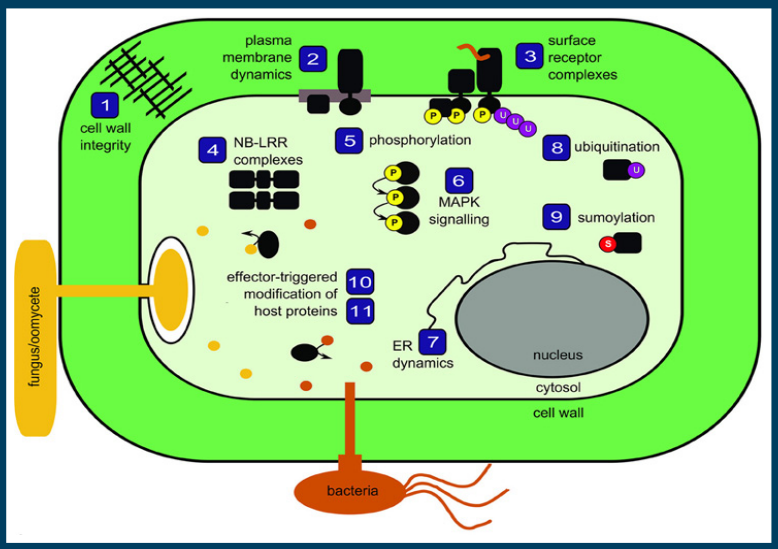
frontiers

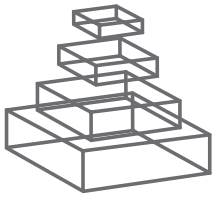
RESEARCH TOPICS



MECHANISMS REGULATING IMMUNITY IN PLANTS

Topic Editors
Alexandra M. E. Jones,
Jacqueline Monaghan and Vardis Ntoukakis

frontiers in PLANT SCIENCE



frontiers

ISSN 1664-8714
ISBN 978-2-88919-166-6
DOI 10.3389/978-2-88919-166-6

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

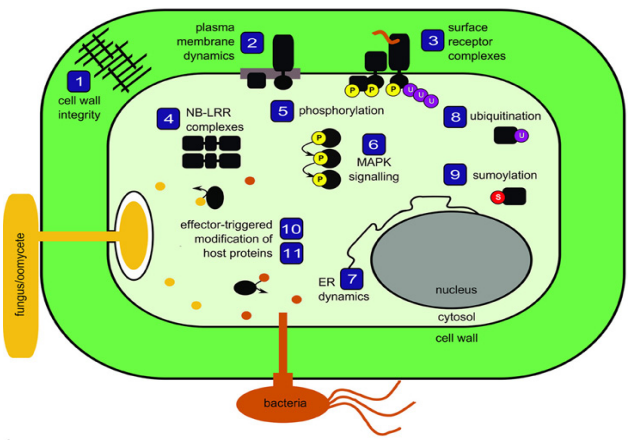
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - 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

MECHANISMS REGULATING IMMUNITY IN PLANTS

Topic Editors:
Alexandra M.E. Jones, The Sainsbury Laboratory, United Kingdom
Jacqueline Monaghan, The Sainsbury Laboratory, United Kingdom
Vardis Ntoukakis, University of Warwick, United Kingdom



Schematic representation of topics covered in the special issue *Mechanisms regulating immunity in plants*. Figure taken from Jones AME, Monaghan J and Ntoukakis V (2013) Editorial: Mechanisms regulating immunity in plants. *Front. Plant Sci.* 4:64. doi: 10.3389/fpls.2013.00064.

The molecular mechanisms underpinning the innate ability of plants to defend against microbial pathogens are of great interest worldwide. In addition to genetic analyses, researchers have increasingly turned to biochemical and proteomic assays to address questions related to plant immunity and pathogen virulence. Protein binding partners and post-translational modifications are central to signal transduction networks resulting in host immune responses, and studying these molecular events is at the forefront of plant-microbe interactions research. In this Research Topic, we will collect Mini-Reviews describing current work on the plant immune system with a focus on protein biology.

Table of Contents

frontiers in
PLANT SCIENCE

EDITORIAL
published: 27 March 2013
doi: 10.3389/fpls.2013.00064

Editorial: Mechanisms regulating immunity in plants

Alexandra M. E. Jones[1]*, Jacqueline Monaghan[1]* and Vardis Ntoukakis[2]*

[1] *The Sainsbury Laboratory, Norwich Research Park, Norwich, UK*
[2] *School of Life Sciences, University of Warwick, Coventry, UK*
**Correspondence: alex.jones@tsl.ac.uk; jacqueline.monaghan@tsl.ac.uk; v.ntoukakis@warwick.ac.uk*

Edited by:
Joshua L. Heazlewood, Lawrence Berkeley National Laboratory, USA

Reviewed by:
Joshua L. Heazlewood, Lawrence Berkeley National Laboratory, USA

Plants are constantly exposed to potential pathogens in their environment. The intimate associations involved in plant-microbe interactions have influenced the evolution of a multi-faceted surveillance system to detect and respond to both the presence of microbes at the cell surface as well as the presence of pathogenic effectors inside the cell. Here, we bring together 11 reviews that discuss current concepts in plant innate immunity with a focus on protein biology and proteomics (**Figure 1**).

To interact with the plant plasma membrane, microbes must first breach the formidable barrier presented by the cell wall. Nühse (2012) introduces the emerging concept of cell wall integrity signaling, noting that both mechanical properties and receptors capable of sensing cellular damage are likely to be involved. In both pathogenic and symbiotic interactions with microbes, the host plasma membrane is substantially modified. Urbanus and Ott (2012) review the dynamic compartmentalization of the plasma membrane and discuss factors, such as alterations to lipid composition and/or anchoring of proteins to the cell wall or cytoskeleton, that contribute to the formation of membrane micro-domains. Embedded within the plasma membrane, pattern recognition receptors (PRRs) can be affected by the formation of micro-domains. Many PRRs are receptor-like kinases that bind ligands derived from microbes and, as discussed by Greeff et al. (2012), rapidly form complexes with other proteins to initiate signaling cascades. Within the plant cell, additional immune receptor complexes detect the presence of pathogenic effector proteins. Bonardi and Dangl (2012) describe pre- and post-activation mechanisms regulating intracellular receptor complexes and recognize the need to use emerging fluorescent protein technologies in parallel to proteomics in order to study spatio-temporal dynamics of immune receptors in living cells.

Of all the molecular events that occur within activated receptor complexes, the most intensely studied using proteomic methods is phosphorylation, both for the amenability of this modification to analysis and for the central role it plays in signal transduction in all organisms. Park et al. (2012) nicely review the role of phosphorylation in all stages of immune signal transduction downstream of PRRs. The authors identify the need to clarify *in vivo* phosphorylation events and they highlight the continued gap in our knowledge between activated receptor complexes and downstream signaling cascades, such as those mediated by mitogen-activated protein kinases, which are discussed in detail by Rasmussen et al. (2012).

The endoplasmic reticulum (ER) is closely connected to defense responses, both as a large intracellular store of calcium and as the site of immune receptor biogenesis. Eichmann and Schäfer (2012) review the integration of stress responses by the ER and its role in initiating programmed cell death through the activation of ER-resident regulatory proteins, drawing comparisons with better characterized animal systems. In addition to ER folding machinery, the turn-over of both plasma membrane-localized and intracellular receptors relies upon ubiquitination. Furlan et al. (2012) review these aspects and explore proteomic methods to identify novel ubiquitination sites. Recent progress has also been made in proteomics to identify modifications by the small ubiquitin-like protein SUMO. Encouragingly, Mazur and van den Burg (2012) describe the use of histidine-tagged SUMO as "routine" and compare proteins identified by these and more advanced methods in plants and animals in the context of SUMO dynamics in abiotic and biotic stress responses.

Adapted pathogens must evade or suppress host immune responses in order to colonize tissues and cause disease, and they deploy numerous effector proteins to secure this objective. Wirthmueller and Banfield (2012) focus on pathogenic mono ADP-ribosyltransferases as important virulence factors acting on host targets in both plant and animal systems. Given the importance of post-translational modifications of proteins in the plant immune system, Howden and Huitema (2012) explore how pathogen effectors modify the post-translational status of host proteins to interfere with defense signaling. The authors also offer insight into experimental approaches for effector/target mining.

Proteomic methods have facilitated the identification of key players involved in plant immunity and have shed light on the significance of post-translational modifications and protein interactions in the regulation and transduction of immune signaling. In future, the use of large-scale and highly sensitive quantitative proteomics in combination with emerging transcriptomic and imaging technologies will play a central role in uncovering the kinetics of immune signaling pathways, which currently remains a challenge. This is an exciting time to be involved in plant immunity research and we hope that this collection of reviews will inform and inspire our readers.

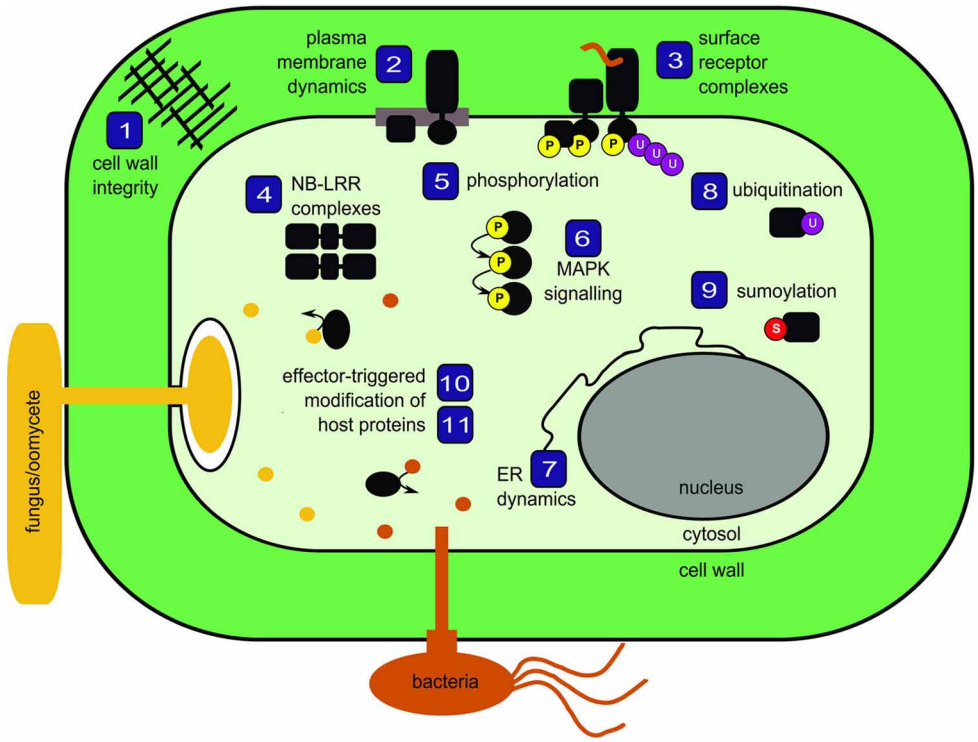


FIGURE 1 | Schematic representation of topics covered in the special issue *Mechanisms regulating immunity in plants*. Numbers correspond to review articles as follows: (1) Nühse (2012), Cell wall integrity signaling and innate immunity in plants; (2) Urbanus and Ott (2012), Plasticity of plasma membrane compartmentalization during plant immune responses; (3) Greeff et al. (2012), Receptor-like kinase complexes in plant innate immunity; (4) Bonardi and Dangl (2012), How complex are intracellular immune receptor signaling complexes? (5) Park et al. (2012), Protein phosphorylation in plant immunity: insights into the regulation of pattern recognition receptor-mediated signaling; (6) Rasmussen et al. (2012), MAP kinase cascades in *Arabidopsis* innate immunity; (7) Eichmann and Schäfer (2012), The endoplasmic reticulum in plant immunity and cell death; (8) Furlan et al. (2012), Regulation of plant immune receptors by ubiquitination; (9) Mazur and van den Burg (2012), Global SUMO proteome responses guide gene regulation, mRNA biogenesis, and plant stress responses; (10) Wirthmueller and Banfield (2012), mADP-RTs: versatile virulence factors from bacterial pathogens of plants and mammals; (11) Howden and Huitema (2012), Effector-triggered post-translational modifications and their role in suppression of plant immunity.

ACKNOWLEDGMENTS

Alexandra M. E. Jones acknowledges support from The Gatsby Charitable Foundation and The Sainsbury Laboratory. Jacqueline Monaghan is a recipient of a long-term fellowship from the European Molecular Biology Organization. Vardis Ntoukakis is supported by The Royal Society.

REFERENCES

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

Eichmann, R., and Schäfer, P. (2012). The endoplasmic reticulum in plant immunity and cell death. *Front. Plant Sci.* 3:200. doi: 10.3389/fpls.2012.00200

Furlan, G., Klinkenberg, J., and Trujillo, M. (2012). Regulation of plant immune receptors by ubiquitination. *Front. Plant Sci.* 3:238. doi: 10.3389/fpls.2012.00238

Greeff, C., Roux, M., Mundy, J., and Petersen, M. (2012). Receptor-like kinase complexes in plant innate immunity. *Front. Plant Sci.* 3:209. doi: 10.3389/fpls.2012.00209

Howden, A. J. M., and Huitema, E. (2012). Effector-triggered post-translational modifications and their role in suppression of plant immunity. *Front. Plant Sci.* 3:160. doi: 10.3389/fpls.2012.00160

Mazur, M. J., and van den Burg, H. A. (2012). Global SUMO proteome responses guide gene regulation, mRNA biogenesis, and plant stress responses. *Front. Plant Sci.* 3:215. doi: 10.3389/fpls.2012.00215

Nühse, T. S. (2012). Cell wall integrity signaling and innate immunity in plants. *Front. Plant Sci.* 3:280. doi: 10.3389/fpls.2012.00280

Park, C.-J., Caddell, D. F., and Ronald, P. C. (2012). Protein phosphorylation in plant immunity: insights into the regulation of pattern recognition receptor-mediated signaling. *Front. Plant Sci.* 3:177. doi: 10.3389/fpls.2012.00177

Rasmussen, M. W., Roux, M., Petersen, M., and Mundy, J. (2012). MAP kinase cascades in Arabidopsis innate immunity. *Front. Plant Sci.* 3:169. doi: 10.3389/fpls.2012.00169

Urbanus, S. L., and Ott, T. (2012). Plasticity of plasma membrane compartmentalization during plant immune responses. *Front. Plant Sci.* 3:181. doi: 10.3389/fpls.2012.00181

Wirthmueller, L., and Banfield, M. J. (2012). mADP-RTs: versatile virulence factors from bacterial pathogens of plants and mammals. *Front. Plant Sci.* 3:142. doi: 10.3389/fpls.2012.00142

Received: 07 March 2013; accepted: 07 March 2013; published online: 27 March 2013.

*Citation: Jones AME, Monaghan J and Ntoukakis V (2013) Editorial: Mechanisms regulating immunity in plants. Front. Plant Sci. **4**:64. doi: 10.3389/fpls.2013.00064*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 11 December 2012
doi: 10.3389/fpls.2012.00280

Cell wall integrity signaling and innate immunity in plants

Thomas S. Nühse*

Faculty of Life Sciences, University of Manchester, Manchester, UK

Edited by:
Alex Jones, The Sainsbury Laboratory, UK

Reviewed by:
Benjamin Schwessinger, University of California at Davis, USA
Milena Roux, University of Copenhagen, Denmark

****Correspondence:***
Thomas S. Nühse, Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK.
e-mail: thomas.nuhse@manchester.ac.uk

All plant pathogens and parasites have had to develop strategies to overcome cell walls in order to access the host's cytoplasm. As a mechanically strong, multi-layered composite exoskeleton, the cell wall not only enables plants to grow tall but also protects them from such attacks. Many plant pathogens employ an arsenal of cell wall degrading enzymes, and it has long been thought that the detection of breaches in wall integrity contributes to the induction of defense. Cell wall fragments are danger-associated molecular patterns or DAMPs that can trigger defense signaling pathways comparable to microbial signals, but the picture is likely to be more complicated. A wide range of defects in cell wall biosynthesis leads to enhanced pathogen resistance. We are beginning to understand the essential role of cell wall integrity surveillance for plant growth, and the connection of processes like cell expansion, plasma membrane–cell wall contact and secondary wall biosynthesis with plant immunity is emerging.

Keywords: cell wall, cell wall integrity, immunity, signaling, danger-associated molecular pattern, receptor-like kinase

The cell wall represents a unique challenge for pathogens specializing in plants. Manipulation of the host, delivery of effectors and suppression of defense responses requires intimate contact between parasite and host. Cell wall polysaccharides – cellulose, pectin, and hemicelluloses such as xyloglucan and arabinoxylan – are potentially a major source of carbon but are difficult to access. Depending on their lifestyle, some pathogens extensively degrade cell walls, such as the macerating necrotrophs *Erwinia* or *Botrytis*; others puncture it with surgical precision, such as biotrophic fungal and oomycete pathogens during the formation of appressoria. Dissolving and rearranging cell walls is also part of the large-scale host manipulation undertaken by plant parasitic nematodes establishing feeding sites (Gheysen and Mitchum, 2011). It seems obvious that such breaches of cell wall integrity (CWI) should alert the host plant to the presence of invaders. Among the potential warning signs are changes in mechanical properties, interference with cell wall proteins or polysaccharides by the binding of effectors, and release of oligosaccharide fragments with DAMP (danger-associated molecular pattern) activity. However, the relative contribution made by each of these signals toward mounting efficient defense responses is still unclear. In the last few years, the concept of CWI signaling in plants has matured. While the close link of this pathway with innate immunity has been instrumental in its discovery, maintenance of mechanical CWI is also a necessary part of controlled cell expansion in healthy plants.

THE NEED FOR CELL WALL MAINTENANCE

Plant cell walls and the pressurized cells within them represent an economic solution for growing a multicellular organism: Without a proportional metabolic investment in cytoplasmic material, cells can grow simply by accumulating water and solutes in the vacuole and then driving expansion of the wall via turgor pressure. Cell walls need to remain strong throughout this expansion and yet yield in a controlled way (Cosgrove, 2005). In some extreme cases, such as the expanding primary root tip or the hypocotyls of etiolating seedlings, this expansion increases the cell surface by an order of magnitude within hours (Beemster and Baskin, 1998). Many other developmental programmes require irreversible cell wall weakening or dissolution, including the emergence of lateral roots and of the radicle from the seed coat; formation of vasculature, stomata, and aerenchyma; abscission, organ separation, and fertilization. The controlled yielding of cell walls during expansion requires a way of feeding back information about wall stability to the cytoplasm so that growth rates can be adjusted if necessary. Root cell elongation, for example, is known to be influenced by a wide range of environmental factors (De Cnodder et al., 2006), confirming that the developmental programme integrates external information rather than unfolding by default. The nature of this surveillance system and the postulated CWI sensors is actively debated (Ringli, 2010; Seifert and Blaukopf, 2010). It is already clear, however, that surveillance of plant cell wall structure and innate immunity are closely linked.

THE CELL WALL AS A BARRIER FOR PATHOGENS

Cell wall degrading enzymes are a major part of the weaponry used by necrotrophic and, to a lesser extent, biotrophic pathogens (Walton, 1994). The tightly packed crystalline arrangement of microfibrils makes cellulose an unattractive target for attack. In contrast, pectin and xylan, major components of type I cell walls in most dicots and type II walls in most grain crops respectively, are easier to access and break down. Enzymes degrading pectin (polygalacturonases, pectate lyases, and pectin methyl esterases) and xylan (endo-xylanases) are key virulence factors for pathogens. In turn, plants counter these attacks with an array of inhibitor proteins (Juge, 2006). Interestingly, the function of polygalacturonase inhibitor proteins seems not primarily to block pectin degradation completely but to shift the breakdown process toward generating larger fragments that are DAMP active (Federici et al., 2006).

Natural infection routes chosen by plant pathogens often reflect how the cell wall acts as a barrier. For example, soil borne fungi typically first colonize a root at the tip but can only invade the root in the elongation zone where walls are temporarily weakened and thinned (Gunawardena and Hawes, 2002). Fruit ripening is another example for easier pathogen entry in areas of developmentally regulated cell wall weakening. Polygalacturonases and pectate lyases contribute substantially to the softening of fruit. Suppression of these enzymes delays fruit softening and at the same time confers enhanced resistance to pathogens like *Botrytis* (summarized in Cantu et al., 2008). Similarly, promoting cell wall stiffness by overexpressing extensin in *Arabidopsis* enhanced resistance to *Pseudomonas syringae* (Wei and Shirsat, 2006). In other cases, changes in cell wall composition increase susceptibility to a pathogen in ways that are more difficult to explain. The receptor-like kinase (RLK) ERECTA is a major determinant of resistance to the necrotrophic pathogens *Ralstonia solanacearum* and *Plectosphaerella cucumerina*. The *erecta* mutant has increased cellulose and uronic acid contents in the cell wall (Godiard et al., 2003; Llorente et al., 2005; Sanchez-Rodriguez et al., 2009). Similarly, mutants in the alpha and beta subunits of heterotrimeric G-proteins are more susceptible to *P. cucumerina* and have a subtly altered cell wall structure including less xylose (Llorente et al., 2005; Delgado-Cerezo et al., 2011). It is unclear how cell wall composition is controlled by these signaling proteins, but the positive correlation of increased uronic acid and decreased xylose with susceptibility to *P. cucumerina* has been confirmed in additional mutants (Sanchez-Rodriguez et al., 2009; Delgado-Cerezo et al., 2011).

DISEASE RESISTANCE TRIGGERED BY CELL WALL DEFECTS

There are many other cases of cell wall alterations or defects that – perhaps counterintuitively – enhance pathogen resistance. Some of these are subtle shifts in polysaccharide composition that may reduce the suitability of the host's wall for pathogen attachment or ingress, i.e., may be susceptibility factors. Several of the *powdery mildew resistant* (*pmr*) mutants may fall into this category (Vogel et al., 2002, 2004). Both *pmr5*, mapped to one member of a large plant-specific gene family related to TRICHOME BIREFRINGENT (Bischoff et al., 2010) and *pmr6*, a pectate lyase mutant, have increased levels of unesterified pectin and activate resistance via an unknown pathway that is independent of the well-studied salicylic acid (SA), ethylene (ET), or jasmonic acid (JA)-responsive paths. The *pmr5* and *pmr6* mutants only have slightly enhanced constitutive defense responses relative to the wild-type. In contrast, resistance to *Erysiphe cichoracearum* in *pmr4*, a callose synthase (Nishimura et al., 2003), and resistance to *Hyaloperonospora parasitica* in *cie1/ mur3*, a putative xyloglucan galactosyltransferase (Tedman-Jones et al., 2008), is based on constitutive activation of SA-dependent defense responses. The clearest indication of a causal link between cell wall defects and activation of defense responses came from the discovery of a series of mutants in cellulose synthase proteins that confer enhanced resistance to either biotrophic or necrotrophic pathogens. Two allelic mutations in the primary wall cellulose synthase gene CesA3 were identified in genetic screens for ectopic lignin deposition in the root (*eli1*) and on the basis of constitutive expression of the JA-induced gene vsp1 (*cev1*), respectively (Cano-Delgado et al., 2000; Ellis et al., 2002). Resistance to powdery mildew is considerably higher in *cev1* than the wild-type and requires JA and ET (Ellis and Turner, 2001). In contrast, defects in cellulose synthase proteins required for secondary cell wall formation (CesA4/IRX5, CesA7/IRX3, and CesA8/IRX1) confer enhanced resistance to the necrotrophic pathogens *P. cucumerina* and *R. solanacearum* in a pathway requiring ABA signaling but neither SA nor JA/ET (Hernandez-Blanco et al., 2007). Several other mutants in cell wall-related genes have since been discovered that also show variable degrees of resistance to pathogens or constitutive expression of defense-related genes (Ko et al., 2006; Vega-Sanchez et al., 2012). Drugs that interfere with cellulose biosynthesis, such as isoxaben and thaxtomin, phenocopy this response (Bischoff et al., 2009; Hamann et al., 2009). These discoveries sparked the idea of cell wall feedback signaling: a dedicated signaling pathway that monitors the physical integrity and functioning of the cell wall and if necessary activates repair responses.

THE CELL WALL INTEGRITY PATHWAY IN PLANTS

Loss of CWI, triggered by genetic defects in polysaccharide biosynthesis or by drugs, reduces cell elongation in etiolated hypocotyls and root tips (Hauser et al., 1995; Desnos et al., 1996; Desprez et al., 2002). If this response is based on a signaling process rather than physical inability to elongate, it should be possible to uncouple cell wall damage from its effect on expansion by blocking the signaling pathway. Experimental evidence shows that this is indeed the case (Refregier et al., 2004; Hematy et al., 2007; Tsang et al., 2011; Wolf et al., 2012). Mutation of the receptor-like kinase THESEUS attenuates the cell expansion defect of *procuste*, a mutant in a primary wall cellulose synthase (Fagard et al., 2000; Hematy et al., 2007). Several other (though not all tested) cell wall-deficient mutants are also rescued in a *the1* mutant background. In seedlings treated with isoxaben, the production of reactive oxygen species and lignin deposition is partially dependent on THE1 (Denness et al., 2011). THESEUS is only one of a whole range of potential cell wall sensors. Many others have been suggested based largely on the predicted (and in a few cases demonstrated) ability to bind cell wall components and transmit a signal to the cytoplasm. The rationale follows the well-characterized CWI pathway in yeast (Levin, 2011). Here, plasma membrane (PM) proteins including Wsc1 and Mid2 extend stiff hyper-glycosylated "antennae" into the wall and transmit signals with their short cytoplasmic domains. In the absence of obvious plant homologs of these sensors, the most attractive candidates are RLKs. In addition to THESEUS, several other members of the CrRLK1L (*Catharanthus roseus* RLK1-like) family of RLKs with an extracellular malectin-like domain have well-documented cell wall-related functions (for review, see Boisson-Dernier et al., 2011): FERONIA and ANXUR are required in the female and male gametophyte, respectively, for successful fertilization. Pollen tube guidance by the synergid cells and sperm release fail in *feronia* while pollen tubes burst prematurely in *anxur1/2* double mutants. FER, THE, and the related HERKULES1 and 2 are brassinosteroid-inducible and have

partially redundant roles in cell expansion throughout the plant (Guo et al., 2009).

Intriguingly, *feronia* mutants are more resistant to powdery mildew infection (Kessler et al., 2010), based perhaps on the mechanistic similarities between fertilization and fungal invasion. Both involve polarization of membrane proteins toward the pollen tube and fungal hyphae/appressoria, respectively. With the exception of the wall-associated kinases (WAKs, see below) and FER, it is not known whether any other candidate cell wall sensors have a role in immunity, such as the leucine-rich repeat (LRR-) RLKs, FEI1 and FEI2. The *fei1fei2* mutant has a characteristic conditional root expansion phenotype and impaired cellulose biosynthesis (Xu et al., 2008) that points to a role in cell wall homoeostasis for these RLKs.

PM–cell wall contacts have a key role in plant resistance to fungal penetration (Mellersh and Heath, 2001). These contacts, visible as Hechtian strands during plasmolysis, can be dissociated by addition of RGD (Arg-Gly-Asp) peptides like in metazoans (Canut et al., 1998). The existence of high-affinity binding sites for the RGD sequence in plants has long been puzzling because plants appeared to have neither fibronectin-like (RGD ligand) nor integrin-like (RGD receptor) proteins. RGD sequence motives are present on several oomycete effector proteins such as IPI-O of *Phytophthora* and are essential for attachment to the host (Senchou et al., 2004). Two recent developments have shed light on the connection: The *Arabidopsis* lectin-like receptor kinase LecRK-I.9 has been identified as a receptor for RGD peptides. Null mutants have reduced membrane–wall contacts, increased susceptibility to *Phytophthora brassicae* and almost no callose deposition in response to effector-disabled *Pseudomonas syringae* or bacterial flagellin. All these effects are phenocopied by overexpression of the RGD-motif effector, IPI-O (Bouwmeester et al., 2011). In a different study, Knepper et al. (2011) showed that NDR1, a PM protein required for several race-specific resistance pathways, also mediated PM–cell wall adhesion depending on its own Asn-Gly-Asp (NGD) motif. It is tempting to speculate that LecRK-I.9 binds to the NGD motif on NDR1, although that leaves the question open how association of two PM proteins establishes contact with the cell wall.

