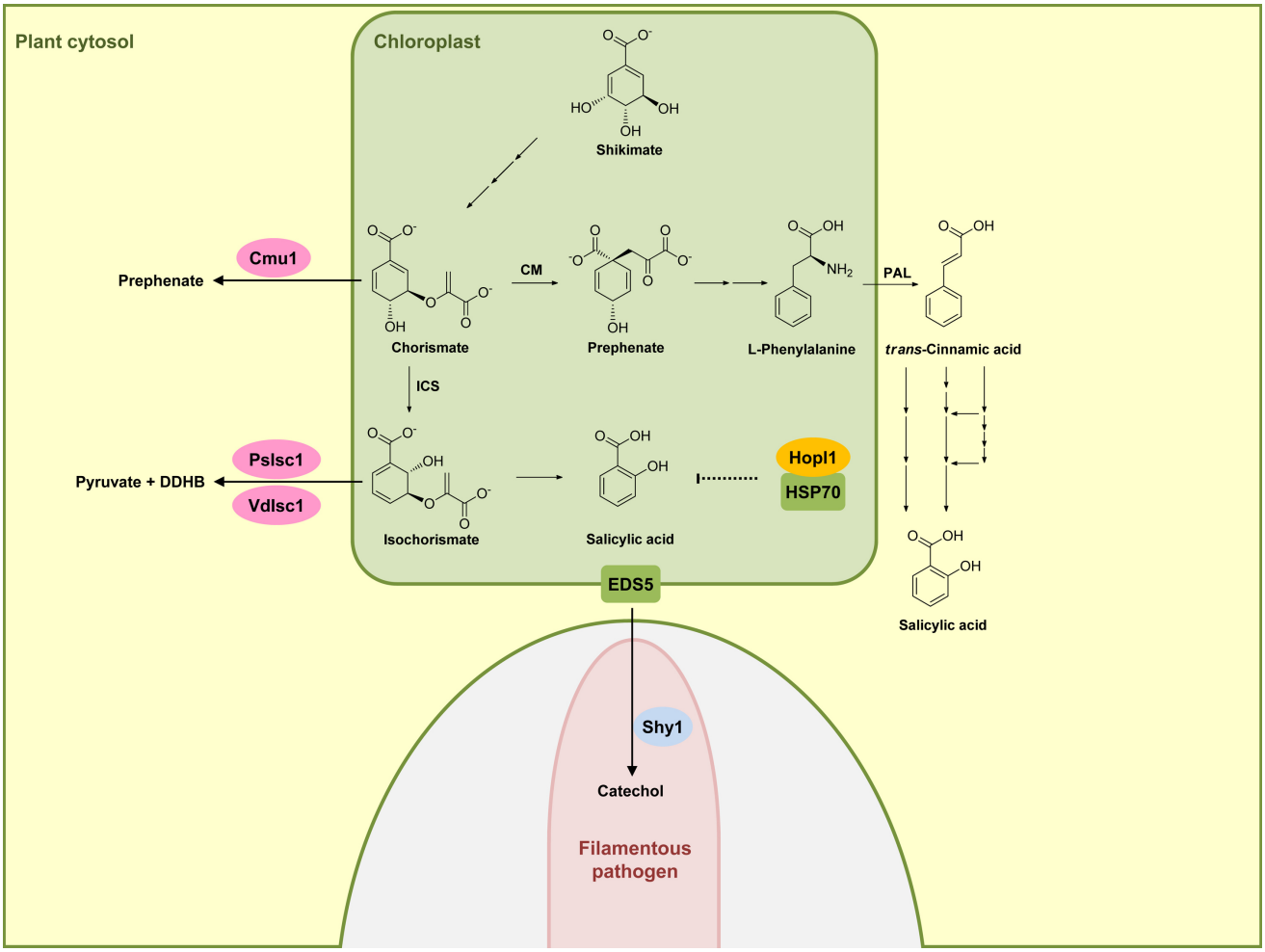


FIGURE 1 | Effectors interfering with salicylic acid biosynthetic pathway. In plants, salicylic acid (SA) is mainly produced via the isochorismate pathway (IC) in plastids (green compartment), but can also be synthesized through phenylalanine ammonia-lyase (PAL) pathway in the cytosol. HopI1 in *P. syringae* interacts with plastidic HSP70 (green), probably affecting SA biosynthesis or transport. Secreted Cmu1 from *U. maydis* is taken up by plant cells and is proposed to rechannel chorismic acid from plastids to the cytosol, thus lowering SA levels. The oomycete *P. sojae* and the fungus *V. dahliae* secrete isochorismatases Pslsc1 and Vdlsc1, respectively, converting isochorismate into 2,3-dihydro-2,3-dihydroxybenzoate (DDHB) and pyruvate. A fungal hypha is indicated in light pink in the lower part. This hypha is surrounded by the apoplast shown enlarged here (light gray) encased by the plant plasma membrane (green). Salicylate hydroxylase Shy1 (light blue) residing in the cytosol of *U. maydis*, can degrade SA. Enzymes are abbreviated: chorismate mutase (CM), isochorismate synthase (ICS). Fungal and oomycete effectors are indicated by pink ovals and bacterial effectors are indicated in dark yellow. Plant proteins are indicated by a green square. Solid arrows represent chemical reactions, dotted arrows indicate indirect inhibition and blunt ended arrows indicate inhibition (for details, see text).

recruitment of cytosolic HSP70 to chloroplasts. It is speculated that HSP70 may be required for assembling/folding components of the SA biosynthesis or transport machinery, although a direct demonstration for this is still lacking (**Figure 1**). The result of HopI1 action is reduced SA accumulation (Jelenska et al., 2007, 2010).

An effector protein directly affecting SA levels is produced by the biotrophic fungus *Ustilago maydis*, which is causing smut disease in maize. Secretome analysis of apoplastic proteins from leaf tissue infected by *U. maydis* identified the secreted chorismate mutase protein Cmu1. Immunoelectron microscopy detected Cmu1 protein not only in the interface between fungal hyphae and surrounding plant plasma membrane but also in the cytosol of invaded plant cells, demonstrating that Cmu1 protein is taken up by host plant cells and functions within the cytoplasm of the plant cells. By activity assays and complementation of an *aro7* mutant of *Saccharomyces cerevisiae*, Cmu1 has been demonstrated to have chorismate mutase activity (Djamei et al., 2011). Metabolic profiling revealed that leaf tissue infected by *cmu1* mutants show increased accumulation of SA. In addition, mutants lacking *cmu1* are reduced in virulence. These results suggest that translocated Cmu1 facilitates the conversion of chorismate to prephenate to lower the availability of chorismate for SA biosynthesis (**Figure 1**). In this way Cmu1 is proposed to suppress SA-dependent plant defense responses, which would be harmful for a biotrophic pathogen like *U. maydis*. Upon transient expression in maize

cells, Cmu1 has also been shown to spread to neighboring cells conceivably priming them for the subsequent infection (Djamei et al., 2011; Djamei and Kahmann, 2012).

A recent report demonstrated that two unrelated hemibiotrophic filamentous pathogens, the oomycete *Phytophtora sojae*, which causes root and stem rot disease in soybean, and the fungus *Verticillium dahlia*, which causes vascular wilt diseases in a large number of different plant species, secrete isochorismatases PsIsc1 and VdIsc1, respectively (Liu et al., 2014). Isochorismatases convert isochorismate into 2,3-dihydro-2,3-dihydroxybenzoate (DDHB) and pyruvate (**Figure 1**), thus eliminating the central precursor for SA production (**Figure 1**). PsIsc1 and VdIsc1 are virulence factors in both *P. sojae* and *V. dahliae* (Liu et al., 2014). Interestingly, these isochorismatases lack predicted signal sequences which direct the protein into the conventional secretory pathway. Nevertheless, these proteins are detected in culture supernatants, suggesting that they are secreted via unconventional secretion pathways. Intracellular expression of these isochorismatases in leaves of *Nicotiana benthamiana* by *Agrobacterium* infiltration significantly has been shown to reduce SA levels and increase levels of DDHB. Furthermore, transient expression of these isochorismatases in *N. benthamiana* elevates susceptibility toward the compatible hemibiotrophic pathogen *P. capsici* with a concomitant decrease in *PR-1* gene expression (Liu et al., 2014), a marker gene of the SA pathway. Thus, these filamentous pathogens attenuate SA-dependent plant defense responses by reducing the level of a crucial intermediate for SA biosynthesis (**Figure 1**).

Salicylic acid has been shown to be transported in the phloem (Rocher et al., 2006), and has been detected also in apoplastic fluid of *V. longisporum*-infected *A. thaliana* plants (Floerl et al., 2012). *U. maydis* has the gene for a putative salicylate hydroxylase NahG-like enzyme (*shy*1) which does not appear to be secreted (Rabe et al., 2013). Recombinant Shy1 protein indeed displays salicylate hydroxylase activity. Subsequent experiments have revealed that *U. maydis* can sense, degrade, and use SA as carbon source. However, this ability could not be linked to virulence (Rabe et al., 2013), which could either reflect redundancy or a contribution to virulence when *U. maydis* infects different plant organs. SA-degrading ability is also reported for the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*, although the protein responsible for SA degradation in this organism has not yet been identified (Penn and Daniel, 2013). These studies illustrate that pathogens have developed different ways to lower SA levels in infected plants and may actually use redundant strategies to accomplish this. To what extent lower SA levels contribute to virulence appears to be variable and may depend on the system and the infection conditions.

Increased SA levels in plants depend on the expression of *ICS1* and components affecting its downstream accumulation. PAMP perception increases intracellular Ca^{2+} concentrations which regulate the activity of calmodulin (CaM) and calcium-dependent protein kinases (CDPKs). The calmodulin binding protein CBP60g positively regulates *ICS1* expression while CBP60a acts as a negative regulator (Truman et al., 2013). SAR DEFICIENT 1 (SARD1) which does not bind CaM acts redundantly with CBP60g in promoting *ICS1* transcription. Both CBP60g and SARD1 are shown to be recruited to the *ICS1* promoter region in response to pathogen attack (Zhang et al., 2010b) and consequently the induction of *ICS1* expression and SA production are significantly impaired in *sard1 cbp60g* double mutants (Zhang et al., 2010b). *ICS1* expression is furthermore positively regulated by a member of the WRKY family of transcription factors, WRKY28, whose DNA binding activity is regulated through phosphorylation (Eulgem et al., 2000; Dempsey et al., 2011; van Verk et al., 2011; Seyfferth and Tsuda, 2014). *ICS1* expression is also negatively regulated by ETHYLENE INSENSITIVE 3 (EIN3) and ETHYLENE INSENSITIVE 3-LIKE 1 (EIL1; Chao et al., 1997). For EIN3, the regulation appears to be direct since EIN3 can specifically bind the *ICS1* promoter (Chen et al., 2009). In addition, NPR1 has been reported to negatively regulate *ICS1* expression via an as yet unknown mechanism (Wildermuth et al., 2001; Zhang et al., 2010a). Up to now no pathogen effectors have been identified that directly target any of the transcriptional regulators for *ICS1* expression. We consider this likely to reflect the highly complex mode of regulation which may make *ICS1* regulation a much less attractive target for effectors than targeting SA accumulation or shifting the balance from SA to JA signaling (see below).