NDR1 and RLKs are not the only candidates for signaling proteins with a cell wall–cytoplasm bridging function. Class I formin homology proteins are membrane-anchored proteins with the ability to organize the actin cytoskeleton. The proline-rich extracellular domain of AtFH1 has been shown to bind to the cell wall (Martiniere et al., 2011). AtFH1 and the closely related AtFH6 are induced in the early stages of giant cell formation triggered by the plant–parasitic root knot nematode, *Meloidogyne incognita* (Favery et al., 2004). These proteins are ideal candidates for transmitting mechanical stress across the PM. The central role of the cytoskeleton in cell wall biosynthesis (Paredez et al., 2006), plant cell morphogenesis (Szymanski and Cosgrove, 2009), and innate immunity (Hardham et al., 2007) is well-recognized. Despite this connection, cytoskeletal functions in plant CWI signaling have not been studied extensively. In the yeast CWI pathway, the formins Bni1 and Bnr1 are key effectors of actin rearrangement and bind directly to the central regulator GTP-Rho1 (Levin, 2011).

The exact nature of the signal that communicates deficient cell walls is a matter of intense debate and may not be (exclusively) based on a direct polysaccharide sensor. Because of the turgor pressure, weakening cell walls will lead to unplanned protoplast expansion and PM stretch. Some responses triggered by inhibition of cellulose biosynthesis do indeed depend on the osmosensors Cre1 and Mca1 (Wormit et al., 2012) while others do not. Oligosaccharide fragments released from wall polysaccharides may represent another damage or danger signal. Specifically in the context of pathogen attack, some of the cell wall degrading enzymes released by microbial parasites have *endo*-activity and will set free such fragments. Short oligogalacturonides (DP 6-16) have long been known to induce rapid and strong defense responses (Doares et al., 1995). Wall-associated protein kinases (WAKs) have now been identified as likely receptors (Kohorn et al., 2009; Brutus et al., 2010). The *WAK*s, a family of RLKs with extracellular fibronectin-type repeats, also play a role in cell wall maintenance in normal plant development (Wagner and Kohorn, 2001; Kohorn et al., 2006), and a differential affinity for low- and high-molecular weight pectins may allow for a dual role in pathogen detection versus cell wall maintenance during growth (Kohorn and Kohorn, 2012). A WAK-like kinase (WAKL22) is a major determinant of resistance to *Fusarium oxysporum* in *Arabidopsis* (Diener and Ausubel, 2005). No specific detection systems for other types of endogenous wall fragments have been identified. Cellodextrins (i.e., β-1,4-linked glucose oligomers conceivably derived from cellulose) and β-1,3-glucan fragments trigger defense responses in grapevine cell cultures (Aziz et al., 2007). However, like oligogalacturonides they only do so in much higher concentrations than comparable "non-self" oligosaccharides such as chitin (Felix et al., 1993). It is likely that sensors for cross-linked cell wall polysaccharides as well as sensors for fragments derived from them play a part in plant CWI signaling, but relative contributions are still completely open.

THE ROLE OF PROTEOMICS IN DECIPHERING THE CWI PATHWAY

Analyzing the subcellular processes during pathogen invasion is difficult with proteomic tools – processes like cell polarization only occur in the attacked cells, and sampling only these is extremely challenging. However, just as the response to bacterial flagellin has been a useful model system for studying defense responses using proteomics and phosphoproteomics (Nühse et al., 2007), low molecular weight compounds can be used to induce cell wall defects (Hamann et al., 2009; Tsang et al., 2011) that phenocopy those observed in cell wall biosynthetic mutants (see above). Signaling proteins identified as differentially phosphorylated in such a setup are very likely to have roles both in normal plant growth and cell wall-based defense against pathogens.

Intriguing links between normal development, cell wall homoeostasis and innate immunity have emerged with the discovery of novel roles for ERECTA and NDR1 (Sanchez-Rodriguez et al., 2009; Knepper et al., 2011). The identification of binding partners (Roux et al., 2011) of these and other proteins, especially putative cell wall sensors, will be a challenge–like mature WAKs, wall-associated proteins may have "the biochemistry of a rock"

(B. Kohorn, unpublished). We need to take on this challenge to advance our understanding of signaling networks connecting immunity and CWI.

ACKNOWLEDGMENT

Thomas S. Nühse was supported by a BBSRC David Phillips fellowship (BB/D020093/1).

REFERENCES

Aziz, A., Gauthier, A., Bezler, A., Poinssot, B., Joubert, J. M., Pugin, A., et al. (2007). Elicitor and resistance-inducing activities of beta-1,4 cellodextrins in grapevine, comparison with beta-1,3 glucans and alpha-1,4 oligogalacturonides. *J. Exp. Bot.* 58, 1463–1472.

Beemster, G. T. S., and Baskin, T. I. (1998). Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. *Plant Physiol.* 116, 1515–1526.

Bischoff, V., Cookson, S. J., Wu, S., and Scheible, W. R. (2009). Thaxtomin A affects CESA-complex density, expression of cell wall genes, cell wall composition, and causes ectopic lignification in *Arabidopsis thaliana* seedlings. *J. Exp. Bot.* 60, 955–965.

Bischoff, V., Nita, S., Neumetzler, L., Schindelasch, D., Urbain, A., Eshed, R., et al. (2010). TRICHOME BIREFRINGENCE and its homolog AT5G01360 encode plant-specific DUF231 proteins required for cellulose biosynthesis in *Arabidopsis*. *Plant Physiol.* 153, 590–602.

Boisson-Dernier, A., Kessler, S. A., and Grossniklaus, U. (2011). The walls have ears: the role of plant CrRLK1Ls in sensing and transducing extracellular signals. *J. Exp. Bot.* 62, 1581–1591.

Bouwmeester, K., De Sain, M., Weide, R., Gouget, A., Klamer, S., Canut, H., et al. (2011). The lectin receptor kinase LecRK-I.9 is a novel Phytophthora resistance component and a potential host target for a RXLR effector. *PLoS Pathog.* 7:e1001327. doi: 10.1371/journal.ppat.1001327

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9452–9457.

Cano-Delgado, A. I., Metzlaff, K., and Bevan, M. W. (2000). The eli1 mutation reveals a link between cell expansion and secondary cell wall formation in *Arabidopsis thaliana*. *Development* 127, 3395–3405.

Cantu, D., Vicente, A. R., Labavitch, J. M., Bennett, A. B., and Powell, A. L. T. (2008). Strangers in the matrix: plant cell walls and pathogen susceptibility. *Trends Plant Sci.* 13, 610–617.

Canut, H., Carrasco, A., Galaud, J. P., Cassan, C., Bouyssou, H., Vita, N., et al. (1998). High affinity RGD-binding sites at the plasma membrane of *Arabidopsis thaliana* links the cell wall. *Plant J.* 16, 63–71.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 6, 850–861.

De Cnodder, T., Verbelen, J.-P., and Vissenberg, K. (2006). "The control of cell size and rate of elongation in the *Arabidopsis* root," in *The Expanding Cell*, eds J. Verbelen and K. Vissenberg (Berlin: Springer), 249–269.

Delgado-Cerezo, M., Sanchez-Rodriguez, C., Escudero, V., Miedes, E., Fernandez, P. V., Jorda, L., et al. (2011). *Arabidopsis* heterotrimeric G-protein regulates cell wall defense and resistance to necrotrophic fungi. *Mol. Plant* 5, 98–114.

Denness, L., Mckenna, J. F., Segonzac, C., Wormit, A., Madhou, P., Bennett, M., et al. (2011). Cell wall damage-induced lignin biosynthesis is regulated by a reactive oxygen species- and jasmonic acid-dependent process in *Arabidopsis*. *Plant Physiol.* 156, 1364–1374.

Desnos, T., Orbovic, V., Bellini, C., Kronenberger, J., Caboche, M., Traas, J., et al. (1996). Procuste1 mutants identify two distinct genetic pathways controlling hypocotyl cell elongation, respectively in dark and light-grown *Arabidopsis* seedlings. *Development* 122, 683–693.

Desprez, T., Vernhettes, S., Fagard, M., Refregier, G., Desnos, T., Aletti, E., et al. (2002). Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. *Plant Physiol.* 128, 482–490.

Diener, A. C., and Ausubel, F. M. (2005). RESISTANCE TO FUSARIUM OXYSPORUM 1, a dominant *Arabidopsis* disease-resistance gene, is not race specific. *Genetics* 171, 305–321.

Doares, S. H., Syrovets, T., Weiler, E. W., and Ryan, C. A. (1995). Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4095–4098.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J. G. (2002). The *Arabidopsis* mutant cev1 links cell wall signaling to jasmonate and ethylene responses. *Plant Cell* 14, 1557–1566.

Ellis, C., and Turner, J. G. (2001). The *Arabidopsis* mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* 13, 1025–1033.

Fagard, M., Desnos, T., Desprez, T., Goubet, F., Refregier, G., Mouille, G., et al. (2000). PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. *Plant Cell* 12, 2409–2423.

Favery, B., Chelysheva, L. A., Lebris, M., Jammes, F., Marmagne, A., De Almeida-Engler, J., et al. (2004). *Arabidopsis* formin AtFH6 is a plasma membrane-associated protein upregulated in giant cells induced by parasitic nematodes. *Plant Cell* 16, 2529–2540.

Federici, L., Di Matte, A., Fernandez-Recio, J., Tsernoglou, D., and Cervone, F. (2006). Polygalacturonase inhibiting proteins: players in plant innate immunity? *Trends Plant Sci.* 11, 65–70.

Felix, G., Regenass, M., and Boller, T. (1993). Specific perception of subnanomolar concentrations of chitin fragments by tomato cells – induction of extracellular alkalinisation, changes in protein phosphorylation and establishment of a refractory state. *Plant J.* 4, 307–316.

Gheysen, G., and Mitchum, M. G. (2011). How nematodes manipulate plant development pathways for infection. *Curr. Opin. Plant Biol.* 14, 415–421.

Godiard, L., Sauviac, L., Torii, K. U., Grenon, O., Mangin, B., Grimsley, N. H., et al. (2003). ERECTA, an LRR receptor-like kinase protein controlling development pleiotropically affects resistance to bacterial wilt. *Plant J.* 36, 353–365.

Gunawardena, U., and Hawes, M. C. (2002). Tissue specific localization of root infection by fungal pathogens: role of root border cells. *Mol. Plant Microbe Interact.* 15, 1128–1136.

Guo, H. Q., Li, L., Ye, H. X., Yu, X. F., Algreen, A., and Yin, Y. H. (2009). Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7648–7653.

Hamann, T., Bennett, M., Mansfield, J., and Somerville, C. (2009). Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. *Plant J.* 57, 1015–1026.

Hardham, A. R., Jones, D. A., and Takemoto, D. (2007). Cytoskeleton and cell wall function in penetration resistance. *Curr. Opin. Plant Biol.* 10, 342–348.

Hauser, M. T., Morikami, A., and Benfey, P. N. (1995). Conditional root expansion mutants of *Arabidopsis*. *Development* 121, 1237–1252.

Hematy, K., Sado, P. E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., et al. (2007). A receptor-like kinase mediates the response of *Arabidopsis* cells to the inhibition of cellulose synthesis. *Curr. Biol.* 17, 922–931.

Hernandez-Blanco, C., Feng, D. X., Hu, J., Sanchez-Vallet, A., Deslandes, L., Llorente, F., et al. (2007). Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *Plant Cell* 19, 890–903.

Juge, N. (2006). Plant protein inhibitors of cell wall degrading enzymes. *Trends Plant Sci.* 11, 359–367.

Kessler, S. A., Shimosato-Asano, H., Keinath, N. F., Wuest, S. E., Ingram, G., Panstruga, R., et al. (2010). Conserved molecular components for pollen tube reception and fungal invasion. *Science* 330, 968–971.

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.

Ko, J. H., Kim, J. H., Jayanty, S. S., Howe, G. A., and Han, K. H. (2006). Loss of function of COBRA, a determinant of oriented cell expansion, invokes cellular defence responses in *Arabidopsis thaliana*. *J. Exp. Bot.* 57, 2923–2936.

Kohorn, B. D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., et al. (2009). Pectin activation of MAP kinase and gene expression is WAK2 dependent. *Plant J.* 60, 974–982.

Kohorn, B. D., Kobayashi, M., Johansen, S., Riese, J., Huang, L. F., Koch, K., et al. (2006). An *Arabidopsis* cell wall-associated kinase required for invertase activity and cell growth. *Plant J.* 46, 307–316.

Kohorn, B. D., and Kohorn, S. L. (2012). The cell wall-associated kinases, WAKs, as pectin receptors. *Front. Plant Sci.* 3:88. doi: 10.3389/fpls.2012.00088

Levin, D. E. (2011). Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. *Genetics* 189, 1145–1175.

Llorente, F., Alonso-Blanco, C., Sanchez-Rodriguez, C., Jorda, L., and

Molina, A. (2005). ERECTA receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. *Plant J.* 43, 165–180.

Martiniere, A., Gayral, P., Hawes, C., and Runions, J. (2011). Building bridges: formin1 of *Arabidopsis* forms a connection between the cell wall and the actin cytoskeleton. *Plant J.* 66, 354–365.

Mellersh, D. G., and Heath, M. C. (2001). Plasma membrane–cell wall adhesion is required for expression of plant defense responses during fungal penetration. *Plant Cell* 13, 413–424.

Nishimura, M. T., Stein, M., Hou, B. H., Vogel, J. P., Edwards, H., and Somerville, S. C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* 301, 969–972.

Nühse, T. S., Bottrill, A. R., Jones, A. M. E., and Peck, S. C. (2007). Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. *Plant J.* 51, 931–940.

Paredez, A. R., Somerville, C. R., and Ehrhardt, D. W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* 312, 1491–1495.

Refregier, G., Pelletier, S., Jaillard, D., and Hofte, H. (2004). Interaction between wall deposition and cell elongation in dark-grown hypocotyl cells in *Arabidopsis*. *Plant Physiol.* 135, 959–968.

Ringli, C. (2010). Monitoring the outside: cell wall-sensing mechanisms. *Plant Physiol.* 153, 1445–1452.

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.

Sanchez-Rodriguez, C., Estevez, J. M., Llorente, F., Hernandez-Blanco, C., Jorda, L., Pagan, I., et al. (2009). The ERECTA receptor-like kinase regulates cell wall-mediated resistance to pathogens in *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 22, 953–963.

Seifert, G. J., and Blaukopf, C. (2010). Irritable walls: the plant extracellular matrix and signaling. *Plant Physiol.* 153, 467–478.

Senchou, V., Weide, R., Carrasco, A., Bouyssou, H., Pont-Lezica, R., Govers, F., et al. (2004). High affinity recognition of a Phytophthora protein by *Arabidopsis* via an RGD motif. *Cell. Mol. Life Sci.* 61, 502–509.

Szymanski, D. B., and Cosgrove, D. J. (2009). Dynamic coordination of cytoskeletal and cell wall systems during plant cell morphogenesis. *Curr. Biol.* 19, R800–R811.

Tedman-Jones, J. D., Lei, R., Jay, F., Fabro, G., Li, X. M., Reiter, W. D., et al. (2008). Characterization of *Arabidopsis* mur3 mutations that result in constitutive activation of defence in petioles, but not leaves. *Plant J.* 56, 691–703.

Tsang, D. L., Edmond, C., Harrington, J. L., and Nuhse, T. S. (2011). Cell wall integrity controls root elongation via a general 1-aminocyclopropane-1-carboxylic acid-dependent, ethylene-independent pathway. *Plant Physiol.* 156, 596–604.

Vega-Sanchez, M. E., Verhertbruggen, Y., Christensen, U., Chen, X. W., Sharma, V., Varanasi, P., et al. (2012). Loss of cellulose synthase-like F6 function affects mixed-linkage glucan deposition, cell wall mechanical properties, and defense responses in vegetative tissues of rice. *Plant Physiol.* 159, 56–69.

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. *Plant Cell* 14, 2095–2106.

Vogel, J. P., Raab, T. K., Somerville, C. R., and Somerville, S. C. (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. *Plant J.* 40, 968–978.

Wagner, T. A., and Kohorn, B. D. (2001). Wall-associated kinases are expressed throughout plant development and are required for cell expansion. *Plant Cell* 13, 303–318.

Walton, J. D. (1994). Deconstructing the cell wall. *Plant Physiol.* 104, 1113–1118.

Wei, G., and Shirsat, A. H. (2006). Extensin over-expression in *Arabidopsis* limits pathogen invasiveness. *Mol. Plant Pathol.* 7, 579–592.

Wolf, S., Mravec, J., Greiner, S., Mouille, G., and Hofte, H. (2012). Plant cell wall homeostasis is mediated by brassinosteroid feedback signaling. *Curr. Biol.* 22, 1732–1737.

Wormit, A., Butt, S. M., Chairam, I., Mckenna, J. F., Nunes-Nesi, A., Kjaer, L., et al. (2012). Osmosensitive changes of carbohydrate metabolism in response to cellulose biosynthesis inhibition. *Plant Physiol.* 159, 105–117.

Xu, S. L., Rahman, A., Baskin, T. I., and Kieber, J. J. (2008). Two leucine-rich repeat receptor kinases mediate signaling, linking cell wall biosynthesis and ACC synthase in *Arabidopsis*. *Plant Cell* 20, 3065–3079.

Conflict of Interest Statement: The author declares 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: 31 August 2012; paper pending published: 25 September 2012; accepted: 26 November 2012; published online: 11 December 2012.

Citation: Nühse TS (2012) Cell wall integrity signaling and innate immunity in plants. Front. Plant Sci. ***3****:280. doi: 10.3389/fpls.2012.00280*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 03 August 2012
doi: 10.3389/fpls.2012.00181

Plasticity of plasma membrane compartmentalization during plant immune responses

Susan L. Urbanus and Thomas Ott*

Institute of Genetics, University of Munich, Martinsried, Germany

Edited by:
Vardis Ntoukakis, University of Warwick, UK

Reviewed by:
John Runions, Oxford Brookes University, UK
Patrick Schäfer, University of Warwick, UK

****Correspondence:***
Thomas Ott, Institute of Genetics, University of Munich, Großhaderner Str. 2-4, 82152 Martinsried, Germany.
e-mail: thomas.ott@biologie.uni-muenchen.de

Plasma membranes require high levels of plasticity to modulate the perception and transduction of extracellular and intracellular signals. Dynamic lateral assembly of protein complexes combined with an independent compositional lipid patterning in both membrane leaflets provide cells the opportunity to decorate this interface with specific proteins in an organized but dynamic manner. Such ability to dynamically reorganize the protein content of the plasma membrane is essential for the regulation of processes such as polarity of transport, development, and microbial infection. While the plant cell wall represents the first physical and mostly unspecific barrier for invading microbes, the plasma membrane is at the forefront of microbial recognition and initiation of defense responses. Accumulating evidence indicating dynamic compartmentalization of plasma membranes in response to environmental cues has increased the interest in the compositional heterogeneity of this bilayer. Here, we elucidate the recruitment of specific proteins into defined membrane structures that ensure functional compartmentalization of the bilayer during infection processes.

Keywords: heterogeneity, compartmentalization, membrane domains, membrane rafts, plant immunity, receptor–ligand interactions

INTRODUCTION

The interface between the cytoplasm and the outer environment in plant cells is comprised of the cell wall and the plasma membrane. In their fluid mosaic model, Singer and Nicolson (1972) proposed that biological membranes, such as the plasma membrane, consist of a phospholipid bilayered matrix that is randomly interspersed with integral proteins. Recent research on plasma membrane components has significantly refined this view. Accumulating evidence indicates that the plasma membrane consists of a phospholipid bilayer that contains dynamic membrane domains, some of which are enriched in sphingolipids, sterols and specific proteins and called membrane rafts (Brown and Rose, 1992; Simons and Ikonen, 1997; Xu et al., 2001). These membrane rafts are able to cluster into more stable (signaling) platforms upon the perception of certain stimuli and crosslinking (Kusumi et al., 2004; Hammond et al., 2005; Lingwood et al., 2008; Hogue et al., 2011). Most of the available knowledge and hypotheses on the function and formation of membrane rafts originates from studies in mammalian and yeast cells (Simson et al., 1995; Sheets et al., 1997; Simons and Ikonen, 1997; Malinska et al., 2003; Kusumi et al., 2004; Fan et al., 2010), where it has among others been shown that membrane rafts play an important role during microbial infection processes (Duncan et al., 2002; Lafont and van der Goot, 2005; van der Meer-Janssen et al., 2010). There is increasing evidence that membrane domains may be similarly involved in the interaction between microbes and plants (Zappel and Panstruga, 2008). Here we discuss the current view on plant plasma membrane remodeling in response to both symbiotic and pathogenic microbes.

DYNAMIC MEMBRANE DOMAINS IN EUKARYOTIC CELLS

Since the *Keystone Symposium on Lipid Rafts and Cell Function*, the consensus definition of membrane rafts is that they are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes (Pike, 2006; Simons and Gerl, 2010). These rather elusive membrane rafts, due to their nanoscale size and dynamic behavior, can be stabilized through protein–protein, protein–lipid, and lipid–lipid interactions to form larger platforms that allow visualization by conventional light microscopy. Two main compartmentalizing forces seem to form membrane domains. The lipid raft model describes how sphingolipids can laterally associate with sterol molecules into close-packed assemblies (liquid-ordered phases; Simons and Ikonen, 1997; Lingwood and Simons, 2010). These close-packed, highly saturated rafts have different properties compared with the surrounding, less ordered and highly unsaturated phospholipid bilayer. Due to these different properties certain transmembrane and membrane-associated proteins preferentially insert into these rafts. In mammalian cells post-translational modifications such as myristoylation and palmitoylation were shown to target proteins preferentially to membrane rafts in the cytoplasmic membrane leaflet, while the addition of a glycosylphosphatidylinositol (GPI) moiety anchors proteins predominantly to membrane rafts at the outer leaflet (Schroeder et al., 1994; Varma and Mayor, 1998; Morrow et al., 2002; Zacharias et al., 2002). Evidence for lipid-dependency of membrane raft formation has been obtained from both artificial membrane models and living cells (Pralle et al., 2000; Hammond et al., 2005; Baumgart et al., 2007; Beck et al., 2007; Roche et al., 2008;

Kaiser et al., 2009). It was also demonstrated that the cortical actin-based cytoskeleton plays a role in the compartmentalization of plasma membranes (Suzuki and Sheetz, 2001; Fujiwara et al., 2002; Kusumi et al., 2004; Lenne et al., 2006). Such impact, however, cannot be tested in artificial membrane models. In the membrane-skeleton fence and anchored-protein picket models the effect of the cytoskeleton on the lateral movement of membrane components is described (Kusumi and Sako, 1996; Sako et al., 1998; Fujiwara et al., 2002; Kusumi et al., 2004). The membrane-skeleton fence model proposes that the cytoplasmic domains of transmembrane and membrane-associated proteins can collide with the cytoskeletal filaments close to the membrane, thereby confining movement of these proteins to compartments that can vary in size from 30 to 250 nm. In the anchored-protein picket model, transmembrane proteins anchor to and line up along the cytoskeletal meshwork as pickets. Due to steric hindrance and hydrodynamic friction-like effects these proteins consequently limit diffusion of other membrane molecules in both membrane leaflets. Since both models affect membrane proteins as well as membrane lipids it can be assumed that dynamic compartmentalization of plasma membranes relies on an interplay between cytoskeleton-mediated and raft-derived effects. Indeed "hop-diffusion" where movement of proteins is temporarily confined by a cytoskeleton fence until the restrictive presence of the actin filaments is loosened by temporary breakdown of the filaments or an increase in the distance between the membrane and the cytoskeletal meshwork, has been described for a number of membrane raft proteins such as the human transferrin receptor (Sako and Kusumi, 1995; Kusumi et al., 2005). Membrane molecules can also move to neighboring compartments when they have gained sufficient kinetic energy to break through the restraining actin filament. The protein FORMIN-1 in *Arabidopsis thaliana* (thale cress) seems to be such a transmembrane picket protein, since it is able to anchor the actin cytoskeleton through the plasma membrane to the cell wall (Martiniere et al., 2011). However, experiments using the fluorescence recovery after photobleaching (FRAP) technique on fluorescently labeled membrane proteins in protoplasts that were re-growing their cell wall, demonstrate that in plants the constraining influence of the cell wall on the mobility of plasma membrane proteins is much greater than the influence of the cytoskeleton (Martiniere et al., 2012).

A frequently used, but much debated method in membrane raft research makes use of the differential packing of molecules in membranes to isolate close-packed assemblies from the plasma membrane through their greater resistance to detergents such as Triton (Heerklotz, 2002; Munro, 2003; Brown, 2006; Kierszniowska et al., 2009; Mongrand et al., 2010; Pathak and London, 2011; Tanner et al., 2011). Isolation of so called detergent-insoluble membranes (DIMs), or detergent-resistant membranes (DRMs), from *Nicotiana tabacum* (tobacco) demonstrated the enrichment of the sphingolipid glycosylceramide and the sterols stigmasterol, 24-methyl cholesterol, sitosterol, and cholesterol compared to detergent-soluble membrane (DSM) fractions. In contrast, relative amounts of phospholipids such as phosphatidylcholine and phosphatidylethanolamine are reduced in DIMs (Mongrand et al., 2004). Similar findings have been reported for DIMs prepared from *A. thaliana*, *Medicago truncatula* (barrel clover), *Phaseolus vulgaris* (common bean), and *Zea mays* (maize; Borner et al., 2005; Lefebvre et al., 2007; Furt et al., 2010; Carmona-Salazar et al., 2011). Proteome analyses of membrane fractions from the above mentioned plant species and additionally from *Solanum tuberosum* (potato), *Sinapis alba* (white mustard), and the monocotyledonous plant species *Avena sativa* (oat), *Secale cereale* (rye), and *Oryza sativa* (rice) demonstrate enrichment of proteins involved in different types of processes in DIMs, including signal transduction and abiotic and biotic stress responses (Shahollari et al., 2004; Morel et al., 2006; Fujiwara et al., 2009; Stanislas et al., 2009; Keinath et al., 2010; Krügel et al., 2012; Takahashi et al., 2012). There seems to be a preference for palmitoylation and myristoylation in proteins involved in signaling processes and stress responses enriched in DIMs, possibly targeting them to membrane rafts in the cytoplasmic membrane leaflet (Morel et al., 2006). Since DIM fractions may only partially, if at all, reflect the composition of individual membrane raft classes, localization of these putative raft-resident molecules in *in planta* membrane domains has to be validated by other techniques to allay concerns about artifactual membrane raft formation and co-purification of non-raft constituents. Additional imaging approaches of putative membrane rafts constituents and testing whether clustering of these putative raft-localized lipids, sterols or proteins is influenced by chemicals such as the sterol extracting methyl-β-cyclodextrin, support the proposed presence of such structures *in planta* (Krügel et al., 2008; Raffaele et al., 2009; Boutte et al., 2011). Furthermore, newly developed imaging techniques such as total internal reflection fluorescence microscopy (TIRFM) or variable-angle epifluorescence microscopy (VAEM) can be successfully applied to cell wall enclosed cells and allow the visualization of mobile proteins located in and around the plasma membrane with very high signal-to-background ratio (Owen et al., 2007; Konopka and Bednarek, 2008; Spira et al., 2012). A recent study showed TIRFM to also be applicable to study single molecule trafficking of PM-resident proteins in plant cells (Martiniere et al., 2012). These techniques will help to analyze domain structures in the plasma membrane in more detail in the future. Whether the plasma membrane domains formed in plant cells can always be categorized as membrane rafts remains to be fully elucidated.