Effectors Interfering with SA-Dependent Signaling and Gene Regulation

NPR1 is the central regulator of the SA signaling pathway and functions as a co-activator for an estimated 95% of the SA-responsive genes. When mutated, SA-dependent transcriptional responses are largely abolished and the corresponding mutants exhibit increased susceptibility to biotrophic and hemibiotrophic pathogens (Aravind and Koonin, 1999; Pajerowska-Mukhtar et al., 2013). In unchallenged plant cells, NPR1 resides largely in the cytosol in an oligomeric state that is stabilized by intermolecular disulfide bonds. Increases in SA levels after pathogen infection alter the cellular redox state (Mou et al., 2003), triggering a reduction of NPR1 by thioredoxins that leads to the dissociation of NPR1 into monomers (Tada et al., 2008). NPR1 monomers are then translocated into the nucleus where they interact with TGA-bZIP transcription factors (Zhang et al., 1999; Zhou et al., 2000), leading to an activation of SA-dependent gene expression including *PR-1* (Fan and Dong, 2002). TGA2 (**Figure 2**), TGA5, and TGA6 are transcriptional repressors of the *PR-1* promoter in the absence of SA and their repressive property may require interaction with additional components like NIMIN1, TOPLESS, and the CBNAC-SNI1 complex (Seyfferth and Tsuda, 2014). Once the transcriptional co-activator NPR1 resides in the nucleus, these previously repressing factors become positive regulators of SA-induced genes (Dong, 2004). NPR1 and related family members NPR3 and NPR4 bind SA and have been proposed to be SA receptors (Fu et al., 2012; Wu et al., 2012). While the true nature of the SA receptor is still debated (Boatwright and Pajerowska-Mukhtar, 2013), it is clear that nuclear localization of NPR1 is crucial for SA-mediated gene expression (Mou et al., 2003). To elicit an appropriate immune function, NPR1 activity in the nucleus needs to be tightly regulated. Nuclear NPR1 has been shown to be

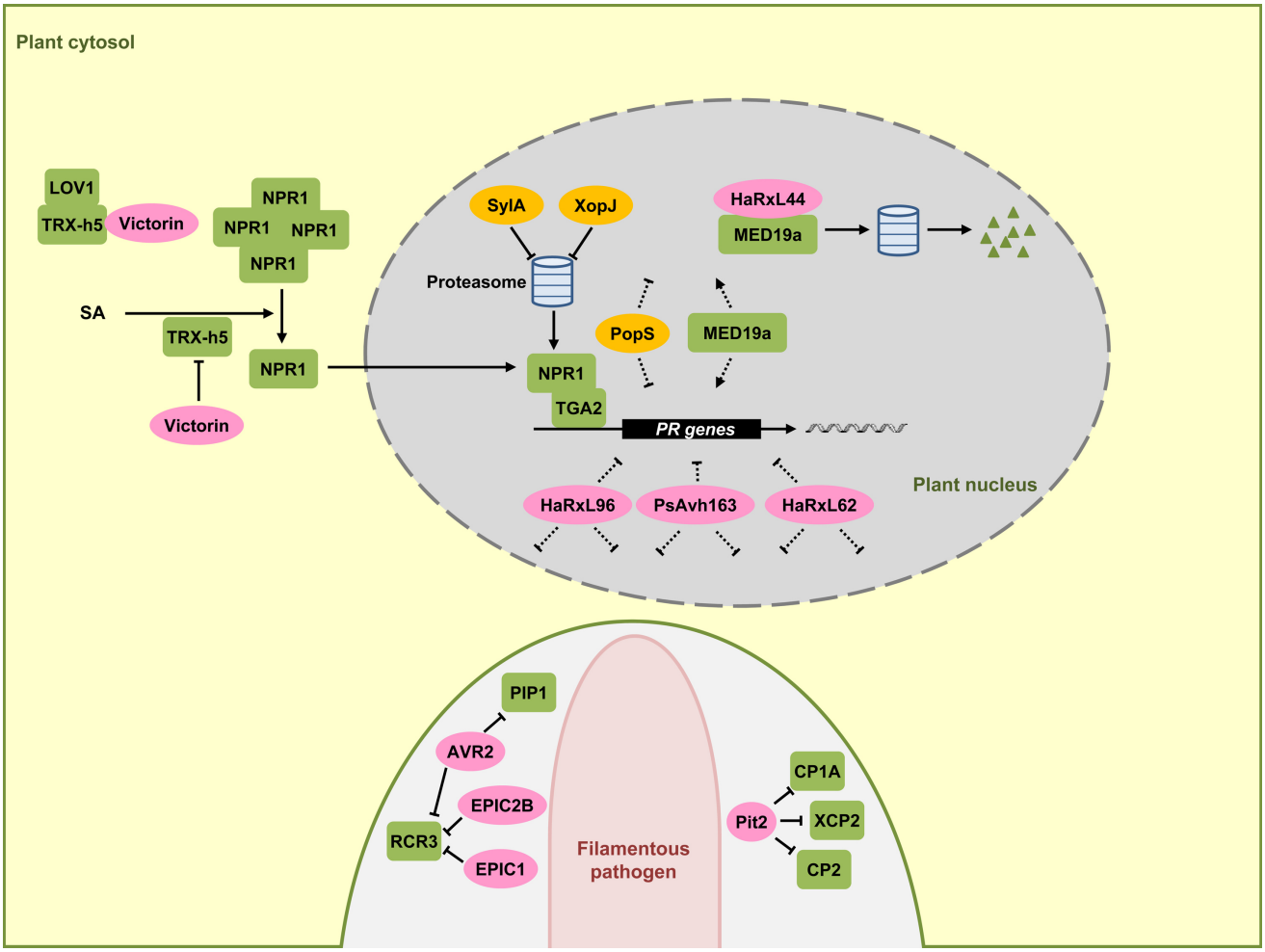


FIGURE 2 | Effectors interfering with SA-dependent signaling and gene regulation. In this signaling scheme, we restrict the presentation to plant components targeted by virulence effectors. NPR1 is a central regulator in SA-dependent signaling pathway, triggering the expression of pathogenesis-related (*PR*) genes in the nucleus (gray) together with the TGA2 transcription factor. SA induces monomerization of cytosolic NPR1 oligomers with the help of thioredoxin TRX-h5. The mycotoxin effector victorin of *C. victoriae* can inhibit NPR1 through binding to TRX-h5 without causing disease. However, in LOV1 expressing plants victorin activates LOV1 with the consequence of cell death which is prerequisite for disease by this necrotrophic pathogen. *P. syringae* effector SylA and *X. campestris* effector XopJ act as proteasome inhibitors to suppress turnover of NPR1 and interrupt SA dependent defenses. HaRxL44 interacts with MED19a, leading to proteasomal degradation of MED19a. Oomycete RxLR effectors HaRxL96, PsAvh163, and HaRxL62, as well as a bacterial effector PopS, inhibit the expression of SA marker gene *PR-1* most likely indirectly. In the lower part a pathogen hypha is indicated in light pink. This hypha is surrounded by the apoplast shown enlarged here (light gray) encased by the plant plasma membrane (green). Effectors AVR2, EPIC1, EPIC2B, and Pit2 are secreted to the apoplastic space where they inhibit plant proteases PIP1, RCR3, CP1A, CP2, and XCP2 all induced by SA. Fungal and oomycete effectors are indicated by pink ovals and bacterial effectors are indicated in dark yellow. Plant components are indicated by green squares. Solid arrows represent direct activation, dotted arrows indicate indirect activation and blunt ended arrows indicate inhibition (for details, see text).

continuously degraded via the proteasome system in naïve cells to prevent untimely activation of immune responses (Spoel et al., 2009). SA stimulation has been shown to trigger phosphorylation of a phosphodegron motif in NPR1 facilitating NPR1 turnover. Phosphorylation-dependent turnover seems to be required for full activation of target gene expression, presumably indicating that NPR1 at the promoter needs to be replaced continuously to maintain gene induction (Spoel et al., 2009).

Although effectors directly targeting NPR1 have not yet been found in plant colonizing microbes, there are examples that some bacterial pathogens may indirectly target NPR1. The toxin syringolin A (SylA) from *P. syringae* pv. *syringae* inhibits proteasome function and the type III effector XopJ from *Xanthomonas campestris* pv. *vesicatoria* interacts with RPT6, a subunit of the proteasome complex crucial for proteasome function. The proposed model is that SlyA and XopJ may negatively influence the proteasome-mediated turnover of NPR1 to compromise SA signaling (**Figure 2**; Schellenberg et al., 2010; Misas-Villamil et al., 2013; Üstün et al., 2013).

The fungal pathogen *Cochliobolus victoriae*, the causal pathogen of Victoria blight disease on oat, also seems to indirectly target NPR1. *C. victoriae* secretes the mycotoxin effector victorin,

an effector evoking defense. In this necrotrophic pathogen defense activation is prerequisite for virulence. Victorin binds to the active site of TRX-h5 (Thioredoxin-h5) inhibiting its activity (Lorang et al., 2012). TRX-h5 has been proposed to act as guard of LOV1, an NB-LRR protein. Production of victorin by the pathogen leads to LOV1 activation (**Figure 2**), resulting in a resistance-like cell death response which promotes disease (Lorang et al., 2012). In plants lacking *LOV1*, victorin treatment leads to reduced *PR-1* expression to levels comparable to *TRX-h5* mutants. This reflects the victorin-induced inhibition of TRX-h5 activity and lack of NPR1 monomerization. As such, victorin canonically functions as a virulence effector molecule in plants lacking *LOV1* by targeting thioredoxin (Lorang et al., 2012).

HopM1 and AvrE are representatives of conserved bacterial effector families which have in common the ability to suppress SA-dependent basal immunity and disease necrosis (DebRoy et al., 2004). The biotrophic bacterial wilt pathogen of tomato, *Ralstonia solanacearum* has the type-III effector PopS which is also a member of the AvrE family. This effector suppresses SA-mediated defense responses but fails to induce cell death (Jacobs et al., 2013). The targets for HopM1 and AvrE-type effectors with respect to SA signaling remain to be discovered.

The oomycete *Hyaloperonospora arabidopsidis* causing downy mildew in *A. thaliana* and *P. sojae* produce the effector proteins HaRxL62, HaRxL96, and PsAvh163, respectively, which are secreted proteins containing a N-terminal RxLR motif that is widely conserved in oomycete effector proteins that are delivered into host cells (Whisson et al., 2007; Anderson et al., 2012; Asai et al., 2014). HaRxL62, HaRxL96, and PsAvh163 effectors, all reduce transcription of the SA marker gene *PR-1* in transgenic plants when these are infected by an avirulent *H. arabidopsidis* strain or treated with SA (Anderson et al., 2012; Asai et al., 2014), suggesting interference with SA signaling. However, it is unclear which component SA-dependent plant defense response is suppressed by these effectors (**Figure 2**).

The nuclear-localized RxLR effector HaRxL44 of *H. arabidopsidis* interacts with the Mediator subunit 19a (MED19a), a positive regulator of plant immunity in *A. thaliana* (Caillaud et al., 2013). Mediator is a highly conserved multi-subunit complex that functions like a molecular bridge to facilitate the interaction between transcription factors at gene enhancer element sequences and RNA polymerase II at transcription initiation sites (Conaway and Conaway, 2011). The interaction of HaRxL44 with MED19a has been shown to induce the destabilization of MED19a by proteasome-dependent degradation (**Figure 2**). Transgenic plants of *A. thaliana* expressing HaRxL44 or *med19a* mutants show weak SA-triggered immunity and strong JA/ET signaling, illustrating that the degradation of MED19a shifts the balance from SA-responsive defense to JA/ET responsive defense, which is typical for many biotrophic pathogens (Caillaud et al., 2013). In addition to MED19a, Mediator subunits MED15 and MED16 are also shown to be required for SA-mediated resistance (Canet et al., 2012; Zhang et al., 2012).