THE ROLE OF MEMBRANE DOMAINS DURING INFECTION PROCESSES

The integral membrane proteins FLOTILLIN-1 and FLOTILLIN-2 (synonymously called REGGIE-2 and REGGIE-1) are frequently used as markers for membrane rafts in the cytoplasmic leaflet of mammalian cells (Lang et al., 1998; Morrow et al., 2002; Blonder et al., 2004; Nixon et al., 2011). In plants and animals, members of this protein family were described to be involved in clathrin-independent endocytosis and are present on host-derived membranes surrounding intracellular microorganisms, indicating raft-mediated endocytosis as a possible entry point for microorganisms (Panter et al., 2000; Dermine et al., 2001; Saalbach et al., 2002; Glebov et al., 2006; Murphy et al., 2007; Li et al., 2008, 2012; Korhonen et al., 2012). Furthermore, evidence that flotillins are interacting and/or co-localizing with many signaling components, such as receptors and mitogen-activated protein kinases (MAPK),

suggests that these proteins may act as scaffolds for a number of signaling processes (Langhorst et al., 2005; Haney et al., 2011; Amaddii et al., 2012). In *A. thaliana* a flotillin homolog was originally identified in DIMs and named AtFLOTILLIN-1 (AtFLOT1; Borner et al., 2005). Recently, a combination of confocal laser scanning microscopy, electron microscopy, and VAEM demonstrated that AtFLOT1 is present in distinct membrane domains, in clathrin-independent invaginations in the plasma membrane, and in endocytic vesicles with a size of approximately 100 nm (Li et al., 2012). Using transgenic plants expressing an artificial microRNA against AtFLOT1, the same study demonstrated that AtFLOT1 is required for meristem and seedling development. In *M. truncatula* MtFLOT2 and especially MtFLOT4 were found to be required for initiation of symbiotic infection structures (infection threads) and their elongation during interactions with the nitrogen-fixating bacterial symbiont *Sinorhizobium meliloti* (Haney and Long, 2010). Upon inoculation of *M. truncatula* roots with *S. meliloti* bacteria the evenly distributed fluorescently labeled MtFLOT4 punctae in the plasma membrane of a root hair cell start to accumulate at the root hair tip. These punctate structures might represent small clusters of membrane rafts that coalesce into a membrane raft platform at the root hair tip to facilitate the entry of the symbiont. Interestingly, fluorophore-tagged MtLYSIN-MOTIF-RECEPTOR-LIKE-KINASE-3 (MtLYK3), an RLK required for bacterial entry into the root hairs, localizes to mobile punctae in root hairs (**Figure 1A**) that become immobilized when the roots are inoculated with *S. meliloti* (Haney et al., 2011). Interestingly, fluorescently labeled MtFLOT4 and MtLYK3 punctae co-localize upon *S. meliloti* inoculation. In addition, the density of MtFLOT4 domains is decreased in the kinase-inactive MtLYK3 *hair curling-1* (*hcl-1*) mutant (Haney et al., 2011). These results suggest that these two proteins assemble into the same membrane domain upon perception of the symbiotic bacteria and possibly even interact with each other. Interestingly, the remorin protein *M. truncatula* SYMBIOTIC-REMORIN-1 (MtSYMREM1), a confirmed interactor of the LYK3 receptor, localizes to distinct domains when over-expressed in transgenic *M. truncatula* roots (**Figure 1B**). Such membrane patterns have also been found on nodular infection threads (**Figure 1C**), at bacterial release sites, and in symbiosome membranes as demonstrated by immunolocalizations and immunogold-labeling electron microscopy (Lefebvre et al., 2010).

Over the years, the plant-specific remorin proteins have become the most widely accepted raft marker proteins in plants. They are highly enriched in DIMs from several plant species (Mongrand et al., 2004; Shahollari et al., 2004; Morel et al., 2006; Lefebvre et al., 2007; Keinath et al., 2010; Krügel et al., 2012; Takahashi et al., 2012) and localize to methyl-β-cyclodextrin-sensitive domains of around 75 nm in the cytoplasmic membrane leaflet in *N. tabacum* cells, as demonstrated by statistical analysis of electron microscopy data (Raffaele et al., 2009). Over-expression of the potato remorin StREM1.3 leads to the labeling of large, immobile membrane domains that might resemble small clusters of membrane rafts (Raffaele et al., 2009) similar to those labeled by MtSYMREM1 (**Figure 1B**). While the biological function of remorins still remains unknown, members of this highly diverse multi-gene family have been shown to regulate viral spreading in leaves (Raffaele et al., 2009), interactions between *M. truncatula* and *S. meliloti* during root nodule symbiosis (Lefebvre et al., 2010), and to serve potential roles during plant–pathogen interactions (Jarsch and Ott, 2011). Additionally, their ability to oligomerize and to interact with signaling proteins such as the symbiotic receptors and the negative regulator of immune responses RPM1-INTERACTING-PROTEIN-4 (RIN4), suggests functions as scaffolding proteins during signal transduction (Liu et al., 2009; Lefebvre et al., 2010; Toth et al., 2012). In *A. thaliana* suspension cells the group 1 remorin AtREM1.3 is phosphorylated in the intrinsically disordered N-terminal domain within 10 min of incubation with flg22, a conserved 22 amino acid peptide from the bacterial-derived elicitor flagellin (Benschop et al., 2007;

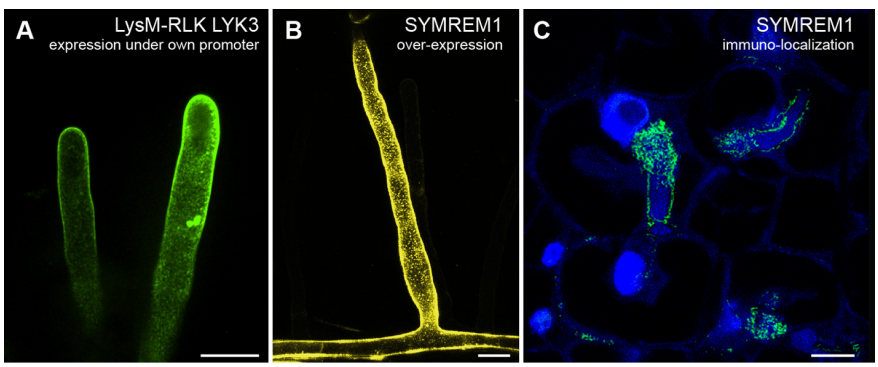


FIGURE 1 | Examples of membrane domain patterning in *Medicago truncatula*. **(A)** Root hair cells expressing GFP-tagged LysM-RLK MtLYK3 under the control of the native *LYK3* promoter in the kinase-inactive LYK3 mutant line *hcl-1*. Membrane domains as described earlier (Haney et al., 2011) can be observed with spinning-disk confocal microscopy. **(B)** Over-expression of the YFP-tagged remorin protein MtSYMREM1 in root hair cells results in the labeling of immobile domains in the plasma membrane that can be imaged with confocal laser scanning microscopy. **(C)** As originally reported (Lefebvre et al., 2010) similar domains, although smaller in size, can also be observed when immuno-localizing the MtSYMREM1 protein in the infection zone of mature root nodules using a specific MtSYMREM1 antibody. The MtSYMREM1 protein (green) resides on the infection thread membrane that surrounds invading rhizobial bacteria and accumulates at sites where these symbionts are released into the host cell. The plant and bacterial DNA is stained with DAPI (blue). Scale bars indicate 20 μm **(A,B)** and 1 μm **(C)**.

Marín and Ott, 2012), suggesting close functional dependency of AtREM1.3 on the functional receptor-like kinase AtFLAGELLIN-SENSITIVE-2 (FLS2). In analogy to MtLYK3, flg22-dependent reduction in the lateral mobility of fluorescently tagged AtFLS2 was observed in *A. thaliana* protoplasts when analyzed by FRAP (Ali et al., 2007). These data suggest that AtFLS2 becomes part of a larger, less mobile complex upon ligand-binding and/or becomes confined to membrane rafts upon ligand-binding. The concept of ligand-dependent recruitment to membrane rafts is supported by the fact that FLS2 is highly recruited to DIMs upon flg22 treatment of *A. thaliana* suspension cells, as demonstrated by quantitative mass spectrometric analyses (Keinath et al., 2010). These findings together with FLS2 being endocytosed upon flg22 treatment in young leaves (Robatzek et al., 2006), support possible links between localization of signaling proteins in membrane domains and endocytic events.

The functional importance of membrane domains during infection processes is also underlined by results from a number of studies on interactions of plants with pathogenic oomycetes or fungi. The NADPH oxidase RESPIRATORY-BURST-OXIDASE-HOMOLOG-D (RBOHD)-mediated production of reactive oxygen species is one of the first signaling responses initiated upon the perception of the oomycete-derived elicitor cryptogein (Simon-Plas et al., 2002). NtRBOHD-mediated H_2O_2-generation was detected in small patches along the plasma membrane in *N. tabacum* cells using transmission electron microscopy after staining with $CeCl_3$ (Lherminier et al., 2009). A possible membrane raft localization of NtRBOHD is also supported by its identification in DIMs from *N. tabacum* after treatment with cryptogein, together with its negative regulator RAC/ROP GTPase NtRAC5 (Mongrand et al., 2004; Morel et al., 2006). *In planta* evidence for dynamic compartmentalization of membrane proteins was also reported upon host cell infection of *Hordeum vulgare* (barley) and *A. thaliana* by the powdery mildew fungus *Blumeria graminis*. Focal accumulations around fungal entry sites were observed for three otherwise evenly distributed fluorescently labeled proteins all involved in powdery mildew penetration resistance, namely MILDEW-RESISTANCE-LOCUS-O (MLO), the syntaxin HvREQUIRED-FOR-MLO-SPECIFIED-RESISTANCE-2 (HvROR2) and its ortholog AtPENETRATION-1 (AtPEN1; Bhat et al., 2005). These focal accumulations may represent membrane raft platforms, especially since they coincide with higher levels of sterols as demonstrated by filipin staining. The host-derived extrahaustorial membrane encasing the haustorial feeding structure of successfully entered fungi or oomycetes is clearly distinct from invaginated extensions of the plasma membrane, as demonstrated by the exclusion of several plasma membrane markers (Koh et al., 2005; Micali et al., 2011; Lu et al., 2012). However, differential presence of plasma membrane proteins such as AtFLS2, AtPEN1, and StREM1.3 in extrahaustorial membranes implies a mechanism to be in place that can actively determine whether proteins are included into the extrahaustorial membrane (Lu et al., 2012). Membrane reorganization may also be triggered and/or enhanced by changes in the lipid patterning of plasma membranes, as the polyphosphoinositide phosphatidylinositol-3-phosphate (PI3P) can be directly targeted by certain fungal and oomycete effectors during plant cell entry (Kale et al., 2010). Polyphosphoinositides have been shown to be enriched in *N. tabacum* DIMs and electron microscopy of immunogold-labeled phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) revealed the presence of distinct domains of approximately 25 nm in the plasma membrane (Furt et al., 2010). However, these domains are not methyl-β-cyclodextrin-sensitive, indicating that these domains are not membrane rafts according to the consensus definition.

CONCLUSIONS AND PERSPECTIVE

Although the concept of membrane rafts still remains to be unequivocally proven in plants, several lines of research clearly demonstrate that the plant plasma membrane is dynamically compartmentalized during biological processes such as infection of host cells by microorganisms. Differently sized domains that could resemble single membrane rafts, small clusters of membrane rafts, large membrane raft platforms, or other types of membrane domains beyond the membrane raft concept, can be observed in the plasma membrane with electron and/or conventional light microscopy. Current data suggest that there is not one but multiple types of membrane domains, that host multimeric signaling complexes which specifically assemble within the plasma membrane in a stimulus-dependent manner. Minor changes in lipid or protein aggregation, such as ligand–receptor complex formation, could induce the assembly of an appropriate membrane domain that can transduce the changing environmental conditions. The presence of signaling components in membrane rafts or other distinct domains may potentially modulate their activity and enhance the interactions between domain-resident components, while reducing interactions with non-domain components. A clear correlation between endocytosis and signaling is apparent in many of the discussed examples, arguing for the concept of membrane rafts serving as signaling hubs that can be exploited by (facultative) intracellular microbes to successfully establish themselves in their host. To gain further knowledge on the behavior of these dynamic plasma membrane domains *in planta*, imaging techniques with high spatial and/or temporal resolution are needed that go beyond conventional light microscopy. For example, electron microscopy and super-resolution fluorescent microscopy could be used to determine the size of plasma membrane domains in fixed samples, Förster resonance energy transfer (FRET)-based live-cell imaging to determine interactions between residents of these domains, and TIRFM or VAEM to visualize the dynamics of these domains in living cells with intact cell wall.

ACKNOWLEDGMENTS

We would like to thank Douglas Cook and Brendan Riely (University of California, Davis, USA) for providing the *M. truncatula* LYK3-GFP lines (Figure 1A), Claudia Popp (LMU Munich, Germany) for donation of *M. truncatula* roots overexpressing a YFP-MtSYMREM1 construct (Figure 1B), and Ton Timmers (LIPM, INRA-CNRS Toulouse, France) for providing the immuno-localization image of MtSYMREM1 (Figure 1C). We also kindly thank the Collaborative Research Centre (Sonderforschungsbereich) SFB924 and the Emmy Noether Program (grant OT423/2-1) funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for financial support.

REFERENCES

Ali, G. S., Prasad, K. V. S. K., Day, I., and Reddy, A. S. N. (2007). Ligand-dependent reduction in the membrane mobility of FLAGELLIN SENSITIVE2, an *Arabidopsis* receptor-like kinase. *Plant Cell Physiol.* 48, 1601–1611.

Amaddii, M., Meister, M., Banning, A., Tomasovic, A., Mooz, J., Rajalingam, K., and Tikkanen, R. (2012). Flotillin-1/Reggie-2 protein plays dual role in activation of receptor-tyrosine kinase/mitogen-activated protein kinase signaling. *J. Biol. Chem.* 287, 7265–7278.

Baumgart, T., Hammond, A. T., Sengupta, P., Hess, S. T., Holowka, D. A., Baird, B. A., and Webb, W. W. (2007). Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3165–3170.

Beck, J. G., Mathieu, D., Loudet, C., Buchoux, S., and Dufourc, E. J. (2007). Plant sterols in "rafts": a better way to regulate membrane thermal shocks. *FASEB J.* 21, 1714–1723.

Benschop, J. J., Mohammed, S., O'Flaherty, M., Heck, A. J. R., Slijper, M., and Menke, F. L. H. (2007). Quantitative phosphoproteomics of early elicitor signaling in *Arabidopsis*. *Mol. Cell. Proteomics* 6, 1198–1214.

Bhat, R. A., Miklis, M., Schmelzer, E., Schulze-Lefert, P., and Panstruga, R. (2005). Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3135–3140.

Blonder, J., Hale, M. L., Lucas, D. A., Schaefer, C. F., Yu, L. R., Conrads, T. R., Issaq, H. J., Stiles, B. G., and Veenstra, T. D. (2004). Proteomic analysis of detergent-resistant membrane rafts. *Electrophoresis* 25, 1307–1318.

Borner, G. H. H., Sherrier, D. J., Weimar, T., Michaelson, L. V., Hawkins, N. D., Macaskill, A., Napier, J. A., Beale, M. H., Lilley, K. S., and Dupree, P. (2005). Analysis of detergent-resistant membranes in *Arabidopsis*. Evidence for plasma membrane lipid rafts. *Plant Physiol.* 137, 104–116.

Boutte, Y., Men, S. Z., and Grebe, M. (2011). Fluorescent in situ visualization of sterols in *Arabidopsis* roots. *Nat. Protoc.* 6, 446–456.

Brown, D. A. (2006). Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology* 21, 430–439.

Brown, D. A., and Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell-surface. *Cell* 68, 533–544.

Carmona-Salazar, L., El Hafidi, M., Enriquez-Arredondo, C., Vazquez-Vazquez, C., De La Vara, L. E. G., and Gavilanes-Ruiz, M. (2011). Isolation of detergent-resistant membranes from plant photosynthetic and non-photosynthetic tissues. *Anal. Biochem.* 417, 220–227.

Dermine, J. F., Duclos, S., Garin, J., St-Louis, F., Rea, S., Parton, R. G., and Desjardins, M. (2001). Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. *J. Biol. Chem.* 276, 18507–18512.

Duncan, M. J., Shin, J. S., and Abraham, S. N. (2002). Microbial entry through caveolae: variations on a theme. *Cell. Microbiol.* 4, 783–791.

Fan, J., Sammalkorpi, M., and Haataja, M. (2010). Formation and regulation of lipid microdomains in cell membranes: theory, modeling, and speculation. *FEBS Lett.* 584, 1678–1684.

Fujiwara, M., Hamada, S., Hiratsuka, M., Fukao, Y., Kawasaki, T., and Shimamoto, K. (2009). Proteome analysis of detergent-resistant membranes (DRMs) associated with OsRac1-mediated innate immunity in rice. *Plant Cell Physiol.* 50, 1191–1200.

Fujiwara, T., Ritchie, K., Murakoshi, H., Jacobson, K., and Kusumi, A. (2002). Phospholipids undergo hop diffusion in compartmentalized cell membrane. *J. Cell Biol.* 157, 1071–1081.

Furt, F., Konig, S., Bessoule, J. J., Sargueil, F., Zallot, R., Stanislas, T., Noirot, E., Lherminier, J., Simon-Plas, F., Heilmann, I., and Mongrand, S. (2010). Polyphosphoinositides are enriched in plant membrane rafts and form microdomains in the plasma membrane. *Plant Physiol.* 152, 2173–2187.

Glebov, O. O., Bright, N. A., and Nichols, B. J. (2006). Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat. Cell Biol.* 8, 46–54.

Hammond, A. T., Heberle, F. A., Baumgart, T., Holowka, D., Baird, B., and Feigenson, G. W. (2005). Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6320–6325.

Haney, C. H., and Long, S. R. (2010). Plant flotillins are required for infection by nitrogen-fixing bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 107, 478–483.

Haney, C. H., Riely, B. K., Tricoli, D. M., Cook, D. R., Ehrhardt, D. W., and Long, S. R. (2011). Symbiotic rhizobia bacteria trigger a change in localization and dynamics of the *Medicago truncatula* receptor kinase LYK3. *Plant Cell* 23, 2774–2787.

Heerklotz, H. (2002). Triton promotes domain formation in lipid raft mixtures. *Biophys. J.* 83, 2693–2701.

Hogue, I. B., Grover, J. R., Soheilian, F., Nagashima, K., and Ono, A. (2011). Gag induces the coalescence of clustered lipid rafts and tetraspanin-enriched microdomains at HIV-1 assembly sites on the plasma membrane. *J. Virol.* 85, 9749–9766.

Jarsch, I. K., and Ott, T. (2011). Perspectives on remorin proteins, membrane rafts, and their role during plant–microbe interactions. *Mol. Plant Microbe Interact.* 24, 7–12.

Kaiser, H. J., Lingwood, D., Levental, I., Sampaio, J. L., Kalvodova, L., Rajendran, L., and Simons, K. (2009). Order of lipid phases in model and plasma membranes. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16645–16650.

Kale, S. D., Gu, B. A., Capelluto, D. G. S., Dou, D. L., Feldman, E., Rumore, A., Arredondo, F. D., Hanlon, R., Fudal, I., Rouxel, T., Lawrence, C. B., Shan, W. X., and Tyler, B. M. (2010). External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell* 142, 284–295.

Keinath, N. F., Kierszniowska, S., Lorek, J., Bourdais, G., Kessler, S. A., Shimosato-Asano, H., Grossniklaus, U., Schulze, W. X., Robatzek, S., and Panstruga, R. (2010). PAMP (pathogen-associated molecular pattern)-induced changes in plasma membrane compartmentalization reveal novel components of plant immunity. *J. Biol. Chem.* 285, 39140–39149.

Kierszniowska, S., Seiwert, B., and Schulze, W. X. (2009). Definition of *Arabidopsis* sterol-rich membrane microdomains by differential treatment with methyl-beta-cyclodextrin and quantitative proteomics. *Mol. Cell. Proteomics* 8, 612–623.

Koh, S., Andre, A., Edwards, H., Ehrhardt, D., and Somerville, S. (2005). *Arabidopsis thaliana* subcellular responses to compatible *Erysiphe cichoracearum* infections. *Plant J.* 44, 516–529.

Konopka, C. A., and Bednarek, S. Y. (2008). Variable-angle epifluorescence microscopy: a new way to look at protein dynamics in the plant cell cortex. *Plant J.* 53, 186–196.

Korhonen, J. T., Puolakkainen, M., Haivala, R., Penttila, T., Haveri, A., Markkula, E., and Lahesmaa, R. (2012). Flotillin-1 (Reggie-2) contributes to *Chlamydia pneumoniae* growth and is associated with bacterial inclusion. *Infect. Immun.* 80, 1072–1078.

Krügel, U., He, H. X., Gier, K., Reins, J., Chincinska, I., Grimm, B., Schulze, W. X., and Kuhn, C. (2012). The potato sucrose transporter StSUT1 interacts with a DRM-associated protein disulfide isomerase. *Mol. Plant* 5, 43–62.

Krügel, U., Veenhoff, L. M., Langbein, J., Wiederhold, E., Liesche, J., Friedrich, T., Grimm, B., Martinoia, E., Poolman, B., and Kuhn, C. (2008). Transport and sorting of the *Solanum tuberosum* sucrose transporter SUT1 is affected by posttranslational modification. *Plant Cell* 20, 2497–2513.

Kusumi, A., Koyama-Honda, I., and Suzuki, K. (2004). Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic* 5, 213–230.

Kusumi, A., Nakada, C., Ritchie, K., Murase, K., Suzuki, K., Murakoshi, H., Kasai, R. S., Kondo, J., and Fujiwara, T. (2005). Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu. Rev. Biophysics. Biomol. Struct.* 34, 351–378.

Kusumi, A., and Sako, Y. (1996). Cell surface organization by the membrane skeleton. *Curr. Opin. Cell Biol.* 8, 566–574.

Lafont, F., and van der Goot, F. G. (2005). Bacterial invasion via lipid rafts. *Cell. Microbiol.* 7, 613–620.

Lang, D. M., Lommel, S., Jung, M., Ankerhold, R., Petrausch, B., Laessing, U., Wiechers, M. F., Plattner, H., and Stuermer, C. A. (1998). Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. *J. Neurobiol.* 37, 502–523.

Langhorst, M. F., Reuter, A., and Stuermer, C. A. (2005). Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. *Cell. Mol. Life Sci.* 62, 2228–2240.

Lefebvre, B., Furt, F., Hartmann, M. A., Michaelson, L. V., Carde, J. P., Sargueil-Boiron, F., Rossignol, M., Napier, J. A., Cullimore, J., Bessoule, J. J., and Mongrand, S. (2007). Characterization of lipid rafts from *Medicago truncatula* root plasma membranes: a proteomic study reveals the presence

of a raft-associated redox system. *Plant Physiol.* 144, 402–418.

Lefebvre, B., Timmers, T., Mbengue, M., Moreau, S., Herve, C., Toth, K., Bittencourt-Silvestre, J., Klaus, D., Deslandes, L., Godiard, L., Murray, J. D., Udvardi, M. K., Raffaele, S., Mongrand, S., Cullimore, J., Gamas, P., Niebel, A., and Ott, T. (2010). A remorin protein interacts with symbiotic receptors and regulates bacterial infection. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2343–2348.

Lenne, P. F., Wawrezinieck, L., Conchonaud, F., Wurtz, O., Boned, A., Guo, X. J., Rigneault, H., He, H. T., and Marguet, D. (2006). Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. *EMBO J.* 25, 3245–3256.

Lherminier, J., Elmayan, T., Fromentin, J., Elaraqui, K. T., Vesa, S., Morel, J., Verrier, J. L., Cailleteau, B., Blein, J. P., and Simon-Plas, F. (2009). NADPH oxidase-mediated reactive oxygen species production: subcellular localization and reassessment of its role in plant defense. *Mol. Plant Microbe Interact.* 22, 868–881.

Li, Q. R., Zhang, Q., Wang, C. Y., Li, N., and Li, J. S. (2008). Invasion of enteropathogenic *Escherichia coli* into host cells through epithelial tight junctions. *FEBS J.* 275, 6022–6032.

Li, R., Liu, P., Wan, Y., Chen, T., Wang, Q., Mettbach, U., Baluska, F., Samaj, J., Fang, X., Lucas, W. J., and Lin, J. (2012). A membrane microdomain-associated protein, *Arabidopsis* Flot1, is involved in a clathrin-independent endocytic pathway and is required for seedling development. *Plant Cell* 24, 2105–2122.

Lingwood, D., Ries, J., Schwille, P., and Simons, K. (2008). Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10005–10010.

Lingwood, D., and Simons, K. (2010). Lipid rafts as a membrane-organizing principle. *Science* 327, 46–50.

Liu, J., Elmore, J. M., Fuglsang, A. T., Palmgren, M. G., Staskawicz, B. J., and Coaker, G. (2009). RIN4 functions with plasma membrane H+-ATPases to regulate stomatal apertures during pathogen attack. *PLoS Biol.* 7, e1000139. doi: 10.1371/journal.pbio.1000139

Lu, Y. J., Schornack, S., Spallek, T., Geldner, N., Chory, J., Schellmann, S., Schumacher, K., Kamoun, S., and Robatzek, S. (2012). Patterns of plant subcellular responses to successful oomycete infections reveal differences in host cell reprogramming and endocytic trafficking. *Cell. Microbiol.* 14, 682–697.

Malinska, K., Malinsky, J., Opekarova, M., and Tanner, W. (2003). Visualization of protein compartmentation within the plasma membrane of living yeast cells. *Mol. Biol. Cell* 14, 4427–4436.

Marín, M., and Ott, T. (2012). Phosphorylation of intrinsically disordered regions in remorin proteins. *Front. Plant Sci.* 3:86. doi: 10.3389/fpls.2012.00086

Martiniere, A., Gayral, P., Hawes, C., and Runions, J. (2011). Building bridges: formin1 of *Arabidopsis* forms a connection between the cell wall and the actin cytoskeleton. *Plant J.* 66, 354–365.

Martiniere, A., Lavagi, I., Nageswaran, G., Rolfe, D. J., Maneta-Peyret, L., Luu, D. T., Botchway, S. W., Webb, S. E., Mongrand, S., Maurel, C., Martin-Fernandez, M. L., Kleine-Vehn, J., Friml, J., Moreau, P., and Runions, J. (2012). Cell wall constrains lateral diffusion of plant plasma-membrane proteins. *Proc. Natl. Acad. Sci. U.S.A.* doi: 10.1073/pnas.1202040109 [Epub ahead of print].

Micali, C. O., Neumann, U., Grunewald, D., Panstruga, R., and O'Connell, R. (2011). Biogenesis of a specialized plant–fungal interface during host cell internalization of *Golovinomyces orontii haustoria*. *Cell. Microbiol.* 13, 210–226.

Mongrand, S., Morel, J., Laroche, J., Claverol, S., Carde, J. P., Hartmann, M. A., Bonneu, M., Simon-Plas, F., Lessire, R., and Bessoule, J. J. (2004). Lipid rafts in higher plant cells – purification and characterization of triton X-100-insoluble microdomains from tobacco plasma membrane. *J. Biol. Chem.* 279, 36277–36286.

Mongrand, S., Stanislas, T., Bayer, E. M., Lherminier, J., and Simon-Plas, F. (2010). Membrane rafts in plant cells. *Trends Plant Sci.* 15, 656–663.

Morel, J., Claverol, S., Mongrand, S., Furt, F., Fromentin, J., Bessoule, J. J., Blein, J. P., and Simon-Plas, F. (2006). Proteomics of plant detergent-resistant membranes. *Mol. Cell. Proteomics* 5, 1396–1411.

Morrow, I. C., Rea, S., Martin, S., Prior, I. A., Prohaska, R., Hancock, J. F., James, D. E., and Parton, R. G. (2002). Flotillin-1/Reggie-2 traffics to surface raft domains via a novel Golgi-independent pathway – identification of a novel membrane targeting domain and a role for palmitoylation. *J. Biol. Chem.* 277, 48834–48841.

Munro, S. (2003). Lipid rafts: elusive or illusive? *Cell* 115, 377–388.

Murphy, S. C., Fernandez-Pol, S., Chung, P. H., Murthy, S. N. P., Milne, S. B., Salomao, M., Brown, H. A., Lomasney, J. W., Mohandas, N., and Haldar, K. (2007). Cytoplasmic remodeling of erythrocyte raft lipids during infection by the human malaria parasite *Plasmodium falciparum*. *Blood* 110, 2132–2139.

Nixon, B., Mitchell, L. A., Anderson, A. L., Mclaughlin, E. A., O'Bryan, M. K., and Aitken, R. J. (2011). Proteomic and functional analysis of human sperm detergent resistant membranes. *J. Cell. Physiol.* 226, 2651–2665.

Owen, D. M., Neil, M. A., French, P. M., and Magee, A. I. (2007). Optical techniques for imaging membrane lipid microdomains in living cells. *Semin. Cell Dev. Biol.* 18, 591–598.

Panter, S., Thomson, R., De Bruxelles, G., Laver, D., Trevaskis, B., and Udvardi, M. (2000). Identification with proteomics of novel proteins associated with the peribacteroid membrane of soybean root nodules. *Mol. Plant Microbe Interact.* 13, 325–333.

Pathak, P., and London, E. (2011). Measurement of lipid nanodomain (raft) formation and size in sphingomyelin/POPC/cholesterol vesicles shows TX-100 and transmembrane helices increase domain size by coalescing preexisting nanodomains but do not induce domain formation. *Biophys. J.* 101, 2417–2425.

Pike, L. J. (2006). Rafts defined: a report on the keystone symposium on lipid rafts and cell function. *J. Lipid Res.* 47, 1597–1598.

Pralle, A., Keller, P., Florin, E. L., Simons, K., and Horber, J. K. H. (2000). Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. *J. Cell Biol.* 148, 997–1007.

Raffaele, S., Bayer, E., Lafarge, D., Cluzet, S., Retana, S. G., Boubekeur, T., Leborgne-Castel, N., Carde, J. P., Lherminier, J., Noirot, E., Satiat-Jeunemaitre, B., Laroche-Traineau, J., Moreau, P., Ott, T., Maule, A. J., Reymond, P., Simon-Plas, F., Farmer, E. E., Bessoule, J. J., and Mongrand, S. (2009). Remorin, a Solanaceae protein resident in membrane rafts and plasmodesmata, impairs potato virus X movement. *Plant Cell* 21, 1541–1555.

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Genes Dev.* 20, 537–542.

Roche, Y., Gerbeau-Pissot, P., Buhot, B., Thomas, D., Bonneau, L., Gresti, J., Mongrand, S., Perrier-Cornet, J. M., and Simon-Plas, F. (2008). Depletion of phytosterols from the plant plasma membrane provides evidence for disruption of lipid rafts. *FASEB J.* 22, 3980–3991.

Saalbach, G., Erik, P., and Wienkoop, S. (2002). Characterisation by proteomics of peribacteroid space and peribacteroid membrane preparations from pea (*Pisum sativum*) symbiosomes. *Proteomics* 2, 325–337.

Sako, Y., and Kusumi, A. (1995). Barriers for lateral diffusion of transferrin receptor in the plasma membrane as characterized by receptor dragging by laser tweezers: fence versus tether. *J. Cell Biol.* 129, 1559–1574.

Sako, Y., Nagafuchi, A., Tsukita, S., Takeichi, M., and Kusumi, A. (1998). Cytoplasmic regulation of the movement of E-cadherin on the free cell surface as studied by optical tweezers and single particle tracking: corralling and tethering by the membrane skeleton. *J. Cell Biol.* 140, 1227–1240.

Schroeder, R., London, E., and Brown, D. (1994). Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins – GPI-anchored proteins in liposomes and cells show similar behavior. *Proc. Natl. Acad. Sci. U.S.A.* 91, 12130–12134.

Shahollari, B., Peskan-Berghofer, T., and Oelmuller, R. (2004). Receptor kinases with leucine-rich repeats are enriched in Triton X-100 insoluble plasma membrane microdomains from plants. *Physiol. Plant.* 122, 397–403.

Sheets, E. D., Lee, G. M., Simson, R., and Jacobson, K. (1997). Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. *Biochemistry* 36, 12449–12458.

Simon-Plas, F., Elmayan, T., and Blein, J. P. (2002). The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells. *Plant J.* 31, 137–147.

Simons, K., and Gerl, M. J. (2010). Revitalizing membrane rafts: new tools and insights. *Nat. Rev. Mol. Cell Biol.* 11, 688–699.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. *Nature* 387, 569–572.

Simson, R., Sheets, E. D., and Jacobson, K. (1995). Detection of temporary lateral confinement

of membrane-proteins using single-particle tracking analysis. *Biophys. J.* 69, 989–993.

Singer, S. J., and Nicolson, G. L. (1972). Fluid mosaic model of structure of cell-membranes. *Science* 175, 720–731.

Spira, F., Mueller, N. S., Beck, G., Von Olshausen, P., Beig, J., and Wedlich-Soldner, R. (2012). Patchwork organization of the yeast plasma membrane into numerous coexisting domains. *Nat. Cell Biol.* 14, 640–648.

Stanislas, T., Bouyssie, D., Rossignol, M., Vesa, S., Fromentin, J., Morel, J., Pichereaux, C., Monsarrat, B., and Simon-Plas, F. (2009). Quantitative proteomics reveals a dynamic association of proteins to detergent-resistant membranes upon elicitor signaling in tobacco. *Mol. Cell. Proteomics* 8, 2186–2198.

Suzuki, K., and Sheetz, M. P. (2001). Binding of cross-linked glycosylphosphatidylinositol-anchored proteins to discrete actin-associated sites and cholesterol-dependent domains. *Biophys. J.* 81, 2181–2189.

Takahashi, D., Kawamura, Y., Yamashita, T., and Uemura, M. (2012). Detergent-resistant plasma membrane proteome in oat and rye: similarities and dissimilarities between two monocotyledonous plants. *J. Proteome Res.* 11, 1654–1665.

Tanner, W., Malinsky, J., and Opekarova, M. (2011). In plant and animal cells, detergent-resistant membranes do not define functional membrane rafts. *Plant Cell* 23, 1191–1193.

Toth, K., Stratil, T. F., Madsen, E. B., Ye, J. Y., Popp, C., Antolin-Llovera, M., Grossmann, C., Jensen, O. N., Schussler, A., Parniske, M., and Ott, T. (2012). Functional domain analysis of the remorin protein LjSYMREM1 in *Lotus japonicus*. *PLoS ONE* 7, e30817. doi: 10.1371/journal.pone.0030817

van der Meer-Janssen, Y. P. M., Van Galen, J., Batenburg, J. J., and Helms, J. B. (2010). Lipids in host–pathogen interactions: pathogens exploit the complexity of the host cell lipidome. *Prog. Lipid Res.* 49, 1–26.

Varma, R., and Mayor, S. (1998). GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* 394, 798–801.

Xu, X. L., Bittman, R., Duportail, G., Heissler, D., Vilcheze, C., and London, E. (2001). Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). *J. Biol. Chem.* 276, 33540–33546.

Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002). Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* 296, 913–916.

Zappel, N. F., and Panstruga, R. (2008). Heterogeneity and lateral compartmentalization of plant plasma membranes. *Curr. Opin. Plant Biol.* 11, 632–640.

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: 27 June 2012; paper pending published: 16 July 2012; accepted: 23 July 2012; published online: 03 August 2012.
*Citation: Urbanus SL and Ott T (2012) Plasticity of plasma membrane compartmentalization during plant immune responses. Front. Plant Sci. **3**:181. doi: 10.3389/fpls.2012.00181*
This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 24 August 2012
doi: 10.3389/fpls.2012.00209

Receptor-like kinase complexes in plant innate immunity

Christiaan Greeff, Milena Roux, John Mundy and Morten Petersen*

Department of Biology, Copenhagen University, Copenhagen, Denmark

Edited by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

Reviewed by:
Pascal Braun, Technical University of Munich, Germany
Lennart Wirthmueller, John Innes Centre, UK

***Correspondence:**
Morten Petersen, Department of Biology, Copenhagen University, Biocenter 3.1.45, Ole Maaløes Vej 5, Copenhagen, Denmark.
e-mail: shutko@bio.ku.dk

Receptor-like kinases (RLKs) are surface localized, transmembrane receptors comprising a large family of well-studied kinases. RLKs signal through their transmembrane and juxtamembrane domains with the aid of various interacting partners and downstream components. The N-terminal extracellular domain defines ligand specificity, and RLK families are sub-classed according to this domain. The most studied of these subfamilies include those with (1) leucine-rich repeat (LRR) domains, (2) LysM domains (LYM), and (3) the *Catharanthus roseus* RLK1-like (CrRLK1L) domain. These proteins recognize distinct ligands of microbial origin or ligands derived from intracellular protein/carbohydrate signals. For example, the pattern-recognition receptor (PRR) AtFLS2 recognizes flg22 from flagellin, and the PRR AtEFR recognizes elf18 from elongation factor (EF-Tu). Upon binding of their cognate ligands, the aforementioned RLKs activate generic immune responses termed pattern-triggered immunity (PTI). RLKs can form complexes with other family members and engage a variety of intracellular signaling components and regulatory pathways upon stimulation. This review focuses on interesting new data about how these receptors form protein complexes to exert their function.

Keywords: receptor-like kinases, complexes, plant immunity, signaling, defense

INTRODUCTION

Autotrophs, like plants, are the source of nutrients for heterotrophs. Plants are members of complex communities and have co-evolved commensal and pathological relationships with microbes. A fine balancing act is required to effectively combat invasion by pathogenic heterotrophs while effectively guarding resources for vegetative and reproductive growth (King and Roughgarden, 1982). This entails appropriately timed activation of defense responses to conserve energy for producing numerous healthy progeny, thus increasing evolutionary fitness through this adaptive plasticity (Sultan, 2000). Detecting harmful heterotrophs and converting this recognition to intracellular signals aimed at combating the intruder and alerting surrounding tissue, is a major challenge, especially since pathogens co-evolve with their hosts to elude discovery (Frank, 1992; Lehti-Shiu et al., 2009).

Plant genomes encode a large number of surface receptor-like kinases (RLKs) to perceive different signals from both distal cells responding to stresses such as herbivore feeding or to the presence of pathogens through detection of non-self molecules (Shiu and Bleecker, 2001). RLKs generally have an extracellular ligand-binding domain, a membrane-spanning region, a juxtamembrane (JM) domain, and a serine/threonine kinase domain. Equivalent mammalian receptors from the Pelle/IRAK family differ in usually employing a cytosolic tyrosine kinase domain (Gish and Clark, 2011). A conserved aspartate in the catalytic loop of most kinases is required for catalytic activity. Ser/Thr kinases mostly have an arginine preceding this catalytic aspartate. Kinases with such residues are termed RD kinases, although most RLKs implicated in microbial detection are non-RD kinases, lacking an arginine preceding the catalytic aspartate. They in general require additional proteins to modulate their function (Johnson et al., 1996; Dardick and Ronald, 2006). An important example is BAK1, which interacts with many Arabidopsis RLKs, and is required for their activity (discussed below).

The plant RLK family has more than 600 members in *Arabidopsis* (Shiu et al., 2004). RLKs are divided into 44 sub-families depending on their N-terminal domains. While RLKs have been implicated in many biologically important processes (Gish and Clark, 2011), this review focuses on RLKs involved in pathogen detection.

Plant RLKs involved in immunity are so-called pattern-recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) and, upon binding of their cognate elicitors, initiate a well-characterized set of defense responses termed PAMP-triggered immunity (PTI). Features of PTI include reactive oxygen species (ROS) production, callose deposition, generation of secondary messengers, and defense gene expression (Jones and Dangl, 2006). RLK elicitation also leads to activation of several mitogen-activated protein (MAP) kinases (Suarez-Rodriguez et al., 2007; Mithoe et al., 2011). PAMPs, and more broadly, microbial-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs), can activate RLKs (Lerouge et al., 1990; Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Krol et al., 2010). Binding of PAMPS and DAMPS to their specific receptors leads to a broad range of downstream signaling events and effects. **Figures 1A–C** gives an overview of some of the complexes of Xa21, FlS2, and EF-Tu receptor (EFR) that will be discussed in this review. **Figure 1D** shows biological effects of FLS2, Xa21 and EFR.

THE LRR FAMILY

The best-studied members of the leucine-rich repeat (LRR)-RLK family are the non-RD kinases AtFLS2, AtEFR, and OsXa21 (Park

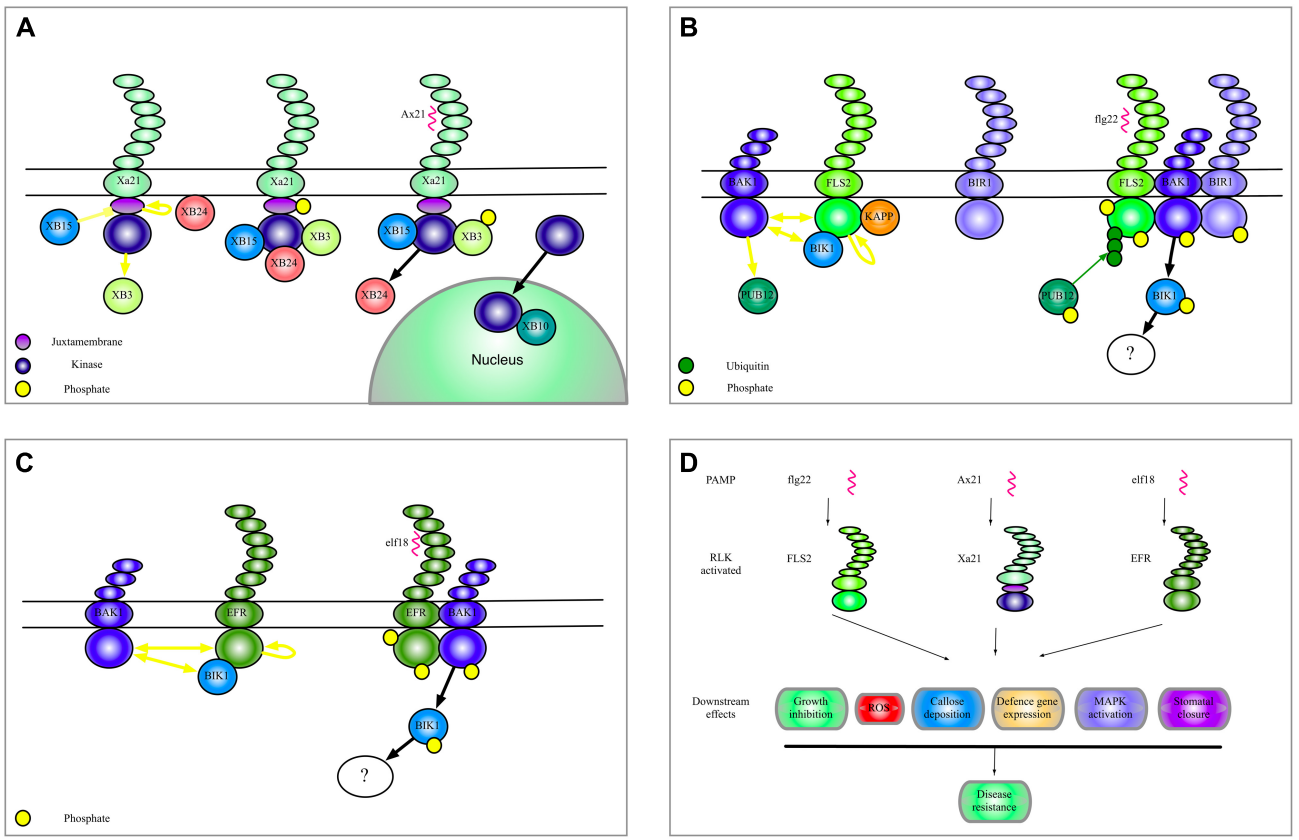


FIGURE 1 | Complexes of Xa21, FLS2, and EFR. (A) A model to summarize the current data regarding Xa21 function as discussed in this manuscript. **(B)** Illustration of the complexes formed by the RLK-FLS2. The yellow dots indicate phosphorylation of a protein. Yellow arrows indicate phosphorylation of a substrate protein. Yellow blunt arrows indicate dephosphorylation of a substrate protein. Green dots and green arrows indicate ubiquitination. Black arrows indicate translocation, association or dissociation. **(C)** Selected interactors of the RLK EFR. **(D)** Shows biological effects of selected RLK activation.

et al., 2010a) and the RD kinase BAK1 (Chinchilla et al., 2007; Heese et al., 2007). These receptors present the core of our current knowledge regarding RLKs involved in defense.

Xa21

The Xa21 extracellular domain is composed of 23 LRRs and was one of the first eukaryotic RLKs found to be involved in resistance (Song et al., 1995; Wang et al., 2006). Xa21 binds the *Xanthomonas oryzae* pv. *oryzae* (Xoo) secreted tyrosine (Tyr) *O*-sulfonation peptide AxY^{S}22 (Lee et al., 2009). Much has been learned about the function of Xa21. For example, the amino acids Ser686, Thr688, and Ser689 in the cytosolic JM domain are important for stability and for endoplasmic reticulum (ER) processing (Xu et al., 2006; Park et al., 2010a). Phosphorylation of residues in the JM domain is also critical for the activation of Xa21 and binding of at least four Xa21-binding proteins named XB3, XB15, XB24, and XB10 (OsWRKY62; Park et al., 2010b) associated with Xa21 via the JM domain. These interactions are all dependent on Thr705 since mutation of this JM domain residue abolishes XB-Xa21 binding (Chen et al., 2010a).

XB3 is an E3 ligase important for Xa21 accumulation and is a substrate for Xa21 kinase activity, although the biological relevance of this relationship is still unclear. After Xa21 binds AxY^{S}22, XB3 is activated by transphosphorylation and likely leads to cleavage of a negative regulator of defense or even of itself, allowing other interactions to take place (Wang et al., 2006).

Xa21 is regulated by two proteins through phosphorylation; XB15, a protein phosphatase 2C (PP2C) and XB24, a protein with intrinsic ATPase activity (Park et al., 2008). XB15 dephosphorylates Xa21 and XB15 over-expression reduces *Xoo* resistance while *xb15* null-mutants exhibit increased cell death and resistance to *Xoo*. This would point to a negative regulatory role of XB15. On the other hand, XB24 promotes autophosphorylation of Xa21 and may be required to prevent proteolytic cleavage of Xa21. The complex between XB24 and Xa21 dissociates upon *Xoo* infection or AxY^{S}22 binding (Chen et al., 2010b). Phosphorylation, especially in the JM domain, plays a critical role in Xa21 stability. It is clear that autophosphorylation of certain residues in Xa21 promotes an inactive state but the exact changes in phosphorylation status upon pathogen infection remain largely unknown.

Xa21 binds to the WRKY transcription factor XB10 and this binding requires an active Xa21 kinase domain. Binding of the AxY^{S}22 peptide to Xa21 leads to translocation of a Xa21 kinase domain-GFP fragment to the nucleus where it interacts with XB10.

The nuclear translocation is important for *Xoo* resistance and the Xa21 kinase domain/XB10 complex probably affects defense gene expression (Park and Ronald, 2012). Whether this or a similar mechanism also applies to other RLKs is currently unknown, but future studies will likely address this issue. Recently, a large-scale yeast two-hybrid study revealed yet another set of Xa21 interacting partners (Seo et al., 2011). Although the biological significance of these discoveries in signaling remains to be seen, they may provide interesting clues to the functions of Xa21 and other RLKs.

To help proteins fold properly, the ER contains a number of chaperones including BiPs (binding immunoglobulin protein) that bind N-glycosylated proteins and direct them to the ER (Molinari and Helenius, 2000). Xa21 is also N-glycosylated and interacts with BiP3, an HSP70-like ATPase located in the ER, and this is important for correct folding and functioning of the protein (Park et al., 2010a). While a pool of Xa21 locates to the PM where AxYS22 ligand is perceived, the majority of the receptor is found in the ER.

AtFLS2

The FLS2 (flagellin sensing 2) receptor recognizes the well-conserved protein flagellin from a broad class of bacterial plant pathogens including *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 (Gómez-Gómez and Boller, 2000). Direct binding of the flagellin-derived peptide flg22 has been shown using ^{125}I-labeled peptides (Chinchilla et al., 2006), but a recent report also implicates FLS2 in unsulfonated *Xoo* Ax21 peptide perception. These two peptides are not sequence related, which makes the finding quite astonishing (Danna et al., 2011).

FLS2 was recently shown to form homo-dimers independently of flg22 binding, but whether this dimerization is important for receptor function is not known (Sun et al., 2012). However, it is well-established that FLS2 forms heterodimers with BRI1-associated kinase 1 (BAK1) (Chinchilla et al., 2007; Schulze et al., 2010) in the presence of bound flg22. BAK1 is a common component in many RLK signaling complexes, and was first identified for its requirement in brassinosteroid signaling via the receptor BRI1 (Li et al., 2002). The essential role of BAK1 in flg22 sensing was revealed by the marked reduction of flg22-induced responses in *bak1* plants (Chinchilla et al., 2007; Heese et al., 2007). Importantly, the BAK1–FLS2 interaction most likely does not compete with other known BAK1 interactors such as BRI1, and the BAK1–FLS2 interaction is therefore not responsible for BR-mediated PAMP defense suppression (Albrecht et al., 2012). BAK1 is a member of the somatic embryogenesis receptor kinase (SERK) family comprising 5 members, SERK1, SERK2, BAK1/SERK3, BAK1-like (BKK1)/SERK4, and SERK5. FLS2 interactions with SERK1, SERK2, SERK5, and BKK have been detected, but its predominant association is with BAK1. BAK1 and BKK1 are thought to act cooperatively in PAMP signaling and resistance to biotrophic pathogens (Roux et al., 2011).

BAK1 and FLS2 also interact with Botrytis-induced kinase 1 (BIK1), which is a receptor-like cytoplasmic kinase (RLCK) implicated in resistance to necrotrophic pathogens (Veronese et al., 2006). BAK1 and FLS2 phosphorylate BIK1 (Lu et al., 2010) and BIK1 in turn phosphorylates both FLS2 and BAK1. This is thought to be an important signal amplification mechanism. However, since FLS2 has been shown to have very low catalytic activity *in vitro* (Schwessinger et al., 2011), BAK1 probably possesses the predominant kinase activity influencing BIK1 phosphorylation. The BIK1–FLS2/BAK1 association is decreased after flg22 sensing, suggesting that BIK1 is released to activate downstream signaling components (Lu et al., 2010). BIK1's role in PTI is dependent on complex interactions with major immune response regulators and may thus provide RLK signaling complexes with the ability to discriminate between biotrophic and necrotrophic pathogens (Laluk et al., 2011). Importantly, *bik1* mutants display enhanced susceptibility to *Pto* DC3000, reduced flg22 responsiveness, as well as compromised flg22-induced resistance to virulent *Pto* DC3000. The BIK1-related kinases, PBS-like kinase 1 (PBL1) and PBL2 also interact with FLS2 and BAK1. *pbl1* mutants show less reduction in PTI responses but the effect seems to be additive to BIK1 function (Zhang et al., 2010).

BAK1, BKK1, SERK1, and SERK2 have also been shown to interact with BIR1 (BAK1-interacting receptor-like kinase 1), an active protein kinase. The *bir1* mutant exhibits increased resistance to biotrophic *Pto* DC3000 and *Hyaloperonospora arabidopsidis* Noco2, due to apparent *R* protein activation (Wang et al., 2011). The *bir1* phenotype is partially rescued in *bir1 pad4* double mutants, and is completely rescued in the *bir1 pad4 sobir* (*suppressor of bir1-1*) triple mutant. Phytoalexin deficient 4 (PAD4) is one of the critical components required for Toll/interleukin-1 receptor (TIR) R protein signaling. Many constitutively active defense phenotypes that result from activated TIR R proteins are suppressed by PAD4 loss of function (Wiermer et al., 2005; Palma et al., 2010; Zhang et al., 2012). The aforementioned results thus indicate that the *bir1* phenotype is partly dependent upon R protein activation, although the majority of defense induction in *bir1* occurs through SOBIR1. SOBIR1 is also a RLK, and over-expression of SOBIR1 leads to activation of cell death. SOBIR1 does not function in flg22 sensing and does not interact with BIR1. Exactly how loss of BIR1 activates SOBIR1 is a mystery (Gao et al., 2009), and it is still uncertain whether BIR1 has a role in the PAMP signaling pathway.

Kinase-associated protein phosphatase (KAPP) interacts with the FLS2 kinase domain (Gómez-Gómez and Boller, 2000), and this interaction may be important for receptor endocytosis upon activation as was found for AtSERK1 (Shah et al., 2002). KAPP has also been found in complexes with other RLKs (Williams et al., 1997; Stone et al., 1998) but whether it functions as a general regulator of a broader spectrum of RLKs needs to be explored.

FLS2 also interacts with E3 ligases that polyubiquitinate the receptor after flg22 signaling. FLS2 is subsequently degraded by the proteasome, which might constitute a mechanism for attenuation as has been described for the mammalian Toll-like receptor 4 (TLR4) and TLR9 (Chuang and Ulevitch, 2004). Plant U-Box 12 (PUB12) and PUB13, both E3 ubiquitin ligases, have been shown to be BAK1 phosphorylation targets, and this modification is required for their association with FLS2. This phosphorylation is reminiscent of the previously mentioned Xa21 phosphorylation of XB3. PUB12 and PUB13 control flg22-dependent, proteasome-mediated degradation of FLS2

(Lu et al., 2011), making this system important for FLS2 signaling attenuation, together with receptor endocytosis (Salomon and Robatzek, 2006).

Despite being a transmembrane protein, FLS2 does not depend critically on N-glycosylation for its function as has been found for EFR (Nekrasov et al., 2009; Saijo et al., 2009; Häweker et al., 2010). However, FLS2 has recently been shown to interact with the reticulon-like proteins RTNLB1 and RTNLB2. RTNLB1/2 are together involved in regulating FLS2 transport from the ER to the plasma membrane (Lee et al., 2011). In addition, stomatal cytokinesis defective 1 (SCD1) was identified by mass spectrometry as an FLS2 interaction partner. *Scd1* mutants display SA-dependent enhanced resistance to infection with *Pto* DC3000, as well as enhanced accumulation of *PR1* transcripts and hydrogen peroxide. However, the same mutants are less sensitive to PAMPs, with reduced seedling growth inhibition and ROS production in response to flg22 or elf18 (Korasick et al., 2010).