Apoplastic proteases constitute a major component in plant defense responses. Benzothiadiazole, a functional analog of SA, facilitates accumulation of active papain-like cysteine proteases including PIP1 and RCR3 in the apoplast of tomato plants (Shabab et al., 2008). *Cladosporium fulvum*, the leaf mold pathogen of tomato, secretes the virulence effector protein AVR2. AVR2 adopts a highly compact structure through disulfide bonds involving its eight cysteine residues. AVR2 inhibits the cysteine protease activity of PIP1 and RCR3 by direct binding (**Figure 2**; Rooney et al., 2005; van Esse et al., 2008; van't Klooster et al., 2011). *P. infestans* secretes EPIC1 and EPIC2B effector proteins that also act as protease inhibitors targeting tomato cysteine protease RCR3 (**Figure 2**; Tian et al., 2007; Song et al., 2009). Consistent with a role in defense, a tomato mutant in RCR3 exhibits enhanced susceptibility to *P. infestans* (Song et al., 2009). This illustrates that the defense-associated cysteine protease RCR3 is targeted by effectors from two unrelated filamentous pathogens (**Figure 2**). In maize, papain-like cysteine proteases also constitute a central component of apoplastic plant defenses. SA treatment of maize leaves strongly induces cysteine protease accumulation in the apoplast. SA-induced apoplastic cysteine proteases and their activity are sufficient to induce *PR-1* gene expression and the activation of plant defenses (van der Linde et al., 2012). Upon infection by *U. maydis*, maize cystatin CC9, a potent inhibitor of maize apoplastic cysteine proteases, is induced. Silencing of the *CC9* gene greatly attenuates *U. maydis* virulence (van der Linde et al., 2012), showing the importance of SA-induced cysteine proteases in maize defense signaling. In addition, the apoplastic virulence effector Pit2 of *U. maydis* (Doehlemann et al., 2011) interacts with and inhibits apoplastic maize cysteine proteases CP1A, CP2, and XCP2 (Mueller et al., 2013). This inhibitory activity depends on a novel 14 amino acid motif in Pit2. This motif is conserved in Pit2 orthologs of related smut fungi but does not exist in AVR2 or cystatins, which also inhibit members of the cysteine protease family (Mueller et al., 2013). SA-induced cysteine proteases are thus emerging as common virulence targets of filamentous pathogens (**Figure 2**). The need to inhibit this class of proteases by pathogen effectors may reflect that these plant proteases target core effectors important for virulence. Alternatively, these proteases could attack critical surface components of the pathogens. Current research aims to identify the important targets of these proteases.

Effectors Targeting the Crosstalk between SA and JA Pathways

There is extensive antagonistic crosstalk between SA and JA pathways which is exploited by pathogens to meet their specific needs (Gimenez-Ibanez and Solano, 2013; Kazan and Lyons, 2014). In the negative crosstalk between SA and JA, the activation of the SA pathway can confer susceptibility to plants upon the attack of pathogens that are restricted by the JA-dependent pathway, and conversely the activation of the JA pathway can suppress the SA pathway in favor of biotrophic pathogens (**Figure 3**). For instance, it has been shown that the NahG plants of *A. thaliana*, which are unable to accumulate SA, show 25-fold higher levels of JA and express JA-responsive genes (Spoel et al., 2003). In addition, several plant proteins regulating the SA–JA crosstalk have already been identified. *npr1* mutants, which are unable to respond to SA, show increased levels of JA and enhanced JA-responsive gene

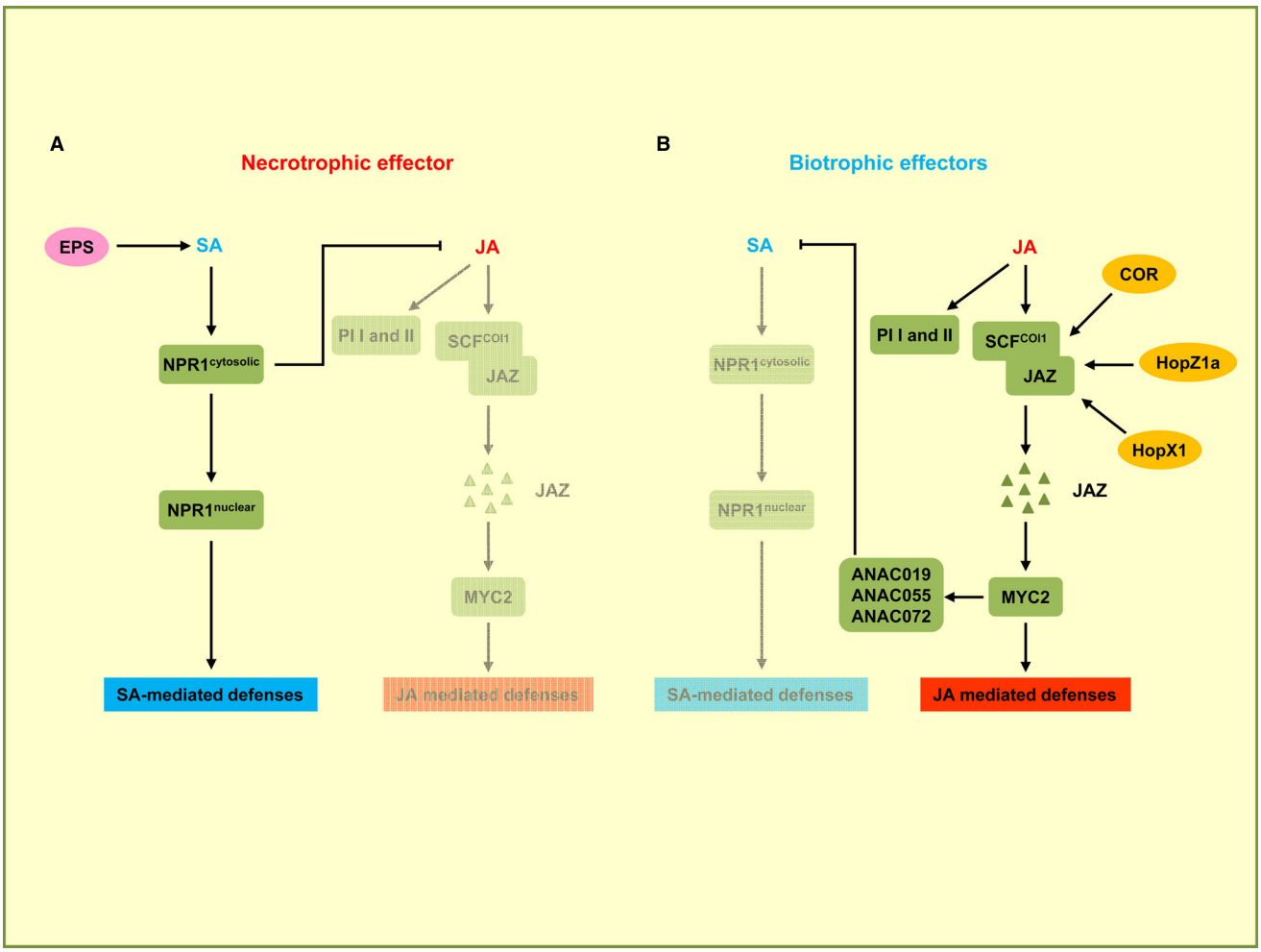


FIGURE 3 | Effectors targeting the crosstalk between SA and JA pathways. In this signaling scheme, we restrict the presentation to plant components targeted by virulence effectors. **(A)** Necrotrophic effector activates the SA pathway while downregulating the JA pathway. *B. cinerea* uses exopolysaccharide EPS (depicted in pink) to activate SA-mediated defenses through NPR1 and to inhibit JA-mediated defenses including the expression of *PI I* and *PI II*. **(B)** Biotrophic effectors activate the JA pathway and suppress the SA pathway. *P. syringae* secretes phytotoxin coronatine (COR) to promote SCF^{COI1} ubiquitin ligase-dependent degradation of JAZ proteins. JAZ degradation activates MYC2, the transcriptional regulator of JA-responsive genes. MYC2 also induces NAC transcription factors ANAC019, ANAC055, and ANAC072 which are repressors of SA production. *P. syringae* uses HopZ1a and HopX1 to directly target JAZ proteins to accelerate their degradation, thus inhibiting SA-mediated defenses. Bacterial effectors are indicated in dark yellow. Plant components are indicated by green squares. Solid arrows represent direct activation, dotted arrows indicate indirect activation, and blunt ended arrows indicate inhibition.

expression, indicating that NPR1 suppresses JA signaling (Spoel et al., 2003). Nuclear localization of NPR1 is not required for the suppression of JA-responsive gene expression, suggesting that cytosolic NPR1 may modulate the crosstalk between SA and JA (**Figure 3**; Spoel et al., 2003). In the JA signaling pathway, JAZ proteins, which are negative regulators for JA-responsive gene expression, are degraded by the E3 ubiquitin ligase SCF^{COI1} complex in response to JA. Subsequently MYC2, the transcriptional regulator of JA-responsive genes is activated. The *MYC2* gene is also required for the repression of SA-mediated defense responses (**Figure 3**; Laurie-Berry et al., 2006). *P. syringae* uses the phytotoxin coronatine (COR), a structural mimic of JA-Ile (the active form of JA in *A. thaliana*), for binding to the JA co-receptor COI1 (Xin and He, 2013). The COR-bound COI1 receptor complex promotes the degradation of JAZ proteins that act as negative regulators of the JA pathway (**Figure 3**). This leads to the activation of JA-responsive genes via MYC2, which also induces the transcription of three homologous NAC family transcription factor genes: *ANAC019*, *ANAC055*, and *ANAC072* (Zheng et al., 2012). These three NAC transcription factors repress the *ICS1* gene leading to a downregulation of SA production and signaling. In this way COR promotes susceptibility to *P. syringae* by suppressing SA signaling (Brooks et al., 2005). The function of COR to induce JA responses can also be carried out by bacterial effector proteins. HopZ1a, an effector of *P. syringae*, directly acetylates JAZ proteins. This leads to COI1-dependent degradation of JAZ proteins (**Figure 3**), resulting in an induction of JA-mediated defenses and a concomitant repression of SA responses (Jiang et al., 2013). The

JAZ proteins are also targets of HopX1, another *P. syringae* effector encoding a cysteine protease that interacts with and promotes the degradation of JAZ proteins (**Figure 3**; Gimenez-Ibanez et al., 2014).

The necrotrophic fungal pathogen *Botrytis cinerea* secretes a non-proteinaceous exopolysaccharide (EPS) effector, β-(1,3)(1,6)-D-glucan (El Oirdi et al., 2011). EPS from *B. cinerea* exploits the antagonism between the SA and JA pathways to promote fungal virulence. Tomato plants pre-treated with the EPS show significantly elevated SA levels and disease susceptibility, and conversely a reduction of JA-dependent defense genes *PI I* and *PI II*. *PI I* and *PI II* code for proteinase inhibitors required for resistance against *B. cinerea*. When EPS is applied to *NPR1*-silenced plants, increased SA accumulation and disease susceptibility are not observed, indicating that EPS-induced disease susceptibility is likely to occur through NPR1. These results demonstrate that *B. cinerea* EPS activates the SA pathway through NPR1 for promoting disease and concomitantly represses the JA pathway that would restrict virulence of this necrotrophic pathogen (El Oirdi et al., 2011).

Conclusions and Outlook

While it is becoming increasingly clear that all biotrophic pathogens (as well as hemibiotrophs during their biotrophic phase) need to suppress SA signaling to cause disease the molecular details of how this is achieved by effectors in the various systems is only beginning to be understood. Given the small number of examples where pathogen effectors targets in these processes have been identified, it is probably not surprising to see little if any overlap between prokaryotic and eukaryotic virulence effector targets. This picture is very likely to change once more effector targets are discovered.

Is there an advantage of interference at the level of SA biosynthesis, SA signal transduction and gene regulation or the antagonistic interplay between SA and JA signaling over interfering with PAMP perception directly at the level of the receptor (Macho and Zipfel, 2015)? We think so, because targeting the activity of a certain PRR would be highly specific while interference with SA signaling further downstream affects the response at a level where signaling pathways have converged. Also, in view of the fact that plants are estimated to have hundreds of PRRs with ligands presently mostly unknown, effector interference at a more downstream level could provide a common response to different PAMP triggers. In addition, effector interference at the level of the PRR might not appropriately allow accommodation of the different life styles of pathogens, i.e., necrotrophs that activate SA signaling, biotrophs that activate JA signaling or hemibiotrophs that switch from one to the other mode of signaling. Thus, maintaining this flexibility may be a key to pathogen success. This is also likely the reason why certain pathogens have developed several effectors interfering with the same pathway, albeit at different levels. Given the more than 10-fold greater abundance of effectors in eukaryotic pathogens compared to bacterial pathogens, we also wonder whether redundancy will suffice as an explanation. In the *U. maydis*-maize system effectors are deployed in an organ-specific manner (Skibbe et al., 2010) explaining different needs for effectors in discrete organs. In addition, eukaryotic pathogens undergo a series of infection-related developmental processes in the infected tissue, which may require a reprogramming of the host in specific ways, conceivably involving alternative effectors. These considerations show that current work on effectors is just scratching the tip of the iceberg, and a lot of exciting science is still to come.