EF-Tu RECEPTOR

EF-Tu receptor is a LRR-RLK that recognizes the peptide elf18 from bacterial elongation factor (EF)-Tu. EFR and BAK1 have also been shown to interact in a ligand-dependent manner (Roux et al., 2011). Indeed, many of the signaling components downstream of EFR and FLS2 are shared. While both EFR and FLS2 are capable of associating with all members of the SERK family, BKK1, SERK1, SERK2 have a stronger association with EFR than with FLS2 (Roux et al., 2011). This might allow EFR to avoid pathogen effector action on the single SERKs. Studies of SERK function have been difficult due to their apparent redundancy and the lethality of some double mutants such as *serk1 serk2* and *bak1-4 bkk1-1* (Colcombet et al., 2005; He et al., 2007). However, the discovery of a novel allele of *bak1*, *bak1-5*, enabled study of non-lethal *bak1-5 bkk1* double mutants. This revealed that BAK1 and BKK1 act cooperatively in PAMP signaling (Roux et al., 2011; Schwessinger et al., 2011).

BIK1 is phosphorylated upon elf18 and flg22 treatment (Lu et al., 2010). Given the many parallels between FLS2 and EFR, it is possible that transphosphorylation of the EFR/BAK1 complex also occurs, although direct proof is still lacking. In contrast to FLS2, but similarly to Xa21, N-glycosylation is critical for EFR function and EFR is subject to ER quality control that requires several chaperones involved in ER-QC for full activity (Häweker et al., 2010).

PEPR1

In contrast to the three receptors described above, Pep1 receptor 1 (PEPR1) binds AtPep1 (Yamaguchi et al., 2006) a DAMP derived from the precursor gene *PROPEP1*. PEPR1 and PEPR2 act redundantly to perceive AtPep1. BAK1 was shown to interact with PEPR1 like FLS2 and EFR (Postel et al., 2010). PEPR1 possesses a putative guanylyl cyclase (GC) domain and cGMP production by the purified RLK was shown *in vitro* (Qi et al., 2010). Interestingly, a GC domain is also present in BRI1 and was shown to have a catalytic function *in vitro* (Kwezi et al., 2007). This cGMP generated after elicitation may trigger a cyclic nucleotide-activated Ca^{2+} channel as part of its signaling activity (Ali et al., 2007).

LysM FAMILY

Chitin elicitor receptor kinase 1 (CERK1) is the best studied Arabidopsis LysM-RLK (Kaku et al., 2006; Miya et al., 2007; Wan et al., 2008), and direct binding of chitin to CERK1 has been detected (Iizasa et al., 2010; Petutschnig et al., 2010). Unlike FLS2 and EFR, CERK1's perception of fungal chitin is BAK1-independent. In rice, Chitin elicitor-binding protein (CeBIP), a LysM domain-containing protein, associates with OsCerk1 and these proteins function together in a hetero-oligomer receptor complex to elicit chitin signaling in a ligand-dependent manner (Shimizu et al., 2010). Two LysM domain proteins, LYM1 and LYM3, have recently been shown to be important for peptidoglycan (PGN), but not chitin recognition. LYM1 and LYM3 are not functionally redundant, and it has been proposed that LYM1, LYM3 and CERK1 may form a complex or complexes. *cerk1* is hypersusceptible to *Pto* DC3000 and shows reduced sensitivity to PGN, phenocopying *lym1/lym3*, however CERK1 does not bind to PGN. Further, given the fact that neither LYM1 nor LYM3 contain a cytoplasmic domain, a LYM1/LYM3/CERK1 complex seems likely (Willmann et al., 2011). RLKs often hetero-oligomerize for optimal functioning as seen in the co-operativity of FLS2/BAK1, EFR/BAK1 and PEPR1/PEPR2.

CrRLK1L FAMILY

Another RLK, FERONIA (FER) was first shown to control pollen tube reception (Escobar-Restrepo et al., 2007). However, the expression of FER throughout the plant suggests a general function not strictly associated with root development or pollen tube reception. Indeed, FER has more recently been shown to aid powdery mildew (PM) penetration into host cells (Kessler et al., 2010) and to be responsible for susceptibility to the oomycete *H. arabidopsidis* (Nibau and Cheung, 2011). It is suspected that FER might play a role in controlling localization of MLO family proteins, known to be important for PM infection (Consonni et al., 2006), as it does for NTA during pollen tube reception. This however still needs to be shown, as well as whether ROS signaling has an effect on MLO localization. Given the many roles of FER it is not surprising to find that it is important for disease resistance as well.

FER appears to exert is signaling functions by controlling ROS production. FER was shown to interact with guanine nucleotide exchange factors (GEFs) that regulate RHO GTPases (RAC/ROPs). RAC/ROP is known play important roles in stress-induced responses. In rice, the binding of a RAC/ROP called Rac GTPase to NADPH oxidases has been characterized, and Rac GTPase was show to be required for PAMP-mediated ROS production (Wong et al., 2007). In *Arabidopsis*, Rop2 was shown to co-immunoprecipitate with FER. In addition, transgenic plants expressing constitutively active, GTP-bound Rop2 displayed increased ROS production (Cheung and Wu, 2011). This indicates that a FER-GEF-RAC/ROP complex is likely able to effect ROS production. While ROS play a role in root development, there are hints that FER is involved in ROS production during PAMP signaling in leaves. For example, FER is enriched in detergent-resistant membranes (DRMs) after flg22 treatment, and FER shows flg22-induced phosphorylation (Benschop et al., 2007). *Fer* mutants also exhibit enhanced ROS production, and aberrant stomatal responses upon flg22 treatment (Keinath et al., 2010). The increase

in ROS production in the *fer* mutant is puzzling given the reduced Rho GTPase activity in this mutant (Duan et al., 2010). The relationship between FLS2 and FER in the control of ROS production is very interesting and should attract attention in the near future.

CONCLUDING REMARKS

There have been enormous advancements in our knowledge about RLK signaling in the last decade, but many questions still remain unanswered. For example, the link between the PRR receptors and production of ROS and activation of MAP kinases is still missing. Nevertheless, a quite comprehensive picture of the route from receptor activation to enhanced defense gene expression has emerged for Xa21 and similar data for FLS2 and EFR are sure to come to light.

ACKNOWLEDGMENTS

This work was supported by grants to Morten Petersen from the Danish Research Council for Technology and Production (11-106302) and the Strategic Research Council (09-067148).

REFERENCES

Albrecht, C., Boutrot, F., Segonzac, C., Schwessinger, B., Gimenez-Ibanez, S., Chinchilla, D., Rathjen, J. P., De Vries, S. C., and Zipfel, C. (2012). Brassinosteroids inhibit pathogen-associated molecular pattern-triggered immune signaling independent of the receptor kinase BAK1. *Proc. Natl. Acad. Sci. U.S.A.* 109, 303–308.

Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsaltas, D., Leng, Q., von Bodman, S., and Berkowitz, G. A. (2007). Death don't have no mercy and neither does calcium: *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. *Plant Cell* 19, 1081–1095.

Benschop, J. J., Mohammed, S., O'Flaherty, M., Heck, A. J. R., Slijper, M., and Menke, F. L. H. (2007). Quantitative phosphoproteomics of early elicitor signaling in *Arabidopsis*. *Mol. Cell Proteomics* 6, 1198–1214.

Chen, X., Chern, M., Canlas, P. E., Jiang, C., Ruan, D., Cao, P., and Ronald, P. C. (2010a). A conserved threonine residue in the juxtamembrane domain of the XA21 pattern recognition receptor is critical for kinase autophosphorylation and XA21-mediated immunity. *J. Biol. Chem.* 285, 10454–10463.

Chen, X., Chern, M., Canlas, P. E., Ruan, D., Jiang, C., and Ronald, P. C. (2010b). An ATPase promotes autophosphorylation of the pattern recognition receptor XA21 and inhibits XA21-mediated immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8029–8034.

Cheung, A. Y., and Wu, H.-M. (2011). THESEUS 1, FERONIA and relatives: a family of cell wall-sensing receptor kinases? *Curr. Opin. Plant Biol.* 14, 632–641.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 18, 465–476.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D. G., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448, 497–500.

Chuang, T.-H., and Ulevitch, R. J. (2004). Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nat. Immunol.* 5, 495–502.

Colcombet, J., Boisson-Dernier, A., Ros-Palau, R., Vera, C. E., and Schroeder, J. I. (2005). *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASES1 and 2 are essential for tapetum development and microspore maturation. *Plant Cell* 17, 3350–3361.

Consonni, C., Humphry, M. E., Hartmann, H. A., Livaja, M., Durner, J., Westphal, L., Vogel, J., Lipka, V., Kemmerling, B., Schulze-Lefert, P., Somerville, S. C., Panstruga, R. (2006). Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. *Nat. Genet.* 38, 716–720.

Danna, C. H., Millet, Y. A., Koller, T., Han, S.-W., Bent, A. F., Ronald, P. C., and Ausubel, F. M. (2011). The *Arabidopsis* flagellin receptor FLS2 mediates the perception of *Xanthomonas* Ax21 secreted peptides. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9286–9291.

Dardick, C., and Ronald, P. (2006). Plant and animal pathogen recognition receptors signal through non-RD kinases. *PLoS Pathog.* 2, e2. doi: 10.1371/journal.ppat.0020002

Duan, Q., Kita, D., Li, C., Cheung, A. Y., and Wu, H.-M. (2010). FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17821–17826.

Escobar-Restrepo, J.-M., Huck, N., Kessler, S., Gagliardini, V., Gheyselinck, J., Yang, W.-C., and Grossniklaus, U. (2007). The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. *Science* 317, 656–660.

Frank, S. A. (1992). Models of plant-pathogen coevolution. *Trends Genet.* 8, 213–219.

Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Cheng, Y. T., Chen, S., Li, X., and Zhang, Y. (2009). Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*. *Cell Host Microbe* 6, 34–44.

Gish, L. A., and Clark, S. E. (2011). The RLK/Pelle family of kinases. *Plant J.* 66, 117–127.

Gómez-Gómez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5, 1003–1011.

Häweker, H., Rips, S., Koiwa, H., Salomon, S., Saijo, Y., Chinchilla, D., Robatzek, S., and von Schaewen, A. (2010). Pattern recognition receptors require N-glycosylation to mediate plant immunity. *J. Biol. Chem.* 285, 4629–4636.

He, K., Gou, X., Yuan, T., Lin, H., Asami, T., Yoshida, S., Russell, S. D., and Li, J. (2007). BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. *Curr. Biol.* 17, 1109–1115.

Heese, A., Hann, D. R., Gimenez-Ibanez, S., Jones, A. M. E., He, K., Li, J., Schroeder, J. I., Peck, S. C., and Rathjen, J. P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12217–12222.

Iizasa, E., Mitsutomi, M., and Nagano, Y. (2010). Direct binding of a plant LysM receptor-like kinase, LysM RLK1/CERK1, to chitin in vitro. *J. Biol. Chem.* 285, 2996–3004.

Johnson, L. N., Noble, M. E. and Owen, D. J. (1996). Active and inactive protein kinases: structural basis for regulation. *Cell* 85, 149–158.

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

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11086–11091.

Keinath, N. F., Kierszniowska, S., Lorek, J., Bourdais, G., Kessler, S. A., Shimosato-Asano, H., Grossniklaus, U., Schulze, W. X., Robatzek, S., and Panstruga, R. (2010). PAMP (pathogen-associated molecular pattern)-induced changes in plasma membrane compartmentalization reveal novel components of plant immunity. *J. Biol. Chem.* 285, 39140–39149.

Kessler, S. A., Shimosato-Asano, H., Keinath, N. F., Wuest, S. E., Ingram, G., Panstruga, R., and Grossniklaus, U. (2010). Conserved molecular components for pollen tube reception and fungal invasion. *Science* 330, 968–971.

King, D., and Roughgarden, J. (1982). Multiple switches between vegetative and reproductive growth in annual plants. *Theor. Popul. Biol.* 21, 194–204.

Korasick, D. A., McMichael, C., Walker, K. A., Anderson, J. C., Bednarek, S. Y., and Heese, A. (2010). Novel functions of stomatal cytokinesis-defective 1 (SCD1) in innate immune responses against bacteria. *J. Biol. Chem.* 285, 23342–23350.

Krol, E., Mentzel, T., Chinchilla, D., Boller, T., Felix, G., Kemmerling, B., Postel, S., Arents, M., Jeworutzki, E., Al-Rasheid, K. A. S., Becker, D., and Hedrich, R. (2010). Perception of the *Arabidopsis* danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. *J. Biol. Chem.* 285, 13471–13479.

Kwezi, L., Meier, S., Mungur, L., Ruzvidzo, O., Irving, H., and Gehring, C. (2007). The *Arabidopsis thaliana* brassinosteroid receptor (AtBRI1) contains a domain that functions as a guanylyl cyclase *in vitro*. *PLoS ONE* 2, e449. doi: 10.1371/journal.pone.0000449

Laluk, K., Luo, H., Chai, M., Dhawan, R., Lai, Z., and Mengiste, T. (2011). Biochemical and genetic requirements for function of the immune response regulator BOTRYTIS-INDUCED KINASE1 in plant growth, ethylene signaling, and PAMP-triggered immunity in

Arabidopsis. *Plant Cell* 23, 2831–2849.

Lee, H. Y., Bowen, C. H., Popescu, G. V., Kang, H.-G., Kato, N., Ma, S., Dinesh-Kumar, S., Snyder, M., and Popescu, S. C. (2011). *Arabidopsis* RTNLB1 and RTNLB2 reticulon-like proteins regulate intracellular trafficking and activity of the FLS2 immune receptor. *Plant Cell* 23, 3374–3391.

Lee, S.-W., Han, S.-W., Sririyanum, M., Park, C.-J., Seo, Y.-S., and Ronald, P. C. (2009). A Type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. *Science* 326, 850–853.

Lehti-Shiu, M. D., Zou, C., Hanada, K., and Shiu, S.-H. (2009). Evolutionary history and stress regulation of plant receptor-like kinase/Pelle genes. *Plant Physiol.* 150, 12–26.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* 344, 781–784.

Li, J., Wen, J., Lease, K. A., Doke, J. T., Tax, F. E., and Walker, J. C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* 110, 213–222.

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., Heese, A., Devarenne, T. P., He, P., and Shan, L. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* 332, 1439–1442.

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.

Mithoe, S. C., Boersema, P. J., Berke, L., Snel, B., Heck, A. J. R., and Menke, F. L. H. (2011). Targeted quantitative phosphoproteomics approach for the detection of phospho-tyrosine signaling in plants. *J. Proteome Res.* 11, 438–448.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19613–19618.

Molinari, M., and Helenius, A. (2000). Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* 288, 331–333.

Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z.-H., Lacombe, S., Rougon, A., Bittel, P., Kiss-Papp, M., Chinchilla, D., van Esse, H. P., Jorda, L., Schwessinger, B., Nicaise, V., Thomma, B. P., Molina, A., Jones, J. D., and Zipfel, C. (2009). Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO J.* 28, 3428–3438.

Nibau, C., and Cheung, A. Y. (2011). New insights into the functional roles of CrRLKs in the control of plant cell growth and development. *Plant Signal. Behav.* 6, 655–659.

Palma, K., Thorgrimsen, S., Malinovsky, F. G., Fiil, B. K., Nielsen, H. B., Brodersen, P., Hofius, D., Petersen, M., and Mundy, J. (2010). Autoimmunity in Arabidopsis *acd11* is mediated by epigenetic regulation of an immune receptor. *PLoS Pathog.* 6, e1001137. doi: 10.1371/journal.ppat.1001137

Park, C.-J., Bart, R., Chern, M., Canlas, P. E., Bai, W., and Ronald, P. C. (2010a). Overexpression of the endoplasmic reticulum chaperone BiP3 regulates XA21-mediated innate immunity in rice. *PLoS ONE* 5, e9262. doi: 10.1371/journal.pone.0009262

Park, C.-J., Han, S.-W., Chen, X., and Ronald, P. C. (2010b). Elucidation of XA21-mediated innate immunity. *Cell. Microbiol.* 12, 1017–1025.

Park, C.-J., Peng, Y., Chen, X., Dardick, C., Ruan, D., Bart, R., Canlas, P. E., and Ronald, P. C. (2008). Rice XB15, a protein phosphatase 2C, negatively regulates cell death and XA21-mediated innate immunity. *PLoS Biol.* 6, e231. doi: 10.1371/journal.pbio.0060231

Park, C.-J., and Ronald, P. C. (2012). Ligand induced cleavage and nuclear localization of the rice XA21 immune receptor. *Nature Precedings*. Available at: http://hdl.handle.net/10101/npre.2012.6776.1.

Petutschnig, E. K., Jones, A. M. E., Serazetdinova, L., Lipka, U., and Lipka, V. (2010). The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. *J. Biol. Chem.* 285, 28902–28911.

Postel, S., Küfner, I., Beuter, C., Mazzotta, S., Schwedt, A., Borlotti, A., Halter, T., Kemmerling, B., and Nürnberger, T. (2010). The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in *Arabidopsis* development and immunity. *Eur. J. Cell Biol.* 89, 169–174.

Qi, Z., Verma, R., Gehring, C., Yamaguchi, Y., Zhao, Y., Ryan, C. A., and Berkowitz, G. A. (2010). Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca2+ channels. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21193–21198.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F. G., Tör, M., de Vries, S., and Zipfel, C. (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.

Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajerowska-Mukhtar, K., Häweker, H., Dong, X., Robatzek, S., and Schulze-Lefert, P. (2009). Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J.* 28, 3439–3449.

Salomon, S., and Robatzek, S. (2006). Induced endocytosis of the receptor kinase FLS2. *Plant Signal. Behav.* 1, 293–295.

Schulze, B., Mentzel, T., Jehle, A. K., Mueller, K., Beeler, S., Boller, T., Felix, G., and Chinchilla, D. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *J. Biol. Chem.* 285, 9444–9451.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A., and Zipfel, C. (2011). Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet.* 7, e1002046. doi: 10.1371/journal.pgen.1002046

Seo, Y.-S., Chern, M., Bartley, L. E., Han, M., Jung, K.-H., Lee, I., Walia, H., Richter, T., Xu, X., Cao, P., Bai, W., Ramanan, R., Amonpant, F., Arul, L., Canlas, P. E., Ruan, R., Park, C. J., Chen, X., Hwang, S., Jeon, J. S., and Ronald, P. C. (2011). Towards establishment of a rice stress response interactome. *PLoS Genet.* 7, e1002020. doi: 10.1371/journal.pgen.1002020

Shah, K., Russinova, E., Gadella, T. W. J. Jr, Willemse, J., and De Vries, S. C. (2002). The *Arabidopsis* kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1. *Genes Dev.* 16, 1707–1720.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64, 204–214.

Shiu, S.-H., and Bleecker, A. B. (2001). Plant receptor-like kinase gene family: diversity, function, and signaling. *Sci. STKE* 2001, re22.

Shiu, S.-H., Karlowski, W. M., Pan, R., Tzeng, Y.-H., Mayer, K. F. X., and Li, W.-H. (2004). Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. *Plant Cell* 16, 1220–1234.

Song, W.-Y., Wang, G.-L., Chen, L.-L., Kim, H.-S., Pi, L.-Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.-X., Zhu, L.-H., Fauquet, C., and Ronald, P. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* 270, 1804–1806.

Stone, J. M., Trotochaud, A. E., Walker, J. C., and Clark, S. E. (1998). Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol.* 117, 1217–1225.

Suarez-Rodriguez, M. C., Adams-Phillips, L., Liu, Y., Wang, H., Su, S.-H., Jester, P. J., Zhang, S., Bent, A. F., and Krysan, P. J. (2007). MEKK1 is required for flg22-induced MPK4 activation in *Arabidopsis* plants. *Plant Physiol.* 143, 661–669.

Sultan, S. E. (2000). Phenotypic plasticity for plant development, function and life history. *Trends Plant Sci.* 5, 537–542.

Sun, W., Cao, Y., Jansen, K. L., Bittel, P., Boller, T., and Bent, A. F. (2012). Probing the *Arabidopsis* flagellin receptor: FLS2-FLS2 association and the contributions of specific domains to signaling function. *Plant Cell* 24, 1096–1113.

Veronese, P., Nakagami, H., Bluhm, B., AbuQamar, S., Chen, X., Salmeron, J., Dietrich, R. A., Hirt, H., and Mengiste, T. (2006). The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. *Plant Cell* 18, 257–273.

Wan, J., Zhang, X.-C., Neece, D., Ramonell, K. M., Clough, S., Kim, S.-Y., Stacey, M. G., and Stacey, G. (2008). A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. *Plant Cell* 20, 471–481.

Wang, Y.-S., Pi, L.-Y., Chen, X., Chakrabarty, P. K., Jiang, J., De Leon,

A. L., Liu, G.-Z., Li, L., Benny, U., Oard, J., Ronald, P. C., and Song, W. Y. (2006). Rice XA21 binding protein 3 is a ubiquitin ligase required for full Xa21-mediated disease resistance. *Plant Cell* 18, 3635–3646.

Wang, Z., Meng, P., Zhang, X., Ren, D., and Yang, S. (2011). BON1 interacts with the protein kinases BIR1 and BAK1 in modulation of temperature-dependent plant growth and cell death in *Arabidopsis*. *Plant J.* 67, 1081–1093.

Wiermer, M., Feys, B. J., and Parker, J. E. (2005). Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* 8, 383–389.

Williams, R. W., Wilson, J. M., and Meyerowitz, E. M. (1997). A possible role for kinase-associated protein phosphatase in the *Arabidopsis* CLAVATA1 signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* 94, 10467–10472.

Willmann, R., Lajunen, H. M., Erbs, G., Newman, M.-A., Kolb, D., Tsuda, K., Katagiri, F., Fliegmann, J., Bono, J.-J., Cullimore, J. V., Jehle, A. K., Götz, F., Kulik, A., Molinaro, A., Lipka, V., Gust, A. A., and Nürnberger, T. (2011). *Arabidopsis* lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19824–19829.

Wong, H. L., Pinontoan, R., Hayashi, K., Tabata, R., Yaeno, T., Hasegawa, K., Kojima, C., Yoshioka, H., Iba, K., Kawasaki, T., and Shimamoto, K. (2007). Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. *Plant Cell* 19, 4022–4034.

Xu, W., Wang, Y., Liu, G., Chen, X., Tinjuangjun, P., Pi, L., and Song, W. (2006). The autophosphorylated Ser686, Thr688, and Ser689 residues in the intracellular juxtamembrane domain of XA21 are implicated in stability control of rice receptor-like kinase. *Plant J.* 45, 740–751.

Yamaguchi, Y., Pearce, G., and Ryan, C. A. (2006). The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in *Arabidopsis*, is functional in transgenic tobacco cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10104–10109.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S.,Mengiste, T., Zhang, Y., and Zhou, J. M. (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.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J., and Zhang, Y. (2012). 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.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D. G., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125, 749–760.

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: 03 May 2012; paper pending published: 01 June 2012; accepted: 16 August 2012; published online: 24 August 2012.

*Citation: Greeff C, Roux M, Mundy J and Petersen M (2012) Receptor-like kinase complexes in plant innate immunity. Front. Plant Sci. **3**:209. doi: 10.3389/fpls.2012.00209*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

GENERAL COMMENTARY
published: 28 November 2012
doi: 10.3389/fpls.2012.00264

Erratum: receptor-like kinase complexes in plant innate immunity

Christiaan Greeff, Milena Roux, John Mundy and Morten Petersen*

Department of Biology, Copenhagen University, Copenhagen, Denmark
**Correspondence: shutko@bio.ku.dk*

Edited by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

Reviewed by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

A commentary on

Receptor-like kinase complexes in plant innate immunity
Greeff et al. (2012). Front. Plant Sci. 3:209. doi: 10.3389/fpls.2012.00209

IN FIGURE 1

After our article was published online, it was brought to our attention that **Figure 1B** could give the impression that the current model is that FLS2 and BIR1 are found together in complexes with BAK1 upon flg22 perception. Since this is not the case and we do not discuss this possibility in the text we have decided to omit BIR1 from **Figure 1B**. It also appears that all the receptors shown in **Figure 1D** share all the outputs upon infection,

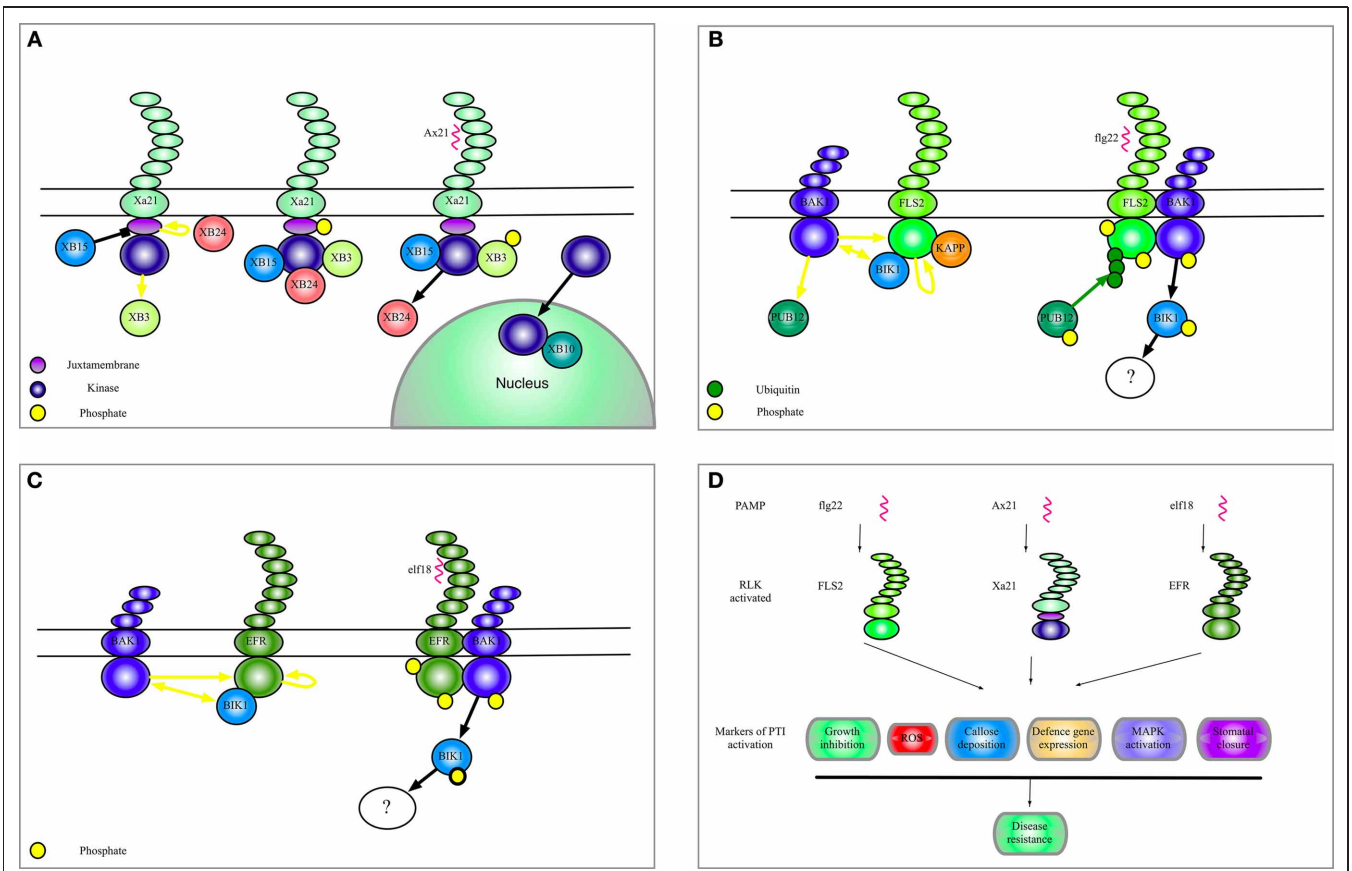


FIGURE 1 | Complexes of Xa21, FLS2, and EFR. (A) A model to summarize the current data regarding Xa21 function as discussed in this manuscript. **(B)** Illustration of the complexes formed by the RLK FLS2. The yellow dots indicate phosphorylation of a protein. Yellow arrows indicate phosphorylation of a substrate protein. Black blunt arrows indicate dephosphorylation of a substrate protein. Green dots and green arrows indicate ubiquitination. Black arrows indicate translocation, association, or dissociation. **(C)** Selected interactors of the RLK EFR. **(D)** Shows biological effects of selected RLK activation.

however, it should be noted that all these responses have not yet been demonstrated for Xa21. We have thus changed the text embedded in the figure to reflect this. In addition we showed in **Figures 1B** and **1C** that FLS and EFR activation results in phosphorylation of BAK1. This, however, is only an effect of receptor activation and is misleading as it stands, therefore have changed the arrow heads. Finally, the yellow block arrow in **Figure 1A** indicating dephosphorylation has been changed to black to make the figure clearer. **Figure 1** legend should then accordingly read "Black blunt arrows indicate dephosphorylation of a substrate protein."