Acknowledgments

We thank Barry Scott for his comments and careful editing of the manuscript. Our work was supported by funds from the Max Planck Society and through the LOEWE program SYNMIKRO of the state of Hesse. XH is supported by China Scholarship Council (CSC).

References

Anderson, R. G., Casady, M. S., Fee, R. A., Vaughan, M. M., Deb, D., Fedkenheuer, K., et al. (2012). Homologous RXLR effectors from *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* suppress immunity in distantly related plants. *Plant J.* 72, 882–893. doi: 10.1111/j.1365-313X.2012.05079.x

Aravind, L., and Koonin, E. V. (1999). Fold prediction and evolutionary analysis of the POZ domain: structural and evolutionary relationship with the potassium channel tetramerization domain. *J. Mol. Biol.* 285, 1353–1361. doi: 10.1006/jmbi.1998.2394

Asai, S., Rallapalli, G., Piquerez, S. J. M., Caillaud, M. C., Furzer, O. J., Ishaque, N., et al. (2014). Expression profiling during *Arabidopsis*/downy mildew interaction reveals a highly-expressed effector that attenuates responses to salicylic acid. *PLoS Pathog.* 10:e1004443. doi: 10.1371/journal.ppat.1004443

Boatwright, J. L., and Pajerowska-Mukhtar, K. (2013). Salicylic acid: an old hormone up to new tricks. *Mol. Plant Pathol.* 14, 623–634. doi: 10.1111/Mpp.12035

Brooks, D. M., Bender, C. L., and Kunkel, B. N. (2005). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Mol. Plant Pathol.* 6, 629–639. doi: 10.1111/J.1364-3703.2005.00311.X

Caillaud, M. C., Asai, S., Rallapalli, G., Piquerez, S., Fabro, G., and Jones, J. D. G. (2013). A downy mildew effector attenuates salicylic acid-triggered immunity in *Arabidopsis* by interacting with the host mediator complex. *PLoS Biol.* 11:e1001732. doi: 10.1371/journal.pbio.1001732

Canet, J. V., Dobon, A., and Tornero, P. (2012). Non-recognition-of-BTH4, an *Arabidopsis* mediator subunit homolog, is necessary for development and response to salicylic acid. *Plant Cell* 24, 4220–4235. doi: 10.1105/tpc.112.103028

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. N. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired-resistance. *Plant Cell* 6, 1583–1592. doi: 10.1105/tpc.6.11.1583

Chao, Q. M., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J. R. (1997). Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 89, 1133–1144. doi: 10.1016/S0092-8674(00)80300-1

Chen, H. M., Xue, L., Chintamanani, S., Germain, H., Lin, H. Q., Cui, H. T., et al. (2009). ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in *Arabidopsis*. *Plant Cell* 21, 2527–2540. doi: 10.1105/tpc.108.065193

Conaway, R. C., and Conaway, J. W. (2011). Function and regulation of the Mediator complex. *Curr. Opin. Genet. Dev.* 21, 225–230. doi: 10.1016/j.gde.2011.01.013

DebRoy, S., Thilmony, R., Kwack, Y. B., Nomura, K., and He, S. Y. (2004). A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity

and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9927–9932. doi: 10.1073/pnas.0401601101

Delaney, T. P., Friedrich, L., and Ryals, J. A. (1995). *Arabidopsis* signal-transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6602–6606. doi: 10.1073/pnas.92.14.6602

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., et al. (1994). A central role of salicylic-acid in plant-disease resistance. *Science* 266, 1247–1250. doi: 10.1126/science.266.5188.1247

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arabidopsis Book* 9, e0156. doi: 10.1199/tab.0156

Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., et al. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8024–8029. doi: 10.1073/pnas.1230660100

Dewdney, J., Reuber, T. L., Wildermuth, M. C., Devoto, A., Cui, J. P., Stutius, L. M., et al. (2000). Three unique mutants of *Arabidopsis* identify *eds* loci required for limiting growth of a biotrophic fungal pathogen. *Plant J.* 24, 205–218. doi: 10.1046/j.1365-313x.2000.00870.x

Djamei, A., and Kahmann, R. (2012). *Ustilago maydis*: dissecting the molecular interface between pathogen and plant. *PLoS Pathog.* 8:e1002955. doi: 10.1371/journal.ppat.1002955

Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahnt, J., et al. (2011). Metabolic priming by a secreted fungal effector. *Nature* 478, 395–398. doi: 10.1038/Nature10454

Dodds, P. N., Lawrence, G. J., Catanzariti, A.-M., Teh, T., Wang, C.-I., Ayliffe, M. A., et al. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8888–8893. doi: 10.1073/pnas.0602577103

Doehlemann, G., Reissmann, S., Assmann, D., Fleckenstein, M., and Kahmann, R. (2011). Two linked genes encoding a secreted effector and a membrane protein are essential for *Ustilago maydis*-induced tumour formation. *Mol. Microbiol.* 81, 751–766. doi: 10.1111/j.1365-2958.2011.07728.x

Doehlemann, G., Requena, N., Schaefer, P., Brunner, F., O'Connell, R., and Parker, J. E. (2014). Reprogramming of plant cells by filamentous plant-colonizing microbes. *New Phytol.* 204, 803–814. doi: 10.1111/Nph.12938

Dong, X. N. (2004). NPR1, all things considered. *Curr. Opin. Plant Biol.* 7, 547–552. doi: 10.1016/j.pbi.2004.07.005

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

El Oirdi, M., Abd El Rahman, T., Rigano, L., El Hadrami, A., Rodriguez, M. C., Daayf, F., et al. (2011). *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *Plant Cell* 23, 2405–2421. doi: 10.1105/tpc.111.083394

Eulgem, T., Rushton, P. J., Robatzek, S., and Somssich, I. E. (2000). The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* 5, 199–206. doi: 10.1016/S1360-1385(00)01600-9

Fan, W. H., and Dong, X. N. (2002). In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in *Arabidopsis*. *Plant Cell* 14, 1377–1389. doi: 10.1105/Tpc.001628

Floerl, S., Majcherczyk, A., Possienke, M., Feussner, K., Tappe, H., Gatz, C., et al. (2012). *Verticillium longisporum* infection affects the leaf apoplastic proteome, metabolome, and cell wall properties in *Arabidopsis thaliana*. *PLoS ONE* 7:e31435. doi: 10.1371/journal.pone.0031435

Fu, Z. Q., Yan, S. P., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/Nature11162

Garcion, C., Lohmann, A., Lamodiere, E., Catinot, J., Buchala, A., Doermann, P., et al. (2008). Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of *Arabidopsis*. *Plant Physiol.* 147, 1279–1287. doi: 10.1104/pp.108.119420

Gimenez-Ibanez, S., Boter, M., Fernandez-Barbero, G., Chini, A., Rathjen, J. P., and Solano, R. (2014). The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in *Arabidopsis*. *PLoS Biol.* 12:e1001792. doi: 10.1371/journal.pbio.1001792

Gimenez-Ibanez, S., and Solano, R. (2013). Nuclear jasmonate and salicylate signaling and crosstalk in defense against pathogens. *Front. Plant Sci.* 4:72. doi: 10.3389/fpls.2013.00072

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Glazebrook, J., Rogers, E. E., and Ausubel, F. M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 143, 973–982.

Ishihara, T., Sekine, K. T., Hase, S., Kanayama, Y., Seo, S., Ohashi, Y., et al. (2008). Overexpression of the *Arabidopsis thaliana* EDS5 gene enhances resistance to viruses. *Plant Biol.* 10, 451–461. doi: 10.1111/j.1438-8677.2008.00050.x

Jacobs, J. M., Milling, A., Mitra, R. M., Hogan, C. S., Ailloud, F., Prior, P., et al. (2013). *Ralstonia solanacearum* requires PopS, an ancient avre-family effector, for virulence and to overcome salicylic acid-mediated defenses during tomato pathogenesis. *mBio* 4:e00875-13. doi: 10.1128/mBio.00875-13

Jelenska, J., van Hal, J. A., and Greenberg, J. T. (2010). *Pseudomonas syringae* hijacks plant stress chaperone machinery for virulence. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13177–13182. doi: 10.1073/pnas.0910943107

Jelenska, J., Yao, N., Vinatzer, B. A., Wright, C. M., Brodsky, J. L., and Greenberg, J. T. (2007). A J domain virulence effector of *Pseudomonas syringae* remodels host chloroplasts and suppresses defenses. *Curr. Biol.* 17, 499–508. doi: 10.1016/j.cub.2007.02.028

Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J.* 19, 4004–4014. doi: 10.1093/emboj/19.15.4004

Jiang, S. S., Yao, J., Ma, K. W., Zhou, H. B., Song, J. K., He, S. Y., et al. (2013). Bacterial effector activates jasmonate signaling by directly targeting JAZ transcriptional repressors. *PLoS Pathog.* 9:e1003715. doi: 10.1371/journal.ppat.1003715

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kazan, K., and Lyons, R. (2014). Intervention of phytohormone pathways by pathogen effectors. *Plant Cell* 26, 2285–2309. doi: 10.1105/tpc.114.125419

Laurie-Berry, N., Joardar, V., Street, I. H., and Kunkel, B. N. (2006). The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. *Mol. Plant Microbe Interact.* 19, 789–800. doi: 10.1094/Mpmi-19-0789

Liu, T. L., Song, T. Q., Zhang, X., Yuan, H. B., Su, L. M., Li, W. L., et al. (2014). Unconventionally secreted effectors of two filamentous pathogens target plant salicylate biosynthesis. *Nat. Commun.* 5, 4686. doi: 10.1038/Ncomms5686

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., et al. (2015). Fungal effectors and plant susceptibility. *Annu. Rev. Plant Biol.* 66, 513–545. doi: 10.1146/annurev-arplant-043014-114623

Lorang, J., Kidarsa, T., Bradford, C. S., Gilbert, B., Curtis, M., Tzeng, S. C., et al. (2012). Tricking the guard: exploiting plant defense for disease susceptibility. *Science* 338, 659–662. doi: 10.1126/science.1226743

Macho, A. P., and Zipfel, C. (2015). Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors. *Curr. Opin. Microbiol.* 23, 14–22. doi: 10.1016/j.mib.2014.10.009

Misas-Villamil, J. C., Kolodziejek, I., Crabill, E., Kaschani, F., Niessen, S., Shindo, T., et al. (2013). *Pseudomonas syringae* pv. syringae uses proteasome inhibitor syringolin A to colonize from wound infection sites. *PLoS Pathog.* 9:e1003281. doi: 10.1371/journal.ppat.1003281

Mou, Z., Fan, W. H., and Dong, X. N. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Mueller, A. N., Ziemann, S., Treitschke, S., Assmann, D., and Doehlemann, G. (2013). Compatibility in the *Ustilago maydis*–maize interaction requires inhibition of host cysteine proteases by the fungal effector Pit2. *PLoS Pathog.* 9:e1003177. doi: 10.1371/journal.ppat.1003177

Nawrath, C., Heck, S., Parinthawong, N., and Metraux, J. P. (2002). EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE transporter family. *Plant Cell* 14, 275–286. doi: 10.1105/Tpc.010376

Nawrath, C., and Metraux, J. P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11, 1393–1404. doi: 10.1105/tpc.11.8.1393

Newman, M. A., Sundelin, T., Nielsen, J. T., and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139. doi: 10.3389/fpls.2013.00139

Pajerowska-Mukhtar, K. M., Emerine, D. K., and Mukhtar, M. S. (2013). Tell me more: roles of NPRs in plant immunity. *Trends Plant Sci.* 18, 402–411. doi: 10.1016/j.tplants.2013.04.004

Penn, C. D., and Daniel, S. L. (2013). Salicylate degradation by the fungal plant pathogen *Sclerotinia sclerotiorum*. *Curr. Microbiol.* 67, 218–225. doi: 10.1007/s00284-013-0349-y

Rabe, F., Ajami-Rashidi, Z., Doehlemann, G., Kahmann, R., and Djamei, A. (2013). Degradation of the plant defence hormone salicylic acid by the biotrophic fungus *Ustilago maydis*. *Mol. Microbiol.* 89, 179–188. doi: 10.1111/Mmi.12269

Rafiqi, M., Ellis, J. G., Ludowici, V. A., Hardham, A. R., and Dodds, P. N. (2012). Challenges and progress towards understanding the role of effectors in plant–fungal interactions. *Curr. Opin. Plant Biol.* 15, 477–482. doi: 10.1016/j.pbi.2012.05.003

Rocher, F., Chollet, J. F., Jousse, C., and Bonnemain, J. L. (2006). Salicylic acid, an ambimobile molecule exhibiting a high ability to accumulate in the phloem. *Plant Physiol.* 141, 1684–1693. doi: 10.1104/pp.106.082537

Rooney, H. C. E., van't Klooster, J. W., van Der Hoorn, R. A. L., Joosten, M. H. A. J., Jones, J. D. G., and de Wit, P. J. G. M. (2005). Cladosporium Avr2 inhibits tomato Rcr3 protease required for Cf-2-dependent disease resistance. *Science* 308, 1783–1786. doi: 10.1126/science.1111404

Schellenberg, B., Ramel, C., and Dudler, R. (2010). *Pseudomonas syringae* virulence factor syringolin A counteracts stomatal immunity by proteasome inhibition. *Mol. Plant Microbe Interact.* 23, 1287–1293. doi: 10.1094/Mpmi-04-10-0094

Seyfferth, C., and Tsuda, K. (2014). Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. *Front. Plant Sci.* 5:697. doi: 10.3389/fpls.2014.00697

Shabab, M., Shindo, T., Gu, C., Kaschani, F., Pansuriya, T., Chintha, R., et al. (2008). Fungal effector protein AVR2 targets diversifying defense-related Cys proteases of tomato. *Plant Cell* 20, 1169–1183. doi: 10.1105/tpc.107.056325

Skibbe, D. S., Doehlemann, G., Fernandes, J., and Walbot, V. (2010). Maize tumors caused by *Ustilago maydis* require organ-specific genes in host and pathogen. *Science* 328, 89–92. doi: 10.1126/science.1185775

Song, J., Win, J., Tian, M. Y., Schornack, S., Kaschani, F., Ilyas, M., et al. (2009). Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. *Proc. Natl. Acad. Sci. U.S.A.* 106, 1654–1659. doi: 10.1073/pnas.0809201106

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770. doi: 10.1105/Tpc.009159

Spoel, S. H., Mou, Z. L., Tada, Y., Spivey, N. W., Genschik, P., and Dong, X. N. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* 137, 860–872. doi: 10.1016/j.cell.2009.03.038

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Tian, M. Y., Win, J., Song, J., van der Hoorn, R., van der Knaap, E., and Kamoun, S. (2007). A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. *Plant Physiol.* 143, 364–377. doi: 10.1104/pp.106.090050

Truman, W., Sreekanta, S., Lu, Y., Bethke, G., Tsuda, K., Katagiri, F., et al. (2013). The CALMODULIN-BINDING PROTEIN60 family includes both negative and positive regulators of plant immunity. *Plant Physiol.* 163, 1741–1751. doi: 10.1104/pp.113.227108

Üstün, S., Bartetzko, V., and Bornke, F. (2013). The *Xanthomonas campestris* type III effector XopJ targets the host cell proteasome to suppress salicylic-acid mediated plant defence. *PLoS Pathog.* 9:e1003427. doi: 10.1371/journal.ppat.1003427

van der Hoorn, R. A. L., and Kamoun, S. (2008). From guard to decoy: a new model for perception of plant pathogen effectors. *Plant Cell* 20, 2009–2017. doi: 10.1105/tpc.108.060194

van der Linde, K., Hemetsberger, C., Kastner, C., Kaschani, F., van der Hoorn, R. A. L., Kumlehn, J., et al. (2012). A maize cystatin suppresses host immunity by inhibiting apoplastic cysteine proteases. *Plant Cell* 24, 1285–1300. doi: 10.1105/tpc.111.093732

van Esse, H. P., van't Klooster, J. W., Bolton, M. D., Yadeta, K. A., Van Baarlen, P., Boeren, S., et al. (2008). The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. *Plant Cell* 20, 1948–1963. doi: 10.1105/tpc.108.059394

van't Klooster, J. W., van der Kamp, M. W., Vervoort, J., Beekwilder, J., Boeren, S., Joosten, M. H. A. J., et al. (2011). Affinity of Avr2 for tomato cysteine protease Rcr3 correlates with the Avr2-triggered Cf-2-mediated hypersensitive response. *Mol. Plant Pathol.* 12, 21–30. doi: 10.1111/j.1364-3703.2010.00647.x

van Verk, M. C., Bol, J. F., and Linthorst, H. J. M. (2011). WRKY transcription factors involved in activation of SA biosynthesis genes. *BMC Plant Biol.* 11:89. doi: 10.1186/1471-2229-11-89

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., et al. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450, 115–118. doi: 10.1038/Nature06203

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Xin, X. F., and He, S. Y. (2013). *Pseudomonas syringae* pv. *tomato DC*3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. *Annu. Rev. Phytopathol.* 51, 473–498. doi: 10.1146/annurev-phyto-082712-102321

Zhang, X. D., Chen, S. X., and Mou, Z. L. (2010a). Nuclear localization of NPR1 is required for regulation of salicylate tolerance, isochorismate synthase 1 expression and salicylate accumulation in *Arabidopsis*. *J. Plant Physiol.* 167, 144–148. doi: 10.1016/j.jplph.2009.08.002

Zhang, Y. X., Xu, S. H., Ding, P. T., Wang, D. M., Cheng, Y. T., He, J., et al. (2010b). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18220–18225. doi: 10.1073/pnas.1005225107

Zhang, X. D., Wang, C. G., Zhang, Y. P., Sun, Y. J., and Mou, Z. L. (2012). The Arabidopsis mediator complex subunit16 positively regulates salicylate-mediated systemic acquired resistance and jasmonate/ethylene-induced defense pathways. *Plant Cell* 24, 4294–4309. doi: 10.1105/tpc.112.103317

Zhang, Y. L., Fan, W. H., Kinkema, M., Li, X., and Dong, X. N. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528. doi: 10.1073/pnas.96.11.6523

Zheng, X. Y., Spivey, N. W., Zeng, W. Q., Liu, P. P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596. doi: 10.1016/j.chom.2012.04.014

Zhou, J. M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., et al. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol. Plant Microbe Interact.* 13, 191–202. doi: 10.1094/Mpmi.2000.13.2.191

Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends Immunol.* 35, 345–351. doi: 10.1016/J.It.2014.05.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 14 January 2015
doi: 10.3389/fpls.2014.00784

AHL-priming functions via oxylipin and salicylic acid

Sebastian T. Schenk and Adam Schikora*

Institute for Phytopathology, Research Centre for Biosystems, Land Use and Nutrition (IFZ), Justus Liebig University Giessen, Giessen, Germany

Edited by:
Hua Lu, University of Maryland Baltimore County, USA

Reviewed by:
Anton Hartmann, Helmholtz Zentrum München – German Research Center for Environmental Health, Germany
Shuishan Song, Biology Institute – Hebei Academy of Sciences, China

***Correspondence:**
Adam Schikora, Institute for Phytopathology, Research Centre for Biosystems, Land Use and Nutrition (IFZ), Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
e-mail: adam.schikora@agrar.uni-giessen.de

Collaborative action between the host plant and associated bacteria is crucial for the establishment of an efficient interaction. In bacteria, the synchronized behavior of a population is often achieved by a density-dependent communication called quorum sensing. This behavior is based on signaling molecules, which influence bacterial gene expression. *N*-acyl homoserine lactones (AHLs) are such molecules in many Gram-negative bacteria. Moreover, some AHLs are responsible for the beneficial effect of bacteria on plants, for example the long chain *N*-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL) can prime *Arabidopsis* and barley plants for an enhanced defense. This AHL-induced resistance phenomenon, named AHL-priming, was observed in several independent laboratories during the last two decades. Very recently, the mechanism of priming with oxo-C14-HSL was shown to depend on an oxylipin and salicylic acid (SA). SA is a key element in plant defense, it accumulates during different plant resistance responses and is the base of systemic acquired resistance. In addition, SA itself can prime plants for an enhanced resistance against pathogen attack. On the other side, oxylipins, including jasmonic acid (JA) and related metabolites, are lipid-derived signaling compounds. Especially the oxidized fatty acid derivative *cis*-OPDA, which is the precursor of JA, is a newly described player in plant defense. Unlike the antagonistic effect of SA and JA in plant–microbe interactions, the recently described pathway functions through a synergistic effect of oxylipins and SA, and is independent of the JA signaling cascade. Interestingly, the oxo-C14-HSL-induced oxylipin/SA signaling pathway induces stomata defense responses and cell wall strengthening thus prevents pathogen invasion. In this review, we summarize the findings on AHL-priming and the related signaling cascade. In addition, we discuss the potential of AHL-induced resistance in new strategies of plant protection.

Keywords: AHL, quorum sensing (QS), oxylipins, SA, priming

AHLs IMPACT ON PLANT PHYSIOLOGY AND DEVELOPMENT

Cell-to-cell signaling is a widespread practice in living organisms. Bacteria use a pheromone-like system called quorum sensing (QS). QS was first described in *Vibrio fischeri*, a bacterium that lives in symbiosis with a squid and produces bioluminescent light at high cell densities (Tomasz, 1965; Kempner and Hanson, 1968; Ruby and Nealson, 1976). *V. fischeri* produces *N*-acyl homoserine lactones (AHLs) that are freely diffusible across the bacterial membranes and accumulate in their surroundings (Kempner and Hanson, 1968). When a threshold concentration of AHLs is achieved, the bacterial population is able to sense (*sensing*) the critical cell density, the so-called *quorum* (Tomasz, 1965). Besides a regulation of the AHL-regulon, a very important feature of QS is the *autoinduction* of AHL-synthase expression. This communication system enables individual bacterial cells to monitor the population density and coordinate a conjoint action(s) (Waters and Bassler, 2005; Antunes and Ferreira, 2009; Teplitski et al., 2010; Nazzaro et al., 2013). In many situations, the ability of bacterial population to behave co-operatively and to communicate with each other brings clear advantages; for example, bacteria benefit from QS for conjugation, symbiotic, or pathogenic interactions with the host, for adaptation and distribution within an ecological niche (efficiency sensing; Hense et al., 2007), or for the production and secretion of secondary metabolites like antibiotics or siderophores (Williams, 2007; Hartmann et al., 2014). In Gram-negative bacteria, QS system is often based on AHLs, it is until now the best characterized bacterial communication system (Engebrecht and Silverman, 1984). AHL molecules can vary in the length of the acyl chain (4-18-carbons) and in the substitutions at the carbon chain. In addition to AHLs, 2-alkyl-4-quinolones, long-chain fatty acids, fatty acid methyl esters, and furanones (autoinducer-2) can be used for bacterial communication (Williams, 2007).