IN TEXT

We would further like to change the following mistake in the text where we write "BAK1 and FLS2 phosphorylate BIK1 (Lu et al., 2010) and BIK1 in turn phosphorylates both FLS2 and BAK1" (page 3, bottom of left column). However, this is incorrect, instead the sentence should read "BAK1 phosphorylate BIK1, and BIK1 phosphorylates BAK1 (Lu et al., 2010)."

REFERENCES

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.

Received: 28 September 2012; accepted: 12 November 2012; published online: 28 November 2012.
*Citation: Greeff C, Roux M, Mundy J and Petersen M (2012) Erratum: receptor-like kinase complexes in plant innate immunity. Front. Plant Sci. **3**:264. doi: 10.3389/fpls.2012.00264*
This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 23 October 2012
doi: 10.3389/fpls.2012.00237

How complex are intracellular immune receptor signaling complexes?

***Vera Bonardi*[1] * *and Jeffery L. Dangl*[1,2,3,4,5]**

[1] *Department of Biology, University of North Carolina, Chapel Hill, NC, USA*
[2] *Howard Hughes Medical Institute, University of North Carolina, Chapel Hill, NC, USA*
[3] *Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC, USA*
[4] *Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC, USA*
[5] *Carolina Center for Genome Sciences, University of North Carolina, Chapel Hill, NC, USA*

Edited by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

Reviewed by:
Vardis Ntoukakis, University of Warwick, UK
Frank L. Takken, University of Amsterdam, Netherlands

***Correspondence:**
Vera Bonardi, Department of Biology, University of North Carolina, CB#3280, 4260 Genome Sciences Building, 250 Bell Tower Drive, Chapel Hill, NC 27599-3280, USA.
e-mail: vbonardi@live.unc.edu

Nucleotide binding leucine-rich repeat proteins (NLRs) are the major class of intracellular immune receptors in plants. NLRs typically function to specifically recognize pathogen effectors and to initiate and control defense responses that severely limit pathogen growth in plants (termed effector-triggered immunity, or ETI). Despite numerous reports supporting a central role in innate immunity, the molecular mechanisms driving NLR activation and downstream signaling remain largely elusive. Recent reports shed light on the pre- and post-activation dynamics of a few NLR-containing protein complexes. Recent technological advances in the use of proteomics may enable high-resolution definition of immune protein complexes and possible activation-relevant post-translational modifications of the components in these complexes. In this review, we focus on research aimed at characterizing pre- and post-activation NLR protein complexes and the molecular events that follow activation. We discuss the use of new or improved technologies as tools to unveil the molecular mechanisms that define NLR-mediated pathogen recognition.

Keywords: NLR, immune system, protein complex, disease resistance, effector, plant

INTRODUCTION

Plants can perceive microbial invaders through two major classes of immune receptors: surface/extracellular receptors, or intracellular immune receptors. Surface receptors, which include receptor-like kinases (RLK) and receptor-like proteins (RLP), detect both microbial-associated molecular patterns (MAMPs), typically conserved within a class of microbe, as well as specific virulence products, or effectors (Monaghan and Zipfel, 2012). Intracellular immune receptors of the nucleotide-binding domain leucine-rich repeat (NLR) protein superfamily play a central role in pathogen recognition and subsequent modulation of immune signaling in both plants and animals. The commonality of domains used by these innate immune receptors is likely the product of convergent evolution (Ausubel, 2005). Thus, NLRs across kingdoms share a common architecture that appears to reflect a common activation mechanism and, to a certain extent, common immune system output functions. Plant NLRs are critical sensors of intracellular pathogen virulence factors, or effectors, whereas their animal counterparts typically sense microbial and endogenous danger signals and link this to the activation of caspase-1 through inflammasome formation (Jones and Dangl, 2006; Franchi et al., 2012). Recent evidence from natural NLR variants and induced mutations (Hayashi et al., 2010; Kofoed and Vance, 2011; Bonardi et al., 2012) demonstrates that this set of sensor functions can be expanded to include a role for some NLRs as "helpers" that transduce signals downstream of some pathogen-activated "sensor" NLRs. As we discuss below, there may be mechanistic divergence between these two broad utilities of the NLR structural platform (Bonardi et al., 2011; Kofoed and Vance, 2011).

NLRs consist of a central nucleotide-binding (NB) domain that modulates sensor NLR activation state through the essential catalytic P-loop motif (Takken and Tameling, 2009), and a C-terminal leucine-rich repeat (LRR) domain which is highly polymorphic and variable in the number of the repeats, and typically confers recognition specificity (**Figure 1A**). Despite a similar domain organization, NLRs are diverse in their N-termini. N-terminal variability of plant NLRs is generally limited to either a coiled-coil (CC) domain, or a Toll/interleukin-1 receptor domain (TIR); occasionally unique extended N-termini can be found in CC–NLRs and TIR–NLRs, as in the case of the tomato protein Prf (Meyers et al., 2003; Mucyn et al., 2006). Conversely, a wider range of domains at the N-termini is observed in animal NLRs (Bonardi et al., 2012).

Although NLRs were originally discovered in plants almost 20 years ago (Bent et al., 1994; Mindrinos et al., 1994), and described in animals soon thereafter (Inohara et al., 1999), the molecular mechanisms by which they sense microbial infection and subsequently transduce defense signaling remain largely elusive. Furthermore, few generalizable analogies exist among the modes of NLR regulation (Eitas and Dangl, 2010; Bonardi et al., 2012). Plant NLRs sense infection by direct recognition of the microbial effector or by sensing microbe-induced modifications of host NLR-associated proteins (Jones and Dangl, 2006). However, the microbial trigger responsible for immune signaling initiation and the molecular mechanisms that control the NLR-dependent signaling events following activation remains unknown for most NLRs.

Here we focus on the molecular dynamics that accompany NLR activation and signaling in animals and plants. We present

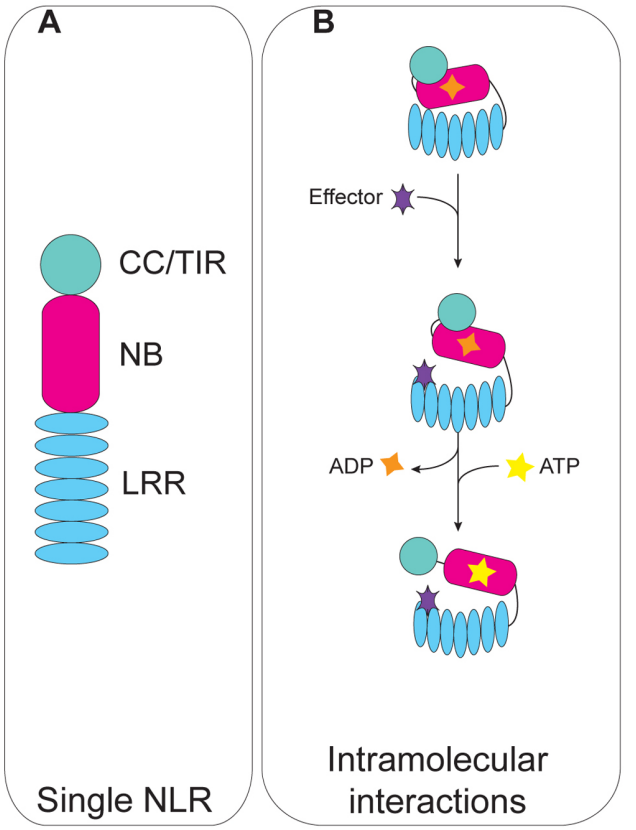


FIGURE 1 | Schematic representation of intramolecular interactions of plant NLRs. (A) Domain modularity of plant NLRs. **(B)** Intramolecular interactions maintain the NLR in an "off" state through the inhibitory function of the LRR domain (top). Effector recognition results in a conformational change that allows nucleotide cycling and NLR activation (middle). Catalytic activity of the NB domain triggers a second conformational change that exposes the N-terminal domain (bottom).

our review in a stepwise manner: from conserved intramolecular interactions aimed to control pre-activation NLR activity, to diverse types of post-activation multimer formation that in some cases ensures appropriate downstream signaling. We focus on molecular changes of intramolecular interactions, homotypic interactions, multimers and higher-order complexes associated to pre- and post-activation states (summarized in **Figure 2**).

INTRAMOLECULAR INTERACTIONS

NLRs must be under tight control to prevent unnecessary ectopic activation of immune responses, which can be detrimental for growth and development. Several examples over the years support a model in which NLR activity is held in check by intramolecular interactions (Takken et al., 2006; Marquenet and Richet, 2007).

Pepper Bs2 and potato Rx were the first NLRs for which intramolecular domain–domain association was demonstrated (Moffett et al., 2002; Leister et al., 2005; Rairdan et al., 2008). While only one intramolecular association between the N-terminal (NX) and the corresponding NB domain was identified in Bs2 (Leister et al., 2005), at least two distinct interactions involving either the CC or the LRR domains with the NB domain were demonstrated for Rx (Moffett et al., 2002). It is noteworthy that intramolecular interactions of Rx are disrupted by the presence of the cognate elicitor, CP (PVX coat protein). By contrast, intramolecular interactions between the NX-NB and the LRR domains of Bs2 are not altered by recognition of the AvrBs2 effector (Leister et al., 2005). Moreover, while the Rx CC–NB interaction requires a functional P-loop (Moffett et al., 2002), the NB–LRR interaction does not. Consistent with this, tomato Mi-1.2 is capable of intramolecular interactions between the CC–NB and the LRR domains, and this association is independent of nucleotide binding activity (van Ooijen et al., 2008). Together, these indicate first, that some or all of nucleotide binding, hydrolysis and exchange are required for maintaining proper pre-activation CC–NB interactions, and second, that CP-dependent activation of Rx requires two consequent molecular rearrangements separated by a nucleotide-binding/hydrolysis/exchange event.

Like Rx, Arabidopsis RPS5 activity is also regulated by intramolecular interactions (CC–NB, NB–LRR), consistent with the hypothesis that the LRR domain maintains the protein in an inactive state to prevent ectopic NLR signaling. This resting state allows subsequent specificity for pathogen recognition (Ade et al., 2007; Bai et al., 2012; Qi et al., 2012). No direct evidence defines intramolecular interactions for barley MLA10. However, molecular dynamics simulation of its CC structure (discussed below) suggests that the EDVID motif within the CC domain (which ensures the Rx CC–NB interaction) might modulate both intra- and intermolecular interactions in MLA10 (Rairdan et al., 2008; Maekawa et al., 2011).

Pre-activation intramolecular domain–domain interactions seem to be a conserved characteristic of NLRs across kingdoms. The inactive resting state of the animal NLR-related protein Apaf-1 is achieved by stacking of the N-terminal caspase recruiting domains (CARD) against a network of intramolecular interactions within the NB domain (Riedl et al., 2005). As demonstrated by structural studies, this packed conformation limits access to the bound ADP molecule, slowing nucleotide hydrolysis/exchange. Furthermore, biophysical characterization of the mammalian NLR NOD2 revealed that the two N-terminal CARDs interact with one another, likely to cooperatively create a binding surface for partner proteins, or alternatively to maintain the NLR in an inactive state (Fridh and Rittinger, 2012).

Based on these studies, the current model for NLR activation involves three steps (**Figure 1B**; Takken et al., 2006): (i) the microbial molecule or effector, or modified-self generated by effector action on a cellular target, are respectively recognized directly or indirectly by the NLR protein; recognition triggers a first molecular rearrangement that releases the inhibitory function of the LRR domain from the NB domain; (ii) the NB becomes accessible for nucleotide exchange, and NLR activation ensues; (iii) continued nucleotide cycling drives a second conformational change that releases the N-terminal domain from the NB domain, and likely makes it accessible as a platform for interactions with downstream signaling partners.

HOMOTYPIC INTERACTIONS

An additional feature of NLR proteins conserved among animals and plants is the potential for self-association. Increasing evidence

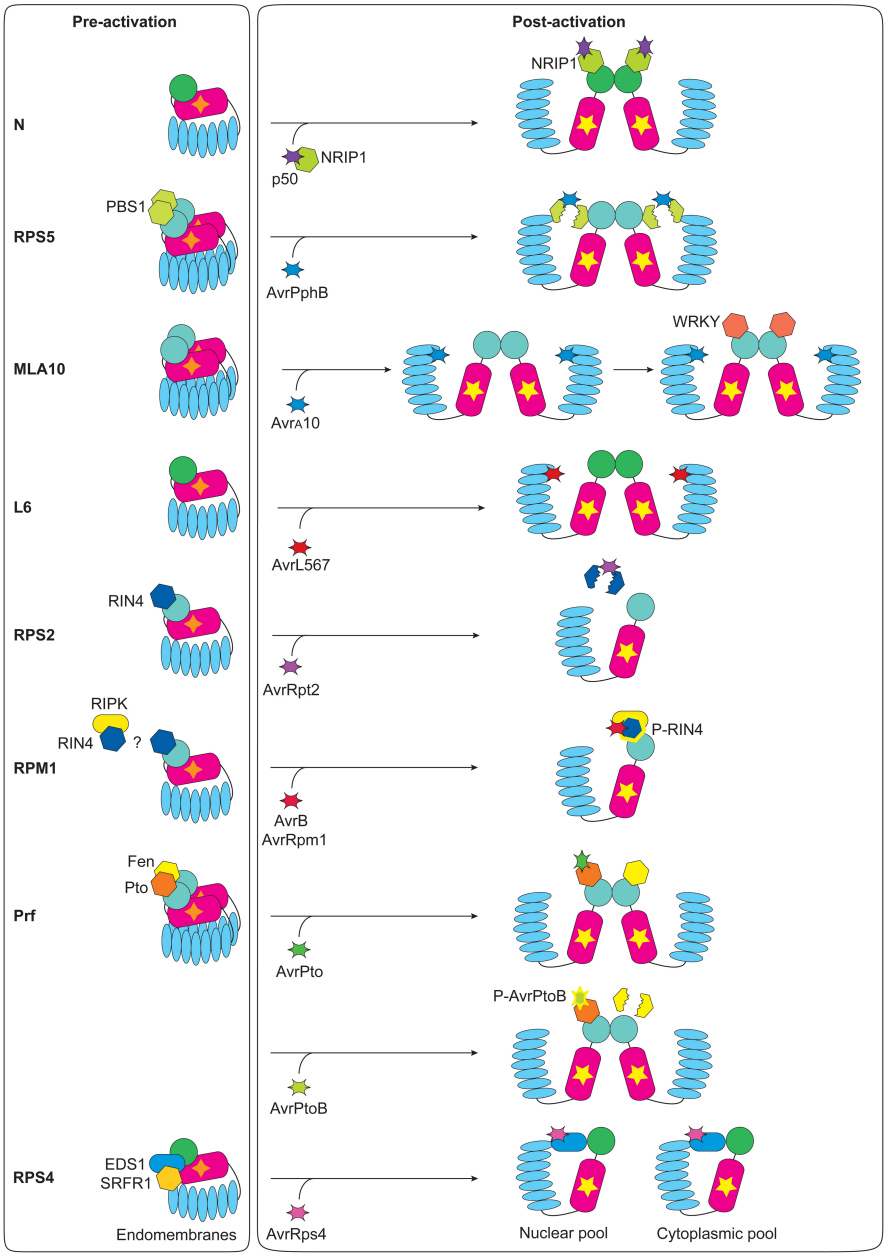


FIGURE 2 | Pre- and post-activation status of NLR immune complexes in plants. The order of the NLRs described reflects the presentation in the text. N exists as monomers prior to activation. p50 sequesters the chloroplastic protein NRIP1 and allows association of NRIP1 to the TIR domain of N, and dimerization of N. RPS5 dimerizes in its resting state and is associated with PBS1 through the RPS5 CC domain. AvrPphB targets and cleaves PBS1, activating RPS5. MLA10 exists in inactive homodimers and recognition of the specific pathogen effector triggers nucleotide-binding/hydrolysis/exchange-dependent conformational changes that allow the recruitment of WRKY transcription factors. L6 is in an inactive monomeric state and upon AvrL567 recognition through the LRR domain, L6 self-associates into dimers through TIR–TIR domain interactions. RPS2 associates with RIN4 prior to activation; no evidence for RPS2 homodimerization exists. AvrRpt2 targets and cleaves and this relieves RIN4-dependent suppression of RPS2 activity. Resting state RPM1 is in a heteromeric protein complex that comprises the guardee RIN4. Moreover RIN4 also associates with RIPK, but whether resting state RIN4, RIPK, and RPM1 are part of the same protein complex, or not, remains unknown. AvrB or AvrRpm1 enhance RIPK-mediated phosphorylation of RIN4, and this drives nucleotide-binding/hydrolysis/exchange-dependent activation of RPM1. Prf forms homodimers that bridge Pto to Fen, or possibly another Pto-family kinase. AvrPto targets Pto and recognition results in a conformational change that activates Prf signaling. AvrPtoB is an E3 ubiquitin ligase that initiates the degradation of Fen, moreover AvrPtoB recognition by Pto results in the phosphorylation of the E3 ligase domain of AvrPtoB by Pto, thus Pto is resistant to AvrPtoB-mediated degradation. No evidence for RPS4 self-association exists, thus the RPS4 inactive state is thought to contain monomeric RPS4, EDS1, and SRFR1. Cleavage of AvrRps4 releases the C-terminus AvrRps4^{C} that interacts with EDS1, thus altering the endomembrane-associated receptor complex. Post-delivery effector processing is a common event, however it is not detailed in this review. Release of the EDS1-containing RPS4 complexes to the cytoplasm and to the nucleus is thought to activate two different defense branches: cell death, and bacterial growth-restriction respectively, and these may occur in different cellular compartments.

suggests that NLR homodimers are the molecular foundation for pre-activation resting state and, in some case, for post-activation signaling events (**Figure 2**).

Potential for dimerization has been described for several plant NLRs, mainly through co-immunoprecipitation analysis of differentially epitope-tagged proteins. Although there is no evidence for self-association of the TIR–NLR N protein in its resting state, detection of the TMV elicitor p50 triggers post-activation N dimerization through association of the TIR domains (Mestre and Baulcombe, 2006; **Figure 2**). Self-association is an early post-activation event that follows pathogen recognition, as an intact P-loop is required for N dimerization (Mestre and Baulcombe, 2006). Similarly, Arabidopsis RPS5 is capable of homotypic association and each domain can interact with itself in the resting state; this association is not affected by activation (Ade et al., 2007; **Figure 2**). In this context, both N and RPS5 are thought to maintain the inactive state through intramolecular interactions, as described above. This state is perturbed by recognition of the specific elicitor, and by subsequent nucleotide cycling, which leads to exposure of the N-terminal TIR- and CC-domains, respectively. Given their diverse N terminal domains, this final event is thought to have different consequences: the N TIR domain becomes a homodimeric signaling platform; whereas the pre-existing RPS5 homodimer, newly exposed CC domains (Ade et al., 2007), might offer a binding-site for as yet unknown interacting proteins.

Given that this limited and conflicting data relies exclusively on co-immunoprecipitation assays, generalizations for the role and the dynamics of self-association may not exist. A major obstacle in the characterization of immune complexes has been the lack of reliable and robust systems to analyze NLR assembly at the molecular level. Recent technical advances begin to overcome this problem. Although to date no full-length NLR structure has been solved, the N-termini of flax L6 and barley MLA10 were recently crystallized (Bernoux et al., 2011; Maekawa et al., 2011). Both the L6 TIR domain and the MLA10 CC domain formed dimers in solution, and in both cases mutations at the dimer interface disrupted homodimerization and signaling activity. Interestingly, missense mutations in the αC helix or the BB loop in the L6 TIR prevented signaling but not dimerization, indicating the potential involvement of the relevant wild-type residues in recruiting post-activation, post-dimer formation signaling partners (Bernoux et al., 2011). Signaling by the MLA10 CC dimer is thought to mimic the microbial elicitor-activated state of the MLA10 homodimer that normally is formed following nucleotide binding/hydrolysis (Bai et al., 2012) potentially via recruitment of WRKY transcription factors for downstream signaling (Shen et al., 2007). L6 is likely to function similarly to N, since self-association does not occur in the resting state, but does accompany activation. Conversely MLA10 dimerization is effector-independent and observed in the resting state, analogous to RPS5 (**Figure 2**). This observation might indicate a functional difference between CC- (MLA10 and RPS5) and TIR- (L6 and N) NLRs. TIR–NLR resting states might be monomeric and, upon pathogen recognition, self-association might provide the N-terminal TIR dimer activation module. Conversely CC–NLRs might constitutively exist as homodimers that undergo activation-dependent conformational rearrangements to expose normally buried surfaces to anchor signaling partners. In support of this theory, the resting state CC–NLR MLA1 was also found in a high-molecular weight complex, and full-length MLA1 co-immunoprecipitated with itself (Maekawa et al., 2011). However, the CC–NLR MLA27 eluted as a monomer *in vitro* after expression and purification from insect cells (Maekawa et al., 2011), suggesting that if this hypothesis is true, homodimerization *in vivo* is mediated by plant-encoded assembly machinery. In light of this evidence, it is tempting to speculate that homodimerization might represent an essential molecular mechanism for the downstream signaling rather than for effector recognition, as indicated by the fact that TIR–NLR homodimerization typically follows NLR activation.

Tomato Prf is a CC–NLR with a unique N-terminal domain that conditions recognition of the bacterial effectors AvrPto and AvrPtoB via its interaction with the host Ser/Thr kinase Pto. While the Pto homolog Fen is marked for degradation by AvrPtoB through the activity of the effector's E3 ubiquitin ligase domain (Rosebrock et al., 2007), Pto itself is resistant to AvrPtoB-dependent degradation. This is because Pto phosphorylates and thus inactivates the AvrPtoB E3 ligase domain (Ntoukakis et al., 2009). Independently, structure-based functional analysis suggested that binding of AvrPto to Pto alters the conformation of Pto, thereby releasing inhibition of Prf and allowing its NB-dependent activation (Xing et al., 2007). Prf is capable of homodimerization, as shown by co-immunoprecipitation; this interaction is primarily mediated by the Prf N-terminal domain and is independent of Pto and AvrPto (Gutierrez et al., 2010), indicating that self-association, similarly to MLA, is constitutive and occurs prior to pathogen detection (**Figure 2**).

Self-association of mammalian NLRs has been widely demonstrated and is a common post-activation event (Hu et al., 1998; Kobayashi et al., 2002; Inohara and Nunez, 2003). Here, NLR activation typically results in inflammasome formation. This physical interaction aids the recruitment of pro-caspase-1 through its direct CARD–CARD interaction with the NLR protein or, in the case of PYD–NLR, through the adaptor protein ASC which bridges the pro-caspase-1 to the inflammasome NLR (Rathinam et al., 2012). Interestingly, homotypic interactions of mammalian NLRs are mediated by the NACHT/NB domain, whereas the diverse N-terminal domains seem to mediate interaction with accessory proteins for the downstream immune signaling (Rathinam et al., 2012). This highlights a potential difference in comparison to the self-association mechanisms adopted by plant immune NLR receptors noted above.

HETEROTYPIC INTERACTIONS

Although structural and functional similarities exist among NLRs within and across kingdoms, mechanistic regulation might rest only on intramolecular interactions to regulate activation, and homotypic interactions to modulate subsequent signaling.

Protein–protein interactions are the foundation of pathogen detection at least for plant sensor NLRs, as initiation of immune responses typically follows direct or indirect association between the NLR and the microbial product. Arabidopsis RPP1 and flax L6 specifically recognize the oomycete ATR1 and the fungal AvrL567 effector, respectively. In both cases, direct interactions

are thought to be determined by the LRR domain as suggested by co-immunoprecipitations of the RPP1 LRR domain to ATR1, or by interaction in yeast as well as structural and mutational analysis for L6 (Dodds et al., 2006; Wang et al., 2007; Krasileva et al., 2010). Direct interaction between pathogen effector and the corresponding immune receptor was recently described in rice, where the *Magnaporthe oryzae* AVR-Pik was found to physically bind the CC domain of Pik (Kanzaki et al., 2012). Plant NLRs can also be activated by effector-induced modifications of an associated host target, as suggested by the guard hypothesis (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). The Arabidopsis CC–NLR RPS5 determines recognition of the bacterial effector AvrPphB, but this event is mediated by the host protein kinase PBS1 (Shao et al., 2003). PBS1–RPS5 physical interaction is a pre-activation event and PBS1 cleavage by the cysteine protease effector AvrPphB is required for RPS5 activation. This presumably causes conformational rearrangements that allow nucleotide exchange (Ade et al., 2007; DeYoung et al., 2012; Qi et al., 2012; **Figure 2**).

Similarly, the Arabidopsis CC–NLRs RPS2 and RPM1 physically associate with different cellular pools of membrane associated RIN4, which is differentially targeted by multiple effectors. AvrB-mediated phosphorylation of RIN4, likely by the receptor-like kinase RIPK (Chung et al., 2011; Liu et al., 2011), or cleavage by the cysteine protease effector AvrRpt2 (Kim et al., 2005) is necessary and sufficient to convert resting state RPM1 and RPS2 respectively into signaling active states (**Figure 2**). Thus, PBS1 and RIN4 function as guardees for the corresponding immune receptors, RPS5 and either RPM1 or RPS2.

Tobacco NRIP1 is a chloroplastic protein that mediates the indirect association of p50 to the TIR domain of the N immune receptor (Caplan et al., 2008). However, NRIP1-N association is not constitutive, but rather requires the formation of the pre-recognition complex of NRIP1 (**Figure 2**). Hence NRIP1–N his interaction might reflect a novel mechanism required for activation of defense signaling where the immune receptor monitors an effector-dependent relocalization of a pre-recognition host complex as a modified self.

Recent high-throughput studies unveiled the potential for protein–protein interactions between immune components. Both an *Arabidopsis* interactome network and the plant-pathogen protein–protein network were identified through yeast-two-hybrid (Arabidopsis Interactome Mapping Consortium, 2011; Mukhtar et al., 2011). Although the obvious constraints of this heterologous system apply, the network revealed a greater than random propensity for indirect interaction between pathogen effectors and host immune receptors. However, whether these indirect interactions reflect a genuine gene-for-gene interaction, or whether they may be biased toward the identification of effector targets over the effector-specific immune receptor remains elusive in the absence of further validation.

HIGHER-ORDER COMPLEXES

Higher-order complex formation has been demonstrated for some plant NLRs, as we discuss below, and is well established for animal NLRs (Franchi et al., 2012). However requirements for diverse accessory partners, and variable oligomer stoichiometry are, thus far, the norm, suggesting the existence of diverse NLR immune complexes that could reflect different activation and/or signaling mechanisms (**Figure 2**).

As described above, tomato Prf is part of a high-molecular complex that contains a Prf dimer, which can bridge Pto and Fen (Gutierrez et al., 2010). This complex likely functions as a regulatory switch to control immune responses and is activated via effector-dependent disruption of negative regulation on Prf (Mucyn et al., 2006, 2009; Gutierrez et al., 2010). Size-exclusion gel filtration analysis combined with mass spectrometry (MS) on the immunoaffinity purified Prf complex allowed the identification of a hetero-multimer that is likely to contain two Prf molecules and two Pto-family kinases (Gutierrez et al., 2010). Additional data suggest that Prf is capable of homotypic and heterotypic interactions with at least Pto and Fen, although two additional Pto-family kinases, Pth2 and Pth3, were also found to be associated with the Prf complex as well (Gutierrez et al., 2010). This multimerization event is thought to bring into close proximity the Prf-associated kinases and thus likely can broaden the specificity of effector recognition events that can activate Prf.

Notably, Prf-dependent defense signaling reflects a pathogen detection mechanism that does not conform simply to the guard hypothesis. Although AvrPto and AvrPtoB recognition leads to Prf-dependent effector-triggered immunity (ETI), these effectors also physically interact with pattern recognition LRR-kinase receptors (PRRs) that typically regulate MAMP-triggered immunity (MTI; Ausubel, 2005), hence, Pto-family kinases and PRR kinases are co-receptors for common effectors. In this context, Prf-mediated ETI evolved as a mechanism to intercept and re-direct effector-triggered suppression of MTI responses into effective ETI (Gohre et al., 2008; Xiang et al., 2008; Gimenez-Ibanez et al., 2009).

Increasing evidence suggests that both products of head-to-head NLR gene pairs are often required for full disease resistance (Eitas and Dangl, 2010; Okuyama et al., 2011). One of the best characterized and most appealing of such cases is the *Arabidopsis* dual resistance gene system of RRS1–RPS4. RRS1-R is an atypical NLR that contains a C-terminal WRKY domain and confers resistance to *Ralstonia solanacearum* expressing PopP2 effector. RPS4 is a TIR–NLR. Physical association between RRS1 and PopP2 has been demonstrated both in yeast cells and in the nucleus of living plant cells (Deslandes et al., 2003; Tasset et al., 2010). Intriguingly, both RRS1 and RPS4 are required for specific AvrRps4-triggered immunity, as well as resistance to *R. solanacearum* and *Colletotrichum higginsianum* (Birker et al., 2009; Narusaka et al., 2009). RPS4 is capable of shuttling to the nucleus and RRS-R levels are enhanced in the nucleus in the presence of PopP2 (Deslandes et al., 2003; Wirthmueller et al., 2007). Thus, an attractive possibility is that effector-mediated activation could result in a yet to be detected physical interaction between RRS1 and RPS4 in the nucleus. This interaction would, in turn, promote transcriptional regulation of target genes.

Although no current evidence for Arabidopsis RPS4 homodimerization exists, it is tempting to speculate that the RPS4–RRS1 heterodimer could be the functional molecule (see above). In addition, the RPS4 TIR–NLR was recently found to physically associate with an immune regulator of basal defense and ETI, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; (Bhattacharjee et al., 2011;

Heidrich et al., 2011). In this context EDS1 is potentially an adaptor protein that could connect effector recognition to downstream defense pathways. Interestingly EDS1 is capable of physical interaction not only with RPS4, but also with the unrelated TIR–NLR RPS6, and the likely transcriptional repressor, SRFR1. However, recognition of the RPS4 and RPS6 activating effectors, AvrRps4 and HopA1 respectively, apparently disrupts the EDS1 interaction with SRFR1 and the TIR–NLR receptors (Bhattacharjee et al., 2011; **Figure 2**). These authors suggested that the association of an NLR immune receptor with EDS1 might underpin a novel mechanism for immune responses where a basal defense regulator coordinates various immune responses that are both effector-dependent and -independent. Thus, EDS1 could be a common virulence target guarded by a number of TIR–NLRs. However, Sohn et al. (2012) were recently unable to reproduce co-immunoprecipitation between AvrRps4 and EDS1. Thus, it remains uncertain whether EDS1 is a *bona fide* guardee.

Besides the large number of heterotypic interactions described for animal NLRs with accessory proteins involved in inflammasome formation, new evidence suggests that NLRs themselves can, in some cases, heterodimerize. The NLRC4 inflammasome is activated by bacterial flagellin and type III secretion system component PrgJ. Recognition of flagellin is specifically mediated by the sensor NLR NAIP5, whereas the NLR NAIP2 serves as the sensor NLR for PrgJ (Kofoed and Vance, 2011; Zhao et al., 2011). The NAIP NLR sensors control ligand-dependent NLRC4 oligomerization in a similar manner: flagellin recognition results in NAIP5–NLRC4 heteromerization, whereas PrgJ recognition drives NAIP2–NLRC4 association (Kofoed and Vance, 2011; Zhao et al., 2011). Thus, NLRC4 is a "helper" NLR for the function of at least two sensor NLRs (Rathinam et al., 2012).

Plant NLR functions were also recently differentiated into "helper" or "sensor" (Bonardi et al., 2011, 2012). Tomato NRC1 and tobacco NRG1 represent the first examples of NLR proteins that function as helper for either Cf-4 or N respectively (Peart et al., 2005; Gabriels et al., 2007). The Arabidopsis CC–NLR ADR1-L2 can act as a helper NLR that regulates signal transduction following effector detection via at least two sensor NLRs. Intriguingly neither ADR1-L2 nor NLRC4 require a functional nucleotide-binding domain to fulfill their helper NLR functions. This suggests that helper NLRs might share common signaling mechanisms (Bonardi et al., 2011; Kofoed and Vance, 2011). A non-functional P-loop variant of NLRC4 abolished both homodimerization and heteromerization with NAIP5 (Zhao et al., 2011), but retained inflammasome-dependent cell death signaling (Kofoed and Vance, 2011). This result could indicate that the non-functional P-loop NLRC4 mutant is unable to coordinate nucleotide binding, but might still retain the ability to function as an adaptor to recruit CASP1 and activate immune signaling downstream from the activated sensor NLRs. Similar to NLRC4, ADR1-L2 might function as an adaptor for effector-activated sensor NLRs, although no clear mechanism exists yet. Because ADR1-L2 coordinates several sorts of immune responses, from effector-dependent to the recognition of conserved microbial compounds, we speculate that various triggers of the plant defense output response might converge on ADR1-L2, possibly via direct physical interaction of this NLR with other defense machinery components. Moreover, the rice Pb1 NLR family, which naturally lacks a P-loop motif, conditions broad spectrum resistance to rice blast, potentially by acting as a helper NLR (Hayashi et al., 2010). There are Pb1 homologs in maize, suggesting evolutionary conservation of function. Further, *Arabidopsis* and *A. lyrata* express NLR proteins that carry degenerate P-loop mutations that are likely to impair the canonical P-loop-dependent activation mechanism (Bonardi et al., 2012). These examples do not fit the current mechanistic activation paradigm outlined above, which relies on nucleotide exchange and hydrolysis to drive intra- and intermolecular rearrangements and activation. Together, these examples support a role of helper NLRs as components of a scaffold machinery for immune responses, and provide a potential mechanistic rationale for the occurrence of co-functional head-to-head NLR genes described above.

A link between the two different receptor tiers of plant immune response signaling was also recently proposed. The immune complex associated with the low abundance plasma membrane localized CC–NLR RPS2 was immunopurified and additional components were identified through chemical cross-linking and MS (Qi and Katagiri, 2011; Qi et al., 2011a,b). Interestingly, RPS2 was found to physically associate with the flagellin receptor FLS2, a PRR that regulates MTI. Furthermore FLS2 was also shown to associate with RPS5 and RPM1 in this system, suggesting that ETI and MTI signaling might be connected (Qi et al., 2011a).

IMPLICATIONS FOR FORMATION OF DIVERSE IMMUNE PROTEIN COMPLEXES

Despite similar autoregulatory mechanisms for pre-activation of NLRs, we hope to have highlighted how variable NLR immune complexes can be. Moreover, in certain cases an NLR might interact with a wide array of partners, although the size of the associated complex thus far observed is likely too small to explain all the possible interactions (Gutierrez et al., 2010). While some of these interactions might be promiscuous and not biologically relevant, many are likely to be associated with differential signaling dynamics.

It was recently reported that plant NLR-mediated cell death, which is a hallmark of successful ETI, and disease resistance measured by pathogen growth restriction, can be uncoupled and that this bifurcation might rest on differential compartmentalization for each signaling branch (Coll et al., 2010; Heidrich et al., 2011; Bai et al., 2012). Therefore, it is straightforward to speculate that an NLR might recruit different partners depending on its cell compartment-specific function. Thus, nucleocytoplasmic partitioning of NLR-containing complexes could result from a different network of interactions (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007; Cheng et al., 2009; Slootweg et al., 2010; Tameling et al., 2010). However, in the absence of insights into cell molecular dynamics during the immune response, the relevance of each interaction cannot yet be assessed.

PROSPECTIVE

An increasing amount of evidence suggests the existence of higher-order molecular complexes associated with NLR proteins. However, whether these interactions are biologically relevant

in plant or animal innate immunity cannot be assessed given the limitations of the techniques used in the majority of these reports. Co-immunoprecipitations from complex mixtures do not discriminate indirect from direct protein–protein interactions, and do not provide evidence for stoichiometry of the molecular complex. Moreover, simple protein–protein interactions do not describe the dynamics of the signaling network upon NLR activation.

Proteomics offers a powerful and indispensable technology in biology as it aids not only in the identification of the components of protein complexes, but also in the determination of post-translational modifications that might shed light on regulatory molecular mechanisms of immune signaling. A large-scale survey of the comparative identification of phosphorylation sites was recently described for plants (Nakagami et al., 2010). Interestingly, many phosphorylated residues in conserved NLR motifs essential for their function were identified, suggesting that phosphorylation might be key for NLR activation and signaling. In the past, MS workflows have been widely improved in their utility and performance, additionally the growing MS-database acquired over the years is deposited in accessible databases (Schmidt et al., 2009; Joshi et al., 2011). Besides Prf and RPS2, as we outlined above, MS on immunoaffinity purified complexes was successfully employed for the identification of novel components of the RIN4 immune complex (Liu et al., 2009). However, one of the main limitations of MS in protein complexes identification rests on how to increase sensitivity to allow monitoring of low-abundance proteins. Recent technical advances for the isolation of low abundance plasma membrane-associated NLRs might be helpful to overcome this limitation (Qi and Katagiri, 2011; Elmore et al., 2012). Moreover, quantification of peptides based on ion abundance rather than spectra counting provides a higher dynamic range of quantification (Patel et al., 2009).

Although MS provides a valuable tool to resolve immune protein complexes, it does not allow elucidation of the molecular mechanisms of protein networks. Only by exploring the 3D structure of the individual NLR proteins, and NLR proteins in complex with effectors and partners, we will be able to investigate their molecular function, to define their direct interaction with additional signaling components, and to provide mechanisms for their control, thus linking NLR structure to a biological relevant signaling system.

As more evidence on protein–protein interactions in innate immune complexes is gathered, we need to critically evaluate not only the validity of the interaction but also the physiological significance of it. Newly emerging fluorescent protein technologies represent an appealing tool to overcome this challenge and to study the spatio-temporal dynamics of the immune receptors in living cells (Miyawaki, 2011). Single molecule fluorescence will greatly advance our understanding of how immune complexes are formed and regulated, as we will likely be able to simultaneously assess protein–protein interaction and protein movement in the cell. These state of the art technologies will direct us toward new questions that can be addressed at spatiotemporal resolution: how is an immune complex formed? How is it regulated upon microbial recognition? How is the complex distributed in the cell upon activation?

Given the likely and unanticipated diversity of NLR functions, it is now essential to elucidate the molecular dynamics of immune complex formation and signaling in a variety of contexts to unveil the spectrum of different mechanisms that regulate NLR activities. Thus resolving the complexity of the NLR immune complexes remains one of the major challenges we face in order to rationally deploy NLR proteins to combat old and emerging plant diseases.

ACKNOWLEDGMENTS

We thank Dr Marc Nishimura, Dr Eui-Hwan Chung, Dr Farid El Kasmi, Dr Laurent Deslandes, and Dr Jonathan Jones for helpful discussions and critical reading of the manuscript. Jeffery L. Dangl is a Howard Hughes Medical Institute-Gordon and Betty Moore Foundation Plant Science Investigator. This work was funded by the National Science Foundation (Arabidopsis 2010 Program Grant IOS-0929410) and the National Institutes of Health (R01GM057171 with an ARRA supplement); Vera Bonardi was supported by the Human Frontier Science Program (LT00905/2006-L).

REFERENCES

Ade, J., DeYoung, B. J., Golstein, C., and Innes, R. W. (2007). Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2531–2536.

Arabidopsis Interactome Mapping Consortium. (2011). Evidence for network evolution in an *Arabidopsis* interactome map. *Science* 333, 601–607.

Ausubel, F. M. (2005). Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* 6, 973–979.

Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., et al. (2012). Structure–function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. *PLoS Pathog.* 8, e1002752. doi: 10.1371/journal.ppat.1002752

Bent, A. F., Kunkel, B. N., Dahlbeck, D., Brown, K. L., Schmidt, R., Giraudat, J., et al. (1994). RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* 265, 1856–1860.

Bernoux, M., Ve, T., Williams, S., Warren, C., Hatters, D., Valkov, E., et al. (2011). Structural and functional analysis of a plant resistance protein TIR domain reveals interfaces for self-association, signaling, and autoregulation. *Cell Host Microbe* 9, 200–211.

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.

Birker, D., Heidrich, K., Takahara, H., Narusaka, M., Deslandes, L., Narusaka, Y., et al. (2009). A locus conferring resistance to *Colletotrichum higginsianum* is shared by four geographically distinct *Arabidopsis* accessions. *Plant J.* 60, 602–613.

Bonardi, V., Cherkis, K., Nishimura, M. T., and Dangl, J. L. (2012). A new eye on NLR proteins: focused on clarity or diffused by complexity? *Curr. Opin. Immunol.* 24, 41–50.

Bonardi, V., Tang, S., Stallmann, A., Roberts, M., Cherkis, K., and Dangl, J. L. (2011). Expanded functions for a family of plant intracellular immune receptors beyond specific recognition of pathogen effectors. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16463–16468.

Burch-Smith, T. M., Schiff, M., Caplan, J. L., Tsao, J., Czymmek, K., and Dinesh-Kumar, S. P. (2007). A novel role for the TIR domain in association with pathogen-derived elicitors. *PLoS Biol.* 5, e68. doi: 10.1371/journal.pbio.0050068

Caplan, J. L., Mamillapalli, P., Burch-Smith, T. M., Czymmek, K., and Dinesh-Kumar, S. P. (2008). Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell* 132, 449–462.

Cheng, Y. T., Germain, H., Wiermer, M., Bi, D., Xu, F., Garcia, A. V., et al. (2009). Nuclear pore complex component MOS7/Nup88 is required for

innate immunity and nuclear accumulation of defense regulators in *Arabidopsis*. *Plant Cell* 21, 2503–2516.

Chung, E. H., da Cunha, L., Wu, A. J., Gao, Z., Cherkis, K., Afzal, A. J., et al. (2011). Specific threonine phosphorylation of a host target by two unrelated type III effectors activates a host innate immune receptor in plants. *Cell Host Microbe* 9, 125–136.

Coll, N. S., Vercammen, D., Smidler, A., Clover, C., Van Breusegem, F., Dangl, J. L., et al. (2010). *Arabidopsis* type I metacaspases control cell death. *Science* 330, 1393–1397.

Dangl, J. L., and Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411, 826–833.

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.

DeYoung, B. J., Qi, D., Kim, S. H., Burke, T. P., and Innes, R. W. (2012). Activation of a plant nucleotide binding-leucine rich repeat disease resistance protein by a modified self protein. *Cell. Microbiol.* 14, 1071–1084.

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.

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

Elmore, J. M., Liu, J., Smith, B., Phinney, B., and Coaker, G. (2012). Quantitative proteomics reveals dynamic changes in the plasma membrane during *Arabidopsis* immune signaling. *Mol. Cell. Proteomics* 11, M111.014555.

Franchi, L., Munoz-Planillo, R., and Nunez, G. (2012). Sensing and reacting to microbes through the inflammasomes. *Nat. Immunol.* 13, 325–332.

Fridh, V., and Rittinger, K. (2012). The tandem CARDs of NOD2: intramolecular interactions and recognition of RIP2. *PLoS ONE* 7, e34375. doi: 10.1371/journal.pone.0034375

Gabriels, S. H., Vossen, J. H., Ekengren, S. K., van Ooijen, G., Abd-El-Haliem, A. M., van den Berg, G. C., et al. (2007). An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins. *Plant J.* 50, 14–28.

Gimenez-Ibanez, S., Ntoukakis, V., and Rathjen, J. P. (2009). The LysM receptor kinase CERK1 mediates bacterial perception in *Arabidopsis*. *Plant Signal. Behav.* 4, 539–541.

Gohre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., et al. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr. Biol.* 18, 1824–1832.

Gutierrez, J. R., Balmuth, A. L., Ntoukakis, V., Mucyn, T. S., Gimenez-Ibanez, S., Jones, A. M., et al. (2010). Prf immune complexes of tomato are oligomeric and contain multiple Pto-like kinases that diversify effector recognition. *Plant J.* 61, 507–518.

Hayashi, N., Inoue, H., Kato, T., Funao, T., Shirota, M., Shimizu, T., et al. (2010). Durable panicle blast-resistance gene Pb1 encodes an atypical CC–NBS–LRR protein and was generated by acquiring a promoter through local genome duplication. *Plant J.* 64, 498–510.

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.

Hu, Y., Ding, L., Spencer, D. M., and Nunez, G. (1998). WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. *J. Biol. Chem.* 273, 33489–33494.

Inohara, N., Koseki, T., Chen, S., Benedict, M. A., and Nunez, G. (1999). Identification of regulatory and catalytic domains in the apoptosis nuclease DFF40/CAD. *J. Biol. Chem.* 274, 270–274.

Inohara, N., and Nunez, G. (2003). NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol.* 3, 371–382.

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

Joshi, H. J., Hirsch-Hoffmann, M., Baerenfaller, K., Gruissem, W., Baginsky, S., Schmidt, R., et al. (2011). MASCP Gator: an aggregation portal for the visualization of *Arabidopsis* proteomics data. *Plant Physiol.* 155, 259–270.

Kanzaki, H., Yoshida, K., Saitoh, H., Fujisaki, K., Hirabuchi, A., Allaux, L., et al. (2012). Arms race co-evolution of *Magnaporthe oryzae* AVR-Pik and rice Pik genes driven by their physical interactions. *Plant J.* doi: 10.1111/j.1365-313X.2012.05110.x [Epub ahead of print].

Kim, H. S., Desveaux, D., Singer, A. U., Patel, P., Sondek, J., and Dangl, J. L. (2005). The *Pseudomonas syringae* effector AvrRpt2 cleaves its C-terminally acylated target, RIN4, from *Arabidopsis* membranes to block RPM1 activation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6496–6501.

Kobayashi, K., Inohara, N., Hernandez, L. D., Galan, J. E., Nunez, G., Janeway, C. A., et al. (2002). RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 416, 194–199.

Kofoed, E. M., and Vance, R. E. (2011). Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477, 592–595.

Krasileva, K. V., Dahlbeck, D., and Staskawicz, B. J. (2010). Activation of an *Arabidopsis* resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. *Plant Cell* 22, 2444–2458.

Leister, R. T., Dahlbeck, D., Day, B., Li, Y., Chesnokova, O., and Staskawicz, B. J. (2005). Molecular genetic evidence for the role of SGT1 in the intramolecular complementation of Bs2 protein activity in *Nicotiana benthamiana*. *Plant Cell* 17, 1268–1278.

Liu, J., Elmore, J. M., Fuglsang, A. T., Palmgren, M. G., Staskawicz, B. J., and Coaker, G. (2009). RIN4 functions with plasma membrane H+-ATPases to regulate stomatal apertures during pathogen attack. *PLoS Biol.* 7, e1000139. doi: 10.1371/journal.pbio.1000139

Liu, J., Elmore, J. M., Lin, Z. J., and Coaker, G. (2011). A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. *Cell Host Microbe* 9, 137–146.

Maekawa, T., Cheng, W., Spiridon, L. N., Toller, A., Lukasik, E., Saijo, Y., et al. (2011). Coiled-coil domain-dependent homodimerization of intracellular barley immune receptors defines a minimal functional module for triggering cell death. *Cell Host Microbe* 9, 187–199.

Marquenet, E., and Richet, E. (2007). How integration of positive and negative regulatory signals by a STAND signaling protein depends on ATP hydrolysis. *Mol. Cell* 28, 187–199.

Mestre, P., and Baulcombe, D. C. (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. *Plant Cell* 18, 491–501.

Meyers, B. C., Kozik, A., Griego, A., Kuang, H., and Michelmore, R. W. (2003). Genome-wide analysis of NBS–LRR-encoding genes in *Arabidopsis*. *Plant Cell* 15, 809–834.

Mindrinos, M., Katagiri, F., Yu, G. L., and Ausubel, F. M. (1994). The *A. thaliana* disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* 78, 1089–1099.

Miyawaki, A. (2011). Proteins on the move: insights gained from fluorescent protein technologies. *Nat. Rev. Mol. Cell Biol.* 12, 656–668.

Moffett, P., Farnham, G., Peart, J., and Baulcombe, D. C. (2002). Interaction between domains of a plant NBS–LRR protein in disease resistance-related cell death. *EMBO J.* 21, 4511–4519.

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15, 349–357.

Mucyn, T. S., Clemente, A., Andriotis, V. M., Balmuth, A. L., Oldroyd, G. E., Staskawicz, B. J., et al. (2006). The tomato NBARC–LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. *Plant Cell* 18, 2792–2806.

Mucyn, T. S., Wu, A. J., Balmuth, A. L., Arasteh, J. M., and Rathjen, J. P. (2009). Regulation of tomato Prf by Pto-like protein kinases. *Mol. Plant Microbe Interact.* 22, 391–401.

Mukhtar, M. S., Carvunis, A. R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., et al. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333, 596–601.

Nakagami, H., Sugiyama, N., Mochida, K., Daudi, A., Yoshida, Y., Toyoda, T., et al. (2010). Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants. *Plant Physiol.* 153, 1161–1174.

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.

Ntoukakis, V., Mucyn, T. S., Gimenez-Ibanez, S., Chapman, H. C., Gutierrez, J. R., Balmuth, A. L., et al. (2009). Host inhibition of a bacterial virulence effector triggers immunity to infection. *Science* 324, 784–787.

Okuyama, Y., Kanzaki, H., Abe, A., Yoshida, K., Tamiru, M., Saitoh, H., et al. (2011). A multifaceted genomics approach allows the isolation of the rice Pia-blast resistance gene consisting of two adjacent NBS–LRR protein genes. *Plant J.* 66, 467–479.

Patel, V. J., Thalassinos, K., Slade, S. E., Connolly, J. B., Crombie, A., Murrell, J. C., et al. (2009). A comparison of labeling and label-free mass spectrometry-based proteomics approaches. *J. Proteome Res.* 8, 3752–3759.

Peart, J. R., Mestre, P., Lu, R., Malcuit, I., and Baulcombe, D. C. (2005). NRG1, a CC–NB–LRR protein, together with N, a TIR–NB–LRR protein, mediates resistance against tobacco mosaic virus. *Curr. Biol.* 15, 968–973.

Qi, D., DeYoung, B. J., and Innes, R. W. (2012). Structure–function analysis of the coiled-coil and leucine-rich repeat domains of the RPS5 disease resistance protein. *Plant Physiol.* 158, 1819–1832.

Qi, Y., and Katagiri, F. (2011). Purification of resistance protein complexes using a biotinylated affinity (HPB) tag. *Methods Mol. Biol.* 712, 21–30.

Qi, Y., Tsuda, K., Glazebrook, J., and Katagiri, F. (2011a). Physical association of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) immune receptors in *Arabidopsis*. *Mol. Plant Pathol.* 12, 702–708.

Qi, Y., Tsuda, K., Nguyen le, V., Wang, X., Lin, J., Murphy, A. S., et al. (2011b). Physical association of *Arabidopsis* hypersensitive induced reaction proteins (HIRs) with the immune receptor RPS2. *J. Biol. Chem.* 286, 31297–31307.

Rairdan, G. J., Collier, S. M., Sacco, M. A., Baldwin, T. T., Boettrich, T., and Moffett, P. (2008). The coiled-coil and nucleotide binding domains of the Potato Rx disease resistance protein function in pathogen recognition and signaling. *Plant Cell* 20, 739–751.

Rathinam, V. A., Vanaja, S. K., and Fitzgerald, K. A. (2012). Regulation of inflammasome signaling. *Nat. Immunol.* 13, 333–332.

Riedl, S. J., Li, W., Chao, Y., Schwarzenbacher, R., and Shi, Y. (2005). Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* 434, 926–933.

Rosebrock, T. R., Zeng, L., Brady, J. J., Abramovitch, R. B., Xiao, F., and Martin, G. B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. *Nature* 448, 370–374.

Schmidt, A., Claassen, M., and Aebersold, R. (2009). Directed mass spectrometry: towards hypothesis-driven proteomics. *Curr. Opin. Chem. Biol.* 13, 510–517.

Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J. E., and Innes, R. W. (2003). Cleavage of Arabidopsis PBS1 by a bacterial type III effector. *Science* 301, 1230–1233.

Shen, Q. H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., et al. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* 315, 1098–1103.

Slootweg, E., Roosien, J., Spiridon, L. N., Petrescu, A. J., Tameling, W., Joosten, M., et al. (2010). Nucleocytoplasmic distribution is required for activation of resistance by the potato NB–LRR receptor Rx1 and is balanced by its functional domains. *Plant Cell* 22, 4195–4215.

Sohn, K. H., Hughes, R. K., Piquerez, S. J., Jones, J. D., and Banfield, M. J. (2012). Distinct regions of the *Pseudomonas syringae* coiled-coil effector AvrRps4 are required for activation of immunity. *Proc. Natl. Acad. Sci. U.S.A.* 109, 16371–16376.

Takken, F. L., Albrecht, M., and Tameling, W. I. (2006). Resistance proteins: molecular switches of plant defence. *Curr. Opin. Plant Biol.* 9, 383–390.

Takken, F. L., and Tameling, W. I. (2009). To nibble at plant resistance proteins. *Science* 324, 744–746.

Tameling, W. I., Nooijen, C., Ludwig, N., Boter, M., Slootweg, E., Goverse, A., et al. (2010). RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the Solanaceae, thereby dictating Rx function. *Plant Cell* 22, 4176–4194.

Tasset, C., Bernoux, M., Jauneau, A., Pouzet, C., Briere, C., Kieffer-Jacquinod, S., et al. (2010). Autoacetylation of the *Ralstonia solanacearum* effector PopP2 targets a lysine residue essential for RRS1-R-mediated immunity in *Arabidopsis*. *PLoS Pathog.* 6, e1001202. doi: 10.1371/journal.ppat.1001202

Van der Biezen, E. A., and Jones, J. D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* 23, 454–456.

van Ooijen, G., Mayr, G., Albrecht, M., Cornelissen, B. J., and Takken, F. L. (2008). Transcomplementation, but not physical association of the CC–NB–ARC and LRR domains of tomato R protein Mi-1.2 is altered by mutations in the ARC2 subdomain. *Mol. Plant* 1, 401–410.

Wang, C. I., Guncar, G., Forwood, J. K., Teh, T., Catanzariti, A. M., Lawrence, G. J., et al. (2007). Crystal structures of flax rust avirulence proteins AvrL567-A and -D reveal details of the structural basis for flax disease resistance specificity. *Plant Cell* 19, 2898–2912.

Wirthmueller, L., Zhang, Y., Jones, J. D., and Parker, J. E. (2007). Nuclear accumulation of the *Arabidopsis* immune receptor RPS4 is necessary for triggering EDS1-dependent defense. *Curr. Biol.* 17, 2023–2029.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., et al. (2008). *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Curr. Biol.* 18, 74–80.