Reports from independent laboratories claimed that the short chain AHLs induce a growth promotion effect due to an impact on the phytohormone auxin (von Rad et al., 2008; Bai et al., 2012; Liu et al., 2012). The first study of AHL impact on plant hormone metabolism was performed with *Medicago truncatula* during the response to AHLs originated from the symbiotic bacterium *Ensifer meliloti* (*Sinorhizobium meliloti*). In this study, authors revealed 150 differentially regulated proteins, within those were several auxin-induced proteins and enzymes that are involved in auxin metabolism. Furthermore, the activation of the β-*Glucuronidase* (GUS) reporter gene under the control of the auxin-responsive *GH3* promoter, indicated the involvement of auxin in the response to AHL (Mathesius et al., 2003). The

possible role of auxin in response to AHL treatment was also suggested by transcriptional analyses. Auxin-associated genes were induced after a treatment with the short chain *N*-hexanoyl-homoserine lactone (C6-HSL) as well as after a pretreatment with the long chain *N*-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL) and a subsequent challenge with the pathogen elicitor flg22 (von Rad et al., 2008; Schenk et al., 2014). In addition, genes involved in cytokinin metabolism, which have an antagonistic function to auxin, were down regulated (von Rad et al., 2008). The same report described an alteration of the free auxin to cytokinin ratio in root and shoot tissues after AHL application, explaining as a consequence the promotion of plant growth (von Rad et al., 2008). Another study showed the involvement of auxin in the AHL-induced growth as a result of the production of hydrogen peroxide and nitric oxide, which are dependent on the cyclic GMP signaling. In the postulated model, the QS molecule *N*-3-oxo-dodecanoyl-L-homoserine lactone (oxo-C10-HSL), induced an enhanced basipetal auxin transport followed by accumulation of H_2O_2 and NO, and stimulated therefore the formation of adventitious roots (Bai et al., 2012). Nevertheless, some publications disagree with the involvement of auxin in the AHL-growth promoting effect on plants. Despite the strong impact of oxo-C10-HSL on primary root growth and in contrast to other findings, lateral root formation, and root hair development was independent of auxin signaling as indicated by the expression analysis of the *GUS*-reporter genes under the control of the auxin-regulated *DR5* promoter (Ortiz-Castro et al., 2008). Moreover, a recent report suggested that the growth promoting effect of AHLs depends on the AHL-derivative L-homoserine, which is produced upon amidolysis of AHLs by the fatty acid amide hydrolase (Palmer et al., 2014). The authors postulated that the increased transpiration induced by L-homoserine, would enhance the water and minerals flow through plant organism and therefore positively influence the growth.

Beside the enhancement of growth, long chain AHLs have impact on plant defense mechanisms (Schikora et al., 2011; Schenk et al., 2012, 2014). In contrast to animals, plants do not have specialized cells for immune responses; for this reason, the attacked plant cell needs to reprogram its regular cellular functions for a defense response. Plants developed specialized local defense mechanisms and specific systemic responses, which are coordinated by systemic signals (Spoel and Dong, 2008). In this coordination, the cross talk between hormones plays a crucial role (Koornneef and Pieterse, 2008). The defense response against necrotrophic pathogens is usually dependent on the plant hormones jasmonic acid (JA) and ethylene (ET), while the defense reactions to biotrophic pathogens are dominantly regulated by salicylic acid (SA; Glazebrook, 2005). The antagonistic interaction between the SA and JA is well characterized (Rojo et al., 2003; Beckers and Spoel, 2006), although some reports claim a synergistic interaction between these two phytohormones (van Wees et al., 2000). An involvement of defense hormones in the AHL-induced resistance was postulated after the observation that an inoculation with the AHL-producing rhizobacterium *Serratia liquefaciens* strain MG1 enhanced systemic defense and the accumulation of SA in tomato plants (Hartmann et al., 2004; Schuhegger et al., 2006). Similar results were observed after a treatment of tomato plants with pure C6- and C4-HSL; the SA- and ET-dependent *Pathogenesis Related1a* (*PR1a*) and two *chitinase* genes were highly expressed after the treatment (Schuhegger et al., 2006). The enhanced expression of those genes in tomato leaves after application of C6-HSL or C4-HSL to the roots suggested that the systemic response functions via an SA-dependent pathway (Schuhegger et al., 2006). Likewise, an application of the long chain AHL (oxo-C14-HSL) on *Arabidopsis* roots induced a systemic response in plant shoots (Schikora et al., 2011). The AHL-induced pathway could therefore depend on SA together with the oxylipin 12-oxo-phytodienoic acid (*cis*-OPDA), as indicated by the accumulation of those two hormones, as well as mutant studies and transcriptional analyses (Schenk et al., 2014), see also chapter on AHL-priming below.

THE SYNERGISTIC ROLE OF SA AND OXYLIPINS IN PLANT DEFENSE

Phyto-oxylipins are a diverse group of lipid-derived compounds including JA and jasmonate-related metabolites like *cis*-OPDA, methyl jasmonate, and the active form of JA, jasmonyl-*l*-isoleucine (JA-Ile). These compounds are unsaturated fatty acids produced by lipoxygenases (LOX) that oxidize the lipid chain at the C9 or C13 position (Andreou and Feussner, 2009). Additionally, oxylipins can be synthesized non-enzymatically via the free radical-catalyzed pathway, which generates similar structures denominated phytoprostanes (Sattler et al., 2006). While a lot is known about the biological function of JA, methyl jasmonate, and JA-Ile, including their perception and signal transduction (reviewed in Browse, 2009), the biological role of oxylipins before their conversion to JA is less understood. Nevertheless, several studies assumed that the precursors of JA play a role in different developing processes and during defense responses (Blee, 2002; Dave and Graham, 2012). For example, 18-cabon divinyl ether fatty acid, colneleic, and colnelenic acids accumulated in potato and tobacco leaves during the late blight disease (Weber et al., 1999). In addition, phytoprostanes accumulated as a consequence of pathogen-induced oxidative stress (ROS-production), induced the activation of Mitogen-Activated Protein Kinases (MAPKs) and glutathione-*S*-transferase (GST), expression of defense genes, and the accumulation of phytoalexin (Thoma et al., 2003). Furthermore, the enzymatically oxidized *cis*-OPDA induced expression of genes related to detoxification, stress responses, and secondary metabolism (Taki et al., 2005; Mueller et al., 2008). Interestingly, the oxylipins-related pathways induced reactions distinct from the JA-induced responses. While the expression of JA-related genes is COI1-dependent, *cis*-OPDA and phytoprostanes (PPA_1 and PPB_1) have been demonstrated to activate gene expression in a COI1-independent manner (Stintzi et al., 2001; Taki et al., 2005; Stotz et al., 2013). Transcriptional analysis of *Arabidopsis* showed that more than 150 genes responded to the application of *cis*-OPDA but not to JA or methyl jasmonate (Taki et al., 2005). The expression of the majority of these genes was regulated through the bZIP TGACG motif-binding transcription factors TGA2, TGA5, and TGA6 (Stotz et al., 2013). Curiously, those transcription factors are also required for the activation of SA-dependent genes (Zhang et al., 1999, 2003). A recent discovery indicated that the oxylipin pathway induced by biotic stress interacts with the SA-dependent

signaling and results in a stomatal defense response (Montillet et al., 2013). The authors postulated that during stomatal defense the activation of MPK3 and MPK6 induced the guard cell lipoxygenase LOX1 and hence the peroxidation of poly unsaturated fatty acids into oxylipins followed by the accumulation of SA. Downstream of this SA accumulation was the regulation of the anion channel SLAC-1, which coordinates the stomatal defense response.

THE PRIMING EFFECT, SENSITIZING FOR FUTURE DEFENSE RESPONSES

One of the consequences of an activated defense mechanism is a high consumption of energy. Therefore, the immune system of higher organisms needs to be coordinated in an efficient manner. In order to lower the cost of defense, plants developed different mechanisms to orchestrate their immune system, among them are negative regulators that suppress the defense response in the absence of a pathogen, or the induction of specific pathways, accordingly to the particular pathogen. Furthermore, plants may use priming as an efficient regulation of defense responses. This mechanism is based on a sensitization of the plant for a stronger and faster response. This phenomenon has been used in agriculture for plant protection since the early 1930s. Priming was usually defined as a part of induced resistance; however, the priming effect is only assessable after a subsequently challenge of the primed tissue (Conrath et al., 2002). Some priming inducers are well characterized, one of them is the non-proteinogenic amino acid β-aminobutyric acid (BABA) and another is SA at low concentrations. BABA priming functions through a SA- and abscisic acid (ABA)-dependent pathway, and induces enhanced callose depositions and tolerance to salt stress (Ton et al., 2005). In addition, BABA-induced resistance interferes with the action of the bacterial toxin coronatine (COR) from the pathogen *Pseudomonas syringae* (Tsai et al., 2011). Yet another priming inducer is the mobile metabolite azelaic acid, which induces a systemic protection via accumulation of SA (Jung et al., 2009).

Besides the accumulation of signaling components, few reports addressed the molecular mechanism of priming and explained the sensitized status of a plant. The first revealed the accumulation of the inactive form of MPK3 that can be rapidly activated upon a subsequent attack (Beckers et al., 2009). The second was the discovery of chromatin modifications on promoters of defense-related genes. In primed plants, histones in promoter regions of the defense-associated transcription factors *WRKY6, WRKY26*, and *WRKY53* are methylated (H3Kme3 and H3K4me2) and acetylated (H3K9, H4K5, and H4K12), which could explain the faster activation and the subsequent stronger stress response (Jaskiewicz et al., 2011). Interestingly, and very important for future research projects, is the fact that the primed status of a plant can be transmitted to next generations. The SA-induced defense and the resistance to the pathogen *P. syringae* were inherited to the offspring by transferring the histone methylation mechanism of relevant genes (Luna et al., 2012). Furthermore, the transgenerational priming was observed in progeny of plants treated with BABA or exposed to insect attack (Rasmann et al., 2012; Slaughter et al., 2012). However, while BABA and *P. syringe* priming are based on SA and SA-depending signaling, the insect-induced transgenerational priming is JA-dependent.

AHL-PRIMING DEPENDS ON AN OXYLIPIN/SA-DEPENDENT PATHWAY

Considering that SA and JA precursors are crucial for long chain AHL-priming, the cross talk between SA and oxylipins seems to be an important feature of the AHL-induced resistance. Evidences that the AHL-induced priming acts via oxylipins/SA-dependent pathway are not restricted to the accumulation of phytohormones after sensitizing the plant with AHLs also genetic evidences support this dependency. Since the *Arabidopsis* mutants *coi1-16* and *jar1* behaved like wild-type plants when tested for AHL-enhanced resistance against *P. syringae*, the effect seems to be independent of the JA perception and the production of JA-Ile (Schenk et al., 2014). However, AHL-priming required Nonexpressor of PR Genes 1 (NPR1), which is the key regulator in SA-dependent defense, as indicated by the high proliferation of *P. syringae* in AHL-pretreated *npr1-1* mutant plants. The same holds true for the triple mutant *tga2/5/6*, which is impaired in the signal transduction in *cis*-OPDA- and SA-signaling cascade(s). Likewise, the AHL effect was lost in the *lox2* mutant (Schenk et al., 2014), missing the Lipoxygenase 2, one of the enzymes required for the oxidation of the unsaturated fatty acids, and hence for the oxylipin response in plants (Blee, 2002; Dave and Graham, 2012). In addition to the genetic studies, evidences that oxo-C14-HSL acts via the oxylipin/SA-induced pathway were observed at transcriptional level. For example, the enhanced expression of *GST6*, *GSTU19*, the stress responding heat shock proteins encoding genes *HSP70* and *HSP17*, and the cytochrome P450 (*CYP81D11*), which was observed after a *cis*-OPDA treatment (Mueller et al., 2008), was also visible during AHL-priming (Schenk et al., 2014). Furthermore, the independency of JA and ET during AHL-priming was strengthened by the expression patterns of prominent JA-responsive genes, *MYC2* and *VSP2,* and the ET-responsive genes *PR3, ERF5,* and *ETR1*, which were not influenced by the AHL pretreatment (Schenk et al., 2014).

STOMATA DEFENSE RESPONSE, ONE OF THE MECHANISMS USED DURING AHL-INDUCED RESISTANCE

Stomata are openings in the epidermal layer of terrestrial plants. These pores are built up of two guard cells that regulate the opening and closure in order to establish the exchange of gasses between the leaf and the environment. This regulation system allows the control of transpiration. During drought stress, the regulation of anion-channels in guard cells is coordinated by ABA. The perception of ABA activates the guard cell-specific, ABA-related protein kinase OST1, which is followed by production of ROS and activation of Ca^{2+}-signaling (Mustilli et al., 2002). Moreover, stomatal closure is tightly controlled by innate immunity as it has a crucial role during prevention of pathogen invasion. This phenomenon is referred to as stomatal defense response and functions as physical barrier against pathogen entry (Melotto et al., 2008).

A report on the inoculation of *Arabidopsis* plants with *P. syringae* showed that guard cells perceive the pathogen, indicating

an active role of guard cells in plant defense (Melotto et al., 2006). However, the guard cells response to biotic stress seems to differ from the response to abiotic stress in respect to the function of the plant defense hormones SA and ABA, as well as the MAP kinases and NPR1 (Melotto et al., 2006; Zeng and He, 2010). Even though, ABA and SA signaling pathways were apparently involved in the stomatal closure induced by the beneficial bacterium *Bacillus subtilis* FB17 (Kumar et al., 2012), the above discussed report on ABA-independent pathway that controls stomatal closure in case of an immune defense response, proposed a signaling pathway, which is induced upon the perception of flg22 and includes the activation of MPK3 and MPK6 (Montillet et al., 2013). The authors observed that it requires the guard-cell-specific LOX1, producing the oxylipin *cis*-OPDA. A high accumulation of *cis*-OPDA after the flg22-elicitation in guard cells was followed by an accumulation of SA (Montillet et al., 2013).

Interestingly, the AHL-induced resistance also depends on SA and *cis*-OPDA and activates the stomatal defense response (**Figure 1**). We observed an increase of closed and reduction of open stomata in oxo-C14-HSL-pretreated *Arabidopsis* plants (Schenk et al., 2014). Furthermore, the expression profile of ABA-dependent genes *RD22*, *RD29*, and *RAB18* revealed no regulation in oxo-C14-HSL-primed plants, which strengthens the postulated hypothesis on ABA-independency in stomatal defense response (Montillet et al., 2013; Schenk et al., 2014).

An important feature of phytopathogenic bacteria is the ability to reopen closed stomata, thus counteract the stomatal defense response. This is usually achieved by the bacterial toxin coronatine (COR) (Melotto et al., 2006; Zeng and He, 2010; Pieterse et al., 2014), which mimics the plant hormone JA-Ile. This virulence factor binds to the JA-receptor complex and activates the antagonistic crosstalk between SA and JA, inhibiting the flg22-triggered immune responses such as ROS production and callose depositions (Yi et al., 2014). In addition, COR suppresses the biosynthesis and accumulation of SA, hence inhibits the local and systemic defense responses (Zheng et al., 2012). Interestingly, the priming agent BABA interferes with the COR impact on stomatal defense responses. BABA-induced resistance activates the SA-dependent responses, while COR was able to suppress this defense reactions as shown by the abolished BABA effect by relatively high concentration of

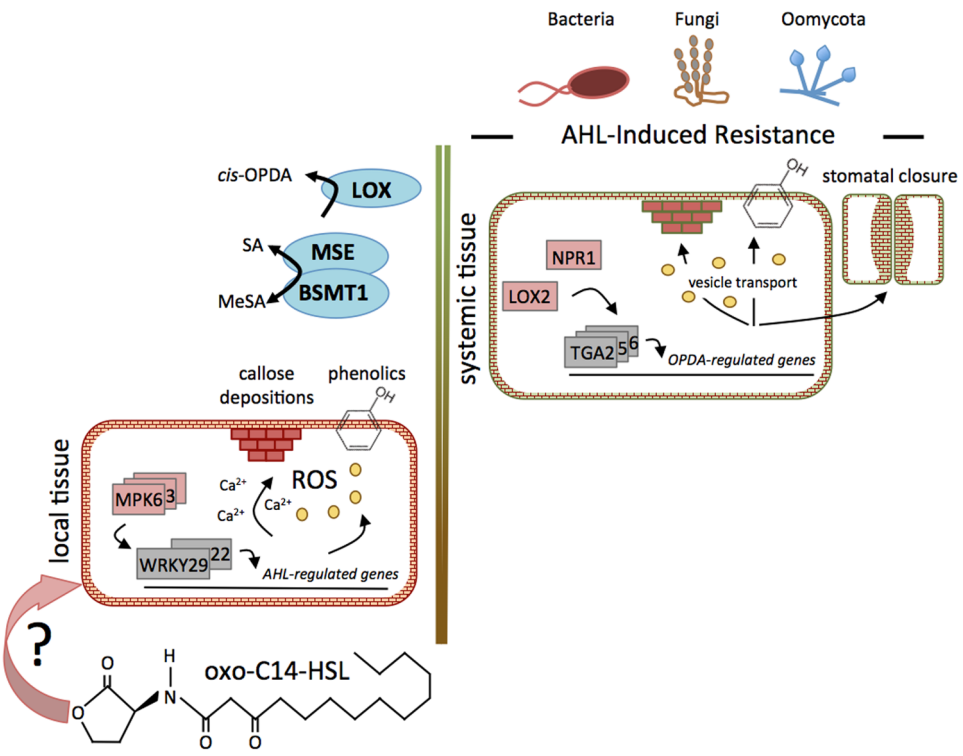


FIGURE 1 | Function of SA and oxylipins in AHL-induced priming. Signaling steps of AHL-induced mechanisms leading to the reinforcement of resistance against several pathogens. The perception mechanism(s) of AHL in plant tissues is not known, indicated by "?". Nonetheless, in local tissues of *Arabidopsis* plants, the oxo-C14-HSL-priming is manifested through the prolonged and stronger activation of MAPKs and the enhanced expression of *WRKY* transcription factors, followed by transcriptional reprogramming of genes related to Ca^{2+}-signaling, defense, G-proteins, cell wall, and flavonoid metabolism. Furthermore, AHLs induced a higher accumulation of ROS, phenolic compounds, and callose in the cell walls. Even though long chain AHLs are not translocated to distal tissues, elevated production of the phytohormones oxylipin (*cis*-OPDA) and SA was observed in distal tissues, indicating that a systemic signaling is involved in this phenomenon. The proteins NPR1 and LOX2, as well as the TGA2/5/6 transcription factors were required. Like in the local tissue, the long chain AHL oxo-C14-HSL induced callose depositions, accumulation of phenolic compounds, and enhanced stomatal closure.

COR, or the failure to prime the *coi1-16* mutant (Tsai et al., 2011). Similarly, in the case of AHL-induced resistance the stomatal defense response seems to depend on SA and could disrupt the function of COR. However, the AHL-priming is still present in the *coi1-16* mutant, which indicates differences between the BABA- and AHL-priming (Schenk et al., 2014).

AGRICULTURAL POTENTIAL OF AHL-PRIMING

Since the beginning of the twenty-first century, the bivalence between avoidance of synthetic pesticides and the performance of crop protection methods is a big challenge in agriculture. To ensure a sufficient food supply, agriculture industry has to develop modern plant protection strategies, which ensure sufficient yield and food quality. Moreover, due to market demands, farmers are under increasing pressure to produce their crops organically, or at least to reduce the chemical impact on the environment. In addition, plant production has to deal with ecological challenges like abiotic or biotic stresses and handle the arable land in the most sustainable manner. Development of new substances, which are useful in both integrated agricultural management and organic farming, is a big challenge. The development of *biologicals* or *biocontrol agents,* which originate from natural products, could be a possible strategy to meet those requirements. For instance the use of *microbial inoculants* of beneficial, soil-born microorganisms could be a competent approach to support agriculture (Berg, 2009). Using the knowledge of microbe–plant interactions, rhizosphere, or root-associated bacteria including *Bacillus*, *Pseudomonas*, and *Serratia* spp. could contribute to the production of new natural products for plant protection (Berg, 2009; Beneduzi et al., 2012; Nadeem et al., 2013). Likewise, microbial metabolites with an impact on plant growth or health have a high potential in this regard (Brader et al., 2014). The bacterial QS molecules are remarkable candidates in such strategies (Hartmann et al., 2014). Purified QS molecules and bacteria with increased production of AHLs, have an impact on plant defense mechanisms and portrait the agricultural potential of homoserine lactones (Zarkani et al., 2013; Hernández-Reyes et al., 2014). Furthermore, the use of N_2-fixating *Rhizobia,* with their positive effects on plant physiology, could be improved by QS molecules. Nodulation efficiency, symbiosome development, exopolysaccharides production, nitrogen fixation, and adaptation to stress are all regulated by QS systems (Gonzalez and Marketon, 2003; Marketon et al., 2003). The promotion of AHL production in *Rhizobia* or *bacterial inoculants* could enhance the beneficial effects (nitrogen fixation, growth promotion, reinforced plant defense) hence, lead to a reduced use of fertilizers or conventional plant protection agents in agriculture, and in this way lower the negative impact of chemicals on the environment. Another strategy was proposed by two independent laboratories, which have bioengineered tobacco and tomato plants with different bacterial AHL-synthesis genes. These transgenic plants foster beneficial plant–bacteria interactions, and alter growth and tolerance to abiotic stress (Scott et al., 2006; Barriuso et al., 2008). However, risks and advantages of AHL-producing plants need to be assessed and require further elucidation.

REFERENCES

Andreou, A., and Feussner, I. (2009). Lipoxygenases – Structure and reaction mechanism. *Phytochemistry* 70, 1504–1510. doi: 10.1016/j.phytochem.2009.05.008

Antunes, L. C., and Ferreira, R. B. (2009). Intercellular communication in bacteria. *Crit. Rev. Microbiol.* 35, 69–80. doi: 10.1080/10408410902733946

Bai, X., Todd, C. D., Desikan, R., Yang, Y., and Hu, X. (2012). N-3-Oxo-Decanoyl-L-Homoserine-Lactone activates auxin-induced adventitious root formation via hydrogen peroxide- and nitric oxide-dependent cyclic GMP signaling in mung bean. *Plant Physiol.* 158, 725–736. doi: 10.1104/pp.111.185769

Barriuso, J., Ramos Solano, B., Fray, R. G., Camara, M., Hartmann, A., and Gutierrez Manero, F. J. (2008). Transgenic tomato plants alter quorum sensing in plant growth-promoting rhizobacteria. *Plant Biotechnol. J.* 6, 442–452. doi: 10.1111/j.1467-7652.2008.00331.x

Beckers, G. J., Jaskiewicz, M., Liu, Y., Underwood, W. R., He, S. Y., Zhang, S., et al. (2009). Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana. Plant Cell* 21, 944–953. doi: 10.1105/tpc.108.062158

Beckers, G. J., and Spoel, S. H. (2006). Fine-tuning plant defence signalling: salicylate versus jasmonate. *Plant Biol. (Stuttg.)* 8, 1–10. doi: 10.1055/s-2005-872705

Beneduzi, A., Ambrosini, A., and Passaglia, L. M. (2012). Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genet. Mol. Biol.* 35, 1044–1051. doi: 10.1590/S1415-47572012000600020

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Blee, E. (2002). Impact of phyto-oxylipins in plant defense. *Trends Plant Sci.* 7, 315–322. doi: 10.1016/S1360-1385(02)02290-2

Brader, G., Compant, S., Mitter, B., Trognitz, F., and Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. *Curr. Opin. Biotechnol.* 27, 30–37. doi: 10.1016/j.copbio.2013.09.012

Browse, J. (2009). Jasmonate passes muster: a receptor and targets for the defense hormone. *Annu. Rev. Plant Biol.* 60, 183–205. doi: 10.1146/annurev.arplant.043008.092007

Conrath, U., Pieterse, C. M., and Mauch-Mani, B. (2002). Priming in plant-pathogen interactions. *Trends Plant Sci.* 7, 210–216. doi: 10.1016/S1360-1385(02)02244-6

Dave, A., and Graham, I. A. (2012). Oxylipin signaling: a distinct role for the jasmonic acid precursor cis-(+)-12-Oxo-Phytodienoic Acid (cis-OPDA). *Front. Plant Sci.* 3:42. doi: 10.3389/fpls.2012.00042

Engebrecht, J., and Silverman, M. (1984). Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. U.S.A.* 81, 4154–4158. doi: 10.1073/pnas.81.13.4154

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Gonzalez, J. E., and Marketon, M. M. (2003). Quorum sensing in nitrogen-fixing rhizobia. *Microbiol. Mol. Biol. Rev.* 67, 574–592. doi: 10.1128/MMBR.67.4.574-592.2003

Hartmann, A., Gantner, S., Schuhegger, R., Steidle, A., Dürr, C., Schmid, M., et al. (2004). "N-acyl homoserine lactones of rhizosphere bacteria trigger systemic resistance in tomato plants," in *Proceedings of the 11th International Congress on Molecular Plant-Microbe Interactions*, eds B. Lugtenberg, I. Tikhonovich, and N. Provorov (St. Paul, MN: APS Press), 554–556.

Hartmann, A., Rothballer, M., Hense, B. A., and Schröder, P. (2014). Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. *Front. Plant Sci.* 5:131. doi: 10.3389/fpls.2014.00131

Hense, B. A., Kuttler, C., Mueller, J., Rothballer, M., Hartmann, A., and Kreft, J. U. (2007). Opinion – Does efficiency sensing unify diffusion and quorum sensing? *Nat. Rev. Microbiol.* 5, 230–239. doi: 10.1038/nrmicro1600

Hernández-Reyes, C., Schenk, S. T., Neumann, C., Kogel, K. H., and Schikora, A. (2014). N-acyl-homoserine lactones-producing bacteria protect plants against plant and human pathogens. *Microb. Biotechnol.* 7, 580–588. doi: 10.1111/1751-7915.12177

Jaskiewicz, M., Conrath, U., and Peterhansel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* 12, 50–55. doi: 10.1038/embor.2010.186

Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91. doi: 10.1126/science.1170025

Kempner, E. S., and Hanson, F. E. (1968). Aspects of light production by *Photobacterium fischeri*. *J. Bacteriol.* 95, 975–979.

Koornneef, A., and Pieterse, C. M. (2008). Cross talk in defense signaling. *Plant Physiol.* 146, 839–844. doi: 10.1104/pp.107.112029

Kumar, A. S., Lakshmanan, V., Caplan, J. L., Powell, D., Czymmek, K. J., Levia, D. F., et al. (2012). Rhizobacteria *Bacillus subtilis* restricts foliar pathogen entry through stomata. *Plant J.* 72, 694–706. doi: 10.1111/j.1365-313X.2012.05116.x

Liu, F., Bian, Z., Jia, Z., Zhao, Q., and Song, S. (2012). The GCR1 and GPA1 participate in promotion of *Arabidopsis* primary root elongation induced by N-acyl-homoserine lactones, the bacterial quorum-sensing signals. *Mol. Plant Microbe Interact.* 25, 677–683. doi: 10.1094/MPMI-10-11-0274

Luna, E., Bruce, T. J., Roberts, M. R., Flors, V., and Ton, J. (2012). Next-generation systemic acquired resistance. *Plant Physiol.* 158, 844–853. doi: 10.1104/pp.111.187468

Marketon, M. M., Glenn, S. A., Eberhard, A., and Gonzalez, J. E. (2003). Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. *J. Bacteriol.* 185, 325–331. doi: 10.1128/JB.185.1.325-331.2003

Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anolles, G., Rolfe, B. G., et al. (2003). Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1444–1449. doi: 10.1073/pnas.262672599

Melotto, M., Underwood, W., and He, S. Y. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu. Rev. Phytopathol.* 46, 101–122. doi: 10.1146/annurev.phyto.121107.104959

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980. doi: 10.1016/j.cell.2006.06.054

Montillet, J. L., Leonhardt, N., Mondy, S., Tranchimand, S., Rumeau, D., Boudsocq, M., et al. (2013). An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in *Arabidopsis*. *PLoS Biol.* 11:e1001513. doi: 10.1371/journal.pbio.1001513

Mueller, S., Hilbert, B., Dueckershoff, K., Roitsch, T., Krischke, M., Mueller, M. J., et al. (2008). General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in *Arabidopsis*. *Plant Cell* 20, 768–785. doi: 10.1105/tpc.107.054809

Mustilli, A. C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J. (2002). *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* 14, 3089–3099. doi: 10.1105/tpc.007906

Nadeem, S. M., Ahmad, M., Zahir, Z. A., Javaid, A., and Ashraf, M. (2013). The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotechnol. Adv.* 32, 429–448. doi: 10.1016/j.biotechadv.2013.12.005

Nazzaro, F., Fratianni, F., and Coppola, R. (2013). Quorum sensing and phytochemicals. *Int. J. Mol. Sci.* 14, 12607–12619. doi: 10.3390/ijms140612607

Ortiz-Castro, R., Martinez-Trujillo, M., and Lopez-Bucio, J. (2008). N-acyl-L-homoserine lactones: a class of bacterial quorum-sensing signals alter post-embryonic root development in *Arabidopsis thaliana*. *Plant Cell Environ.* 31, 1497–1509. doi: 10.1111/j.1365-3040.2008.01863.x

Palmer, A. G., Senechal, A. C., Mukherjee, A., Ane, J. M., and Blackwell, H. E. (2014). Plant responses to bacterial N-acyl L-homoserine lactones are dependent on enzymatic degradation to L-homoserine. *ACS Chem. Biol.* 9, 1834–1845. doi: 10.1021/cb500191a

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Rasmann, S., De Vos, M., Casteel, C. L., Tian, D., Halitschke, R., Sun, J. Y., et al. (2012). Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant Physiol.* 158, 854–863. doi: 10.1104/pp.111.187831

Rojo, E., Solano, R., and Sanchez-Serrano, J. J. (2003). Interactions between signaling compounds involved in plant defense. *J. Plant Growth Regul.* 22, 82–98. doi: 10.1007/s00344-003-0027-6

Ruby, E. G., and Nealson, K. H. (1976). Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*; a model of symbiosis based on bacterial studies. *Biol. Bull.* 151, 574–586. doi: 10.2307/1540507

Sattler, S. E., Mene-Saffrane, L., Farmer, E. E., Krischke, M., Mueller, M. J., and Dellapenna, D. (2006). Nonenzymatic lipid peroxidation reprograms gene expression and activates defense markers in *Arabidopsis* tocopherol-deficient mutants. *Plant Cell* 18, 3706–3720. doi: 10.1105/tpc.106.044065

Schenk, S. T., Hernandez-Reyes, C., Samans, B., Stein, E., Neumann, C., Schikora, M., et al. (2014). N-acyl-homoserine lactone primes plants for cell wall reinforcement and induces resistance to bacterial pathogens via the salicylic acid/oxylipin pathway. *Plant Cell* 26, 2708–2723. doi: 10.1105/tpc.114.126763

Schenk, S. T., Stein, E., Kogel, K. H., and Schikora, A. (2012). *Arabidopsis* growth and defense are modulated by bacterial quorum sensing molecules. *Plant Signal. Behav.* 7, 178–181. doi: 10.4161/psb.18789

Schikora, A., Schenk, S. T., Stein, E., Molitor, A., Zuccaro, A., and Kogel, K. H. (2011). N-acyl-homoserine lactone confers resistance towards biotrophic and hemibiotrophic pathogens via altered activation of AtMPK6. *Plant Physiol.* 157, 1407–1418. doi: 10.1104/pp.111.180604

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., et al. (2006). Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ.* 29, 909–918. doi: 10.1111/j.1365-3040.2005.01471.x

Scott, R. A., Weil, J., Le, P. T., Williams, P., Fray, R. G., Von Bodman, S. B., et al. (2006). Long- and short-chain plant-produced bacterial N-acyl-homoserine lactones become components of phyllosphere, rhizosphere, and soil. *Mol. Plant Microbe Interact.* 19, 227–239. doi: 10.1094/MPMI-19-0227

Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B., and Mauch-Mani, B. (2012). Descendants of primed *Arabidopsis* plants exhibit resistance to biotic stress. *Plant Physiol.* 158, 835–843. doi: 10.1104/pp.111.191593

Spoel, S. H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune responses. *Cell Host Microbe* 3, 348–351. doi: 10.1016/j.chom.2008.05.009

Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E. E. (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12837–12842. doi: 10.1073/pnas.211311098

Stotz, H. U., Mueller, S., Zoeller, M., Mueller, M. J., and Berger, S. (2013). TGA transcription factors and jasmonate-independent COI1 signalling regulate specific plant responses to reactive oxylipins. *J. Exp. Bot.* 64, 963–975. doi: 10.1093/jxb/ers389

Taki, N., Sasaki-Sekimoto, Y., Obayashi, T., Kikuta, A., Kobayashi, K., Ainai, T., et al. (2005). 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in *Arabidopsis*. *Plant Physiol.* 139, 1268–1283. doi: 10.1104/pp.105.067058

Teplitski, M., Mathesius, U., and Rumbaugh, K. P. (2010). Perception and degradation of N-acyl homoserine lactone quorum sensing signals by mammalian and plant cells. *Chem. Rev.* 111, 100–116. doi: 10.1021/cr100045m

Thoma, I., Loeffler, C., Sinha, A. K., Gupta, M., Krischke, M., Steffan, B., et al. (2003). Cyclopentenone isoprostanes induced by reactive oxygen species trigger defense gene activation and phytoalexin accumulation in plants. *Plant J.* 34, 363–375. doi: 10.1046/j.1365-313X.2003.01730.x

Tomasz, A. (1965). Control of the competent state in pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria. *Nature* 208, 155–159. doi: 10.1038/208155a0

Ton, J., Jakab, G., Toquin, V., Flors, V., Iavicoli, A., Maeder, M. N., et al. (2005). Dissecting the beta-aminobutyric acid-induced priming phenomenon in *Arabidopsis*. *Plant Cell* 17, 987–999. doi: 10.1105/tpc.104.029728

Tsai, C. H., Singh, P., Chen, C. W., Thomas, J., Weber, J., Mauch-Mani, B., et al. (2011). Priming for enhanced defence responses by specific inhibition of the *Arabidopsis* response to coronatine. *Plant J.* 65, 469–479. doi: 10.1111/j.1365-313X.2010.04436.x

van Wees, S. C., De Swart, E. A., van Pelt, J. A., van Loon, L. C., and Pieterse, C. M. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8711–8716. doi: 10.1073/pnas.130425197

von Rad, U., Klein, I., Dobrev, P. I., Kottova, J., Zazimalova, E., Fekete, A., et al. (2008). Response of *Arabidopsis thaliana* to N-hexanoyl-DL-homoserine-lactone,

a bacterial quorum sensing molecule produced in the rhizosphere. *Planta* 229, 73–85. doi: 10.1007/s00425-008-0811-4

Waters, C. M., and Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* 21, 319–346. doi: 10.1146/annurev.cellbio.21.012704.131001

Weber, H., Chételat, A., Caldelari, D., and Farmer, E. E. (1999). Divinyl ether fatty acid synthesis in late blight-diseased potato leaves. *Plant Cell* 11, 485–493. doi: 10.1105/tpc.11.3.485

Williams, P. (2007). Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* 153, 3923–3938. doi: 10.1099/mic.0.2007/012856-0

Yi, S. Y., Shirasu, K., Moon, J. S., Lee, S. G., and Kwon, S. Y. (2014). The activated SA and JA signaling pathways have an influence on flg22-triggered oxidative burst and callose deposition. *PLoS ONE* 9:e88951. doi: 10.1371/journal.pone.0088951

Zarkani, A. A., Stein, E., Rohrich, C. R., Schikora, M., Evguenieva-Hackenberg, E., Degenkolb, T., et al. (2013). Homoserine lactones influence the reaction of plants to rhizobia. *Int. J. Mol. Sci.* 14, 17122–17146. doi: 10.3390/ijms140817122

Zeng, W., and He, S. Y. (2010). A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to *Pseudomonas syringae* pv tomato DC3000 in *Arabidopsis. Plant Physiol.* 153, 1188–1198. doi: 10.1104/pp.110.157016

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528. doi: 10.1073/pnas.96.11.6523

Zhang, Y. L., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653. doi: 10.1105/tpc.014894

Zheng, X. Y., Spivey, N. W., Zeng, W., Liu, P. P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596. doi: 10.1016/j.chom.2012.04.014

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The reviewer, Anton Hartmann declares that, despite having collaborated with the author Adam Schikora, the review process was handled objectively and no conflict of interest exists.

Received: 13 October 2014; accepted: 17 December 2014; published online: 14 January 2015.

*Citation: Schenk ST and Schikora A (2015) AHL-priming functions via oxylipin and salicylic acid. Front. Plant Sci. **5**:784. doi: 10.3389/fpls.2014.00784*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on