Xing, W., Zou, Y., Liu, Q., Liu, J., Luo, X., Huang, Q., et al. (2007). The structural basis for activation of plant immunity by bacterial effector protein AvrPto. *Nature* 449, 243–247.

Zhao, Y., Yang, J., Shi, J., Gong, Y. N., Lu, Q., Xu, H., et al. (2011). The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 477, 596–600.

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: 02 September 2012; paper pending published: 20 September 2012; accepted: 08 October 2012; published online: 23 October 2012.

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

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 03 August 2012
doi: 10.3389/fpls.2012.00177

Protein phosphorylation in plant immunity: insights into the regulation of pattern recognition receptor-mediated signaling

Chang-Jin Park, Daniel F. Caddell and Pamela C. Ronald*

Department of Plant Pathology and the Genome Center, University of California at Davis, Davis, CA, USA

Edited by:
Vardis Ntoukakis, University of Warwick, UK

Reviewed by:
Christof Rampitsch, Agriculture and Agri-Food Canada, Canada
Sixue Chen, University of Florida, USA

****Correspondence:***
Pamela C. Ronald, Department of Plant Pathology and the Genome Center, University of California at Davis, One Shields Avenue, Davis, CA 95616, USA.
e-mail: pcronald@ucdavis.edu

Plants are continuously challenged by pathogens including viruses, bacteria, and fungi. The plant immune system recognizes invading pathogens and responds by activating an immune response. These responses occur rapidly and often involve post-translational modifications (PTMs) within the proteome. Protein phosphorylation is a common and intensively studied form of these PTMs and regulates many plant processes including plant growth, development, and immunity. Most well-characterized pattern recognition receptors (PRRs), including *Xanthomonas* resistance 21, flagellin sensitive 2, and elongation factor-Tu receptor, possess intrinsic protein kinase activity and regulate downstream signaling through phosphorylation events. Here, we focus on the phosphorylation events of plant PRRs that play important roles in the immune response. We also discuss the role of phosphorylation in regulating mitogen-associated protein kinase cascades and transcription factors in plant immune signaling.

Keywords: EFR, FLS2, pattern recognition receptor, plant immunity, post-translational modifications, protein phosphorylation, XA21

INTRODUCTION

Proteins can undergo various post-translational modifications (PTMs) that affect their conformation, activity, stability, and localization. These PTMs, which are often reversible, are highly specific regulators of many cellular processes (Jensen, 2004). Currently, more than 300 types of PTMs have been described including ubiquitination, sumoylation, sulfation, glycosylation, and phosphorylation (Stulemeijer and Joosten, 2008; Ghelis, 2011). Phosphorylation is one of the most predominant PTMs and one-third of all eukaryotic proteins are thought to be phosphorylated (Olsen et al., 2006). Protein phosphorylation in eukaryotes predominantly occurs on serine (Ser) and threonine (Thr) residues, whereas phosphorylation on tyrosine (Tyr) residues is much less abundant (de la Fuente van Bentem and Hirt, 2009). Based on a recent large-scale phosphorylation study, the relative abundances of pSer, pThr, and pTyr were estimated to be 82.7, 13.1, and 4.2% in *Arabidopsis* and 84.8, 12.3, and 2.9% in rice (Sugiyama et al., 2008; Nakagami et al., 2010). Phosphorylation occurring on unusual residues such as histidine, lysine, and arginine (Besant and Attwood, 2005; Ciesla et al., 2011) will not be reviewed, because their involvement in plant immunity has not yet been elucidated.

A large body of evidence demonstrates that phosphorylation is essential for immune responses in animals and plants. For example, in animals, nearly 7,000 phosphorylation sites on more than 1,800 phosphoproteins were identified in response to lipopolysaccharide activation (Weintz et al., 2010). In *Arabidopsis*, more than 1,170 phosphopeptides from 472 phosphoproteins were identified after treatments with flg22 or xylanase, both of which elicit immune responses in *Arabidopsis* cell cultures (Benschop et al., 2007). These results indicate that many proteins are differentially phosphorylated and that the phosphorylation events are essential to both animal and plant immune responses. In this review, we focus primarily on phosphorylation events mediated by plant pattern recognition receptors (PRRs) that play important roles in the immune response.

PATTERN RECOGNITION RECEPTORS IN RICE AND *ARABIDOPSIS*

Plant innate immunity is controlled by a set of defined receptors referred to as PRRs. A more detailed description of PRRs can be found in recent reviews (Ronald and Beutler, 2010; Schwessinger and Ronald, 2012). In general, recognition of conserved microbial signatures (also called pathogen-associated molecular patterns, PAMP) by PRRs triggers mitogen-associated protein kinase (MAPK) activation, production of reactive oxygen species (ROS), Ca^{2+} burst, transcriptional reprogramming, hormone biosynthesis, and deposition of callose in the cell wall (Nurnberger et al., 2004; Ronald and Beutler, 2010; Segonzac and Zipfel, 2011).

The rice PRR, *Xanthomonas* resistance 21 (XA21), recognizes a conserved sulfated peptide called $AxY^{S}22$, derived from the *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) protein Ax21 (activator of XA21-mediated immunity; Lee et al., 2009). In *Arabidopsis*, two additional plant PRRs have been well-characterized. These are flagellin sensitive 2 (FLS2) and elongation factor (EF)-Tu receptor (EFR), which recognize the flg22 peptide from flagellated bacteria and the EF-Tu-derived peptide elf18, respectively (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). These PRRs consist of an extracellular leucine-rich repeat (LRR) domain, a transmembrane (TM) domain, a juxtamembrane

(JM) domain, and an intracellular non-arginine–aspartate (non-RD) kinase domain (**Figure 1**; Dardick and Ronald, 2006; Schwessinger and Ronald, 2012).

Non-RD kinases typically carry a cysteine (C), or glycine (G) before the conserved catalytic aspartate (D) residue. All plant receptor kinases (RKs) characterized to date that carry the non-RD kinase motif are involved in recognition of conserved microbial signatures (Schwessinger and Ronald, 2012). In contrast, the larger group of RD kinases have an arginine (R) immediately preceding the conserved catalytic aspartate (D). RD kinases are known to perform more diverse functions and are often associated with developmental processes. RD kinases also work in partnership with non-RD kinases to transduce immune responses. In *Arabidopsis*, brassinosteroid insensitive 1 (BRI1)-associated kinase 1 (BAK1), an RD kinase, was initially identified as a positive regulator of brassinosteroid responses. BAK1 forms an *in vivo* ligand-dependent complex with the BRI1 receptor (Li et al., 2002; Nam and Li, 2002). Further research revealed that BAK1 is also involved in PRR-mediated signaling, physically interacting with the non-RD kinases FLS2 and EFR (Chinchilla et al., 2007, 2009; Schwessinger et al., 2011). BAK1 null mutants are compromised in their responsiveness to several other conserved microbial signatures including HrpZ (hypersensitive response and pathogenicity Z), lipopolysaccharides, and peptidoglycans (Heese et al., 2007; Shan et al., 2008). The rice ortholog of BAK1, XA21-associated kinase 1 (XAK1), is required for XA21-mediated immunity (Chen et al., unpublished). These results demonstrate that PRRs utilize coregulatory receptors carrying RD kinases as signaling partners to transduce the immune response.

PHOSPHORYLATIONS OF PATTERN RECOGNITION RECEPTORS

In accordance with an essential role of phosphorylation in immune signaling, phosphorylation of FLS2 is the first step in the FLS2-mediated intracellular signaling events (Boller and Felix, 2009). *De novo* phosphorylation of a FLS2/BAK1 complex is clearly detectable in cells 15 s after the addition of flg22 using *in vivo* labeling with short pulses of [^{33}P]orthophosphate (Schulze et al., 2010). Treatment with protein kinase inhibitors is able to block a broad spectrum of early defense responses (Lecourieux et al., 2002; Navazio et al., 2002; Kadota et al., 2004).

In animals, signal transduction is often regulated by phosphorylation of residues in the JM domain of RKs (Aifa et al., 2006; Thiel and Carpenter, 2007). It is now becoming clear that plant PRRs, at least XA21 and FLS2, are also phosphorylated on residues in their JM domains (**Figure 1**; **Table 1**). Targeted mutagenesis of the XA21 JM domain indicated that amino acids Ser^{686}, Thr^{688}, and Ser^{689} are autophosphorylated and required to maintain XA21 protein stability (Xu et al., 2006). Transgenic rice carrying XA21 mutants with alanine replacement of these three sites display partially compromised resistance compared to wildtype XA21 plants (Xu et al., 2006). Thr^{705} in the XA21 JM domain is also an important phosphorylation site and also affects the autophosphorylation activity of XA21 (Chen et al., 2010b). The XA21 mutant derivatives, XA21^{T705A} and XA21^{T705E}, are both unable to transduce the XA21-mediated immune response. The importance of the JM domain in XA21-mediated immunity was also demonstrated through isolation of XA21-binding proteins (XBs). For example, the protein phosphatase 2C XB15

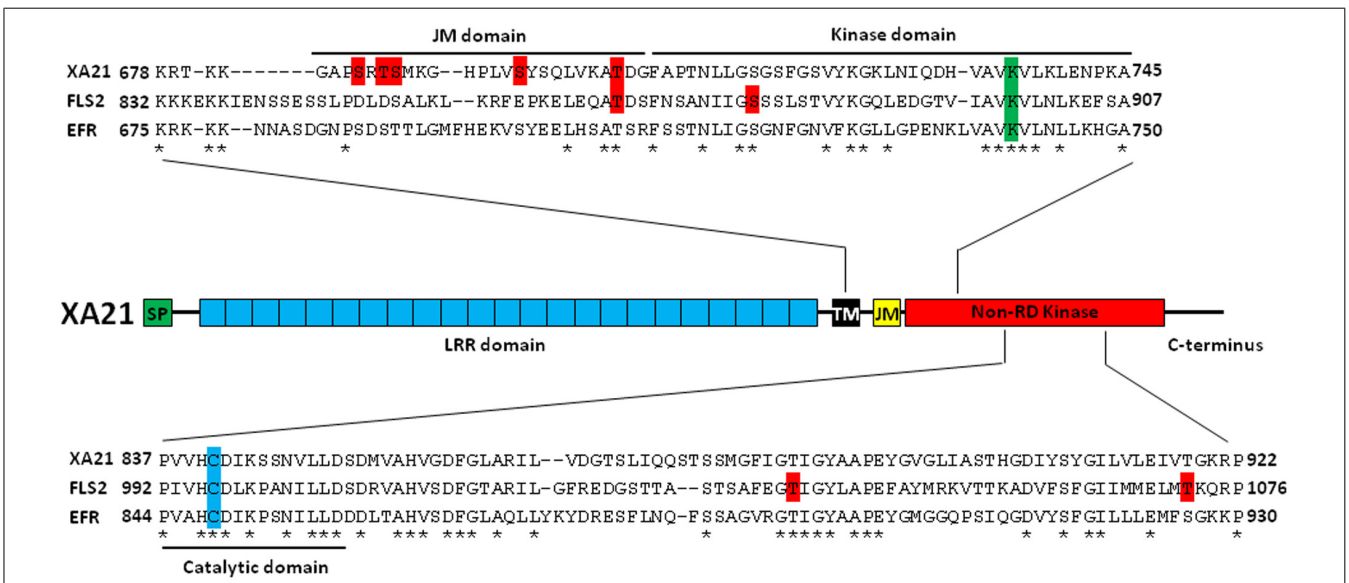


FIGURE 1 | Characterized Ser/Thr residues of pattern recognition receptors in plants. *Top*: Identified and proposed autophosphorylation sites on rice XA21 and *Arabidopsis* FLS2 and EFR are highlighted in red. The conserved lysine that is essential for autophosphorylation is highlighted in green. The JM, kinase, and catalytic domains are indicated by black brackets. *Center*: The domain structure of rice XA21. *Bottom*: Alignment of the catalytic domains of XA21, FLS2, and EFR. The cysteine that replaces the R in these non-RD kinases is highlighted in blue. Putative autophosphorylation sites of FLS2 are highlighted in red. Amino acids that are conserved between XA21, FLS2, and EFR are marked as "*". SP, signal peptide; LRR domain, 23 leucine-rich repeats domain; TM, transmembrane domain; JM, juxtamembrane domain; non-RD kinase, non-arginine–aspartate kinase; XA21, rice *Xanthomonas* resistance 21; FLS2, *Arabidopsis* flagellin sensitive 2; EFR, *Arabidopsis* elongation factor-Tu receptor.

Table 1 | Summary of rice and *Arabidopsis* PRRs.

Organism	PRR	PRR class	Phosphorylation site	Ligand	Interacting protein	Protein class
Rice	XA21	LRR RK, non-RD kinase	S686[1]	Ax21	XAK1[4]	LRR RK
			T688[1]	(AxY^S22)[3]	XB3[5]	E3 ubiquitin ligase
			S689[1]		XB10[6]	WRKY transcription factor
			T705[2]		XB15[7]	Protein phosphatase 2C
					XB24[8]	ATPase
					ROX1[9]	Thiamine pyrophosphokinase
					ROX2[9]	NOL1/NOP2/sun protein
	CEBiP	LysM	NA	Chitin oligosaccharide[10]	OsCERK1[11,12]	LysM RK, RD kinase
Arabidopsis	FLS2	LRR RK, non-RD kinase	T867[13]	Flagellin	BAK1[15]	LRR RK
			S878[13]	(flg22)[14]	BIK1[16]	Cytoplasmic kinase
			T1040[13]		KAPP[17]	Protein phosphatase 2C
			T1072[13]		BKK1, SERK1, SERK2[18]	LRR RK
					PUB12, PUB13[19]	E3 ubiquitin ligase
					SCD1[20,21]	DENN domain
					ACA8[22]	Calcium ATPase
	EFR	LRR RK, non-RD kinase	NA	Elongation factor-Tu (elf18)[23]	BAK1[15]	LRR RK
					BIK1, PBL1[16,24]	Cytoplasmic kinase
					BKK1, SERK1, SERK2[18]	LRR RK
					SCD1[20,21]	DENN domain

NA, not available; ACA, autoinhibited Ca^{2+}-ATPase; BIK, Botrytis-induced kinase; BKK, BAK1-like kinase; NOL/NOP, nucleolar protein; CERK, chitin elicitor receptor kinase; PBL, PBS1-like; PUB, plant U-box; ROX, regulator of XA21; SCD, stomatal cytokinesis-defective; SERK, somatic-embryogenesis receptor-like kinase; SUN, Sad1-UNC-84 homology.

References: [1] *Xu et al. (2006)*, [2] *Chen et al. (2010b)*, [3] *Lee et al. (2009)*, [4] *Chen et al., unpublished*, [5] *Wang et al. (2006)*, [6] *Peng et al. (2008)*, [7] *Park et al. (2008)*, [8] *Chen et al. (2010c)*, [9] *Lee et al. (2011)*, [10] *Kaku et al. (2006)*, [11] *Shimizu et al. (2010)*, [12] *Schwessinger and Ronald (2012)*, [13] *Robatzek et al. (2006)*, [14] *Chinchilla et al. (2006)*, [15] *Chinchilla et al. (2007)*, [16] *Lu et al. (2010)*, [17] *Gomez-Gomez et al. (2001)*, [18] *Roux et al. (2011)*, [19] *Lu et al. (2011)*, [20] *Korasick et al. (2010)*, [21] *Monaghan and Zipfel (2012)*, [22] *Frei Dit Frey et al. (2012)*, [23] *Zipfel et al. (2006)*, *and* [24] *Zhang et al. (2010)*.

no longer interacts with $XA21^{S697A}$, indicating that Ser697 in the JM domain is critical for interaction with XB15 (Park et al., 2008). Autophosphorylated XA21 is dephosphorylated by XB15 *in vitro*, suggesting that the function of XB15 is to attenuate the XA21-mediated innate immune response. The ATPase XB24 also associates with the XA21 JM domain and uses ATP to promote phosphorylation of certain Ser/Thr sites on XA21, keeping the XA21 protein in an inactive state. Upon recognition of sulfated Ax21, the XA21 kinase disassociates from XB24 and is activated, triggering downstream defense responses (Chen et al., 2010c; **Figure 2**).

In *Arabidopsis*, the FLS2 JM residue Thr^{867} appears to be analogous to Thr^{705} in XA21 (**Figure 1**; **Table 1**; Chen et al., 2010b) and is also essential for the function of FLS2 (Robatzek et al., 2006). $FLS2^{T867V}$ inhibits FLS2 internalization and response to flg22, indicating that both processes are intimately connected (Robatzek et al., 2006). Although the $FLS2^{T867V}$ mutation had no effect on flg22-binding, $FLS2^{T867V}$ mutant lines were insensitive to flg22 and displayed an enhanced disease susceptibility phenotype when challenged with pathogenic *Pseudomonas syringae*. Microscopic analysis of transgenic plants expressing $FLS2^{T867V}$-GFP showed normal cell membrane localization of the mutant FLS2 protein. However, $FLS2^{T867V}$ endocytosis is strongly reduced after flg22 treatment, suggesting that phosphorylation of $FLS2^{T867}$ plays an important role in endocytosis. Further study is needed to determine if Thr^{867} of FLS2 is essential for FLS2 autophosphorylation in *Arabidopsis* and if Thr^{705} of XA21 is critical for XA21 endocytosis in rice.

Four FLS2 amino acids were shown to be critical to FLS2 function using site-directed mutagenesis. Seedling growth of *Arabidopsis* transgenic lines expressing $FLS2^{T867V}$, $FLS2^{T1040A}$, $FLS2^{S878A}$, and $FLS2^{T1072A}$ were inhibited by flg22 treatment. Three of these mutations ($FLS2^{T867V}$, $FLS2^{T1040A}$, and $FLS2^{T1072A}$) also abolished flg22-induced generation of ROS (Robatzek et al., 2006). It is not known if these sites are phosphorylated or if they are required for kinase activity.

In all protein kinases, it is well known that a conserved lysine residue is responsible for a phosphotransfer reaction (Carrera et al., 1993). The importance of this lysine for kinase function has been demonstrated for plant PRRs. For example, the Lys^{736} residue inside the XA21 kinase domain is essential for XA21 autophosphorylation (Liu et al., 2002). However, although catalytic activity of XA21 is essential for full resistance levels, the catalytically impaired XA21 mutant maintains partial resistance activity (Andaya and

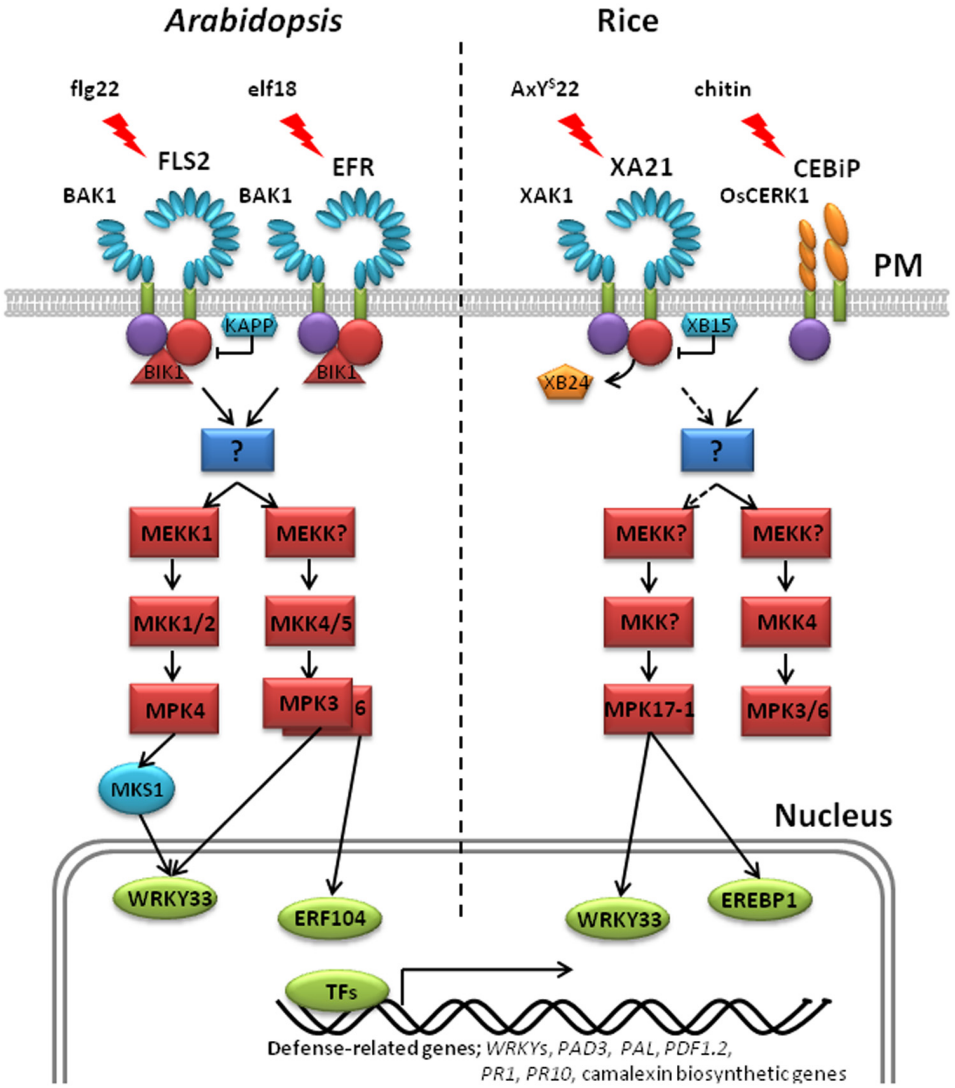


FIGURE 2 | Models for pattern recognition receptor-mediated phosphorylation pathways in *Arabidopsis* and rice. *Left*: *Arabidopsis* FLS2 and BAK1 associate with the membrane-associated cytoplasmic kinase *Botrytis*-induced kinase 1 (BIK1) *in vitro* and *in vivo* (Lu et al., 2010). In the resting state, *Arabidopsis* FLS2 interacts with BIK1. Flg22 perception induces FLS2 and BAK1 association and phosphorylation. Activated BAK1 phosphorylates BIK1, which in turn transphosphorylates the FLS2/BAK1 complex. Phosphorylated BIK1 is released from the FLS2/BAK1 complex to activate downstream intracellular signaling. No direct phosphorylation targets of FLS2 have yet been identified. At least two MAPK cascades are initiated downstream of activated FLS2, leading to the phosphorylation of the adaptor protein MKS1 and the transcription factors, AtWRKY33 and ERF104. Kinase-associated protein phosphatase (KAPP), a PP2C, blocks the activated FLS2 signaling and attenuates the downstream immune response. EFR-mediated immunity is believed to trigger the same MAPK cascades as FLS2. *Right*: In the resting state, rice XA21 forms an *in vivo* complex with the ATPase, XB24, and the XA21-associated kinase, XAK1. Association of XAK1 and XA21 requires the XA21 JM and kinase domains. Binding of AxY^{S}22 to XA21 induces dissociation of XA21 from XB24 and activates XA21, triggering autophosphorylation. Activated XA21 likely activates a MAPK cascade that includes MPK17-1, leading to the phosphorylation of the transcription factors, OsWRKY33 and OsEREBP1. Recruitment of XB15 to the XA21 JM domain and subsequent dephosphorylation of phosphorylated residue(s) attenuates XA21 signaling. Cleavage of XA21 and translocalization of the intracellular kinase domain to the nucleus is required for the XA21-mediated immune response (Park and Ronald, 2012). Unlike other well-characterized PRRs, the rice receptors CEBiP and OsCERK1 contain extracellular LysM (lysine motif) domains in place of LRR domains (Kaku et al., 2006; Shimizu et al., 2010). Upon chitin perception, the CEBiP and OsCERK1 complex activates MAPK cascades. In *Arabidopsis* and rice, phosphorylation of transcription factors leads to large-scale transcriptional reprogramming, including the activation of *WRKY*s, *PAD3*, *PAL*, *PR*s, *PDF1.2*, and camalexin biosynthetic genes.

Ronald, 2003). The partial resistance is comparable to that of transgenic lines expressing XA21D, an XA21 family member consisting of an LRR domain but lacking a kinase domain, indicating that XA21 catalytic activity is not absolutely required for function. In *Arabidopsis*, a mutation in Lys^{898} of FLS2, which is analogous to Lys^{736} in XA21, abolishes MPK3 and MPK6 activation by flg22 when transiently overexpressed in protoplasts (Asai et al., 2002). Similarly, a kinase inactive mutation at Lys^{741} of EFR is unable to confer elf18-triggered ROS burst when transiently expressed in *Nicotiana benthamiana* (Schwessinger et al., 2011).

MITOGEN-ACTIVATED PROTEIN KINASES SERVE AS INTERNODES IN PRR-MEDIATED IMMUNITY

Mitogen-associated protein kinase cascades are important for transmitting signals generated by receptors into cellular responses. Multiple studies support central roles for MAPK cascades in the immunity of *Arabidopsis*, parsley, tobacco, tomato, and rice (Frye et al., 2001; Zhang and Klessig, 2001; Cardinale et al., 2002; del Pozo et al., 2004; Pitzschke et al., 2009; Jung et al., 2010). Generally, MAP kinase kinase kinases (MAP3Ks, also called MEKKs) are activated by RKs. MAP3Ks activate downstream MAP kinase kinases (MAP2Ks, also called MKKs or MEKs) that in turn activate MAPKs (also called MPKs). MAPKs then target various proteins, which include other kinases, enzymes, and transcription factors (Khokhlatchev et al., 1998; Rodriguez et al., 2010). Genome-sequencing of *Arabidopsis* and rice have revealed the existence of approximately 60 MAP3Ks, 10 MAP2Ks, and 20 MAPKs in *Arabidopsis* (Group, 2002) and at least 75 MAP3Ks, 8 MAP2Ks, and 17 MAPKs in rice (Reyna and Yang, 2006; Rao et al., 2010).

In *Arabidopsis*, many studies have shown that activated FLS2 triggers MAPK signaling cascades (Asai et al., 2002; Chinchilla et al., 2007; Pitzschke et al., 2009; **Figure 2**). Initially, MEKK1 activates MPK4 which was previously shown to negatively regulate the defense response (Andreasson et al., 2005). At the MAPK kinase level, flg22-induced activation of MPK3/4/6 is dependent on MKK1/2, while MPK3 and MPK6 are also activated by MKK4 (Meszaros et al., 2006; Gao et al., 2008; Qiu et al., 2008b). Thus, two simultaneous MAPK cascades are postulated. The first consists of an unknown MEKK–MKK4/5–MPK3/6 and acts positively on FLS2-mediated signaling. The other consists of MEKK1–MKK1/2–MPK4 and acts negatively on the pathway (Nicaise et al., 2009). A physical interaction between MEKK1 and FLS2 has not been observed. Therefore, researchers are searching for signaling intermediates that function upstream of MEKK1 that would link FLS2 with the key MAPK cascades.

Elongation factor-Tu receptor-mediated signaling in *Arabidopsis* is thought to utilize a similar signal transduction pathway with FLS2. In-gel assays detect a rapid activation of MAPKs in EFR-mediated immune response after elf18 treatment (Zipfel et al., 2006). Treatment with both flg22 and elf18 at the same time induces the same MAPKs without an additive effect, indicating that these kinases belong to the same cellular pool of enzymes. These results suggest that PRR-mediated signaling induced by the two conserved microbial signatures, elf18 and flg22, converge at a step upstream of these kinases.

The role of MAPK cascades in PRR-mediated immunity has also been investigated in rice. For example, OsMPK3 [previously named OsBIMK1 (Song and Goodman, 2002)] and OsMPK17-1 [previously named OsBWMK1 (He et al., 1999)] both interact with XBs, suggesting that these MAP kinases are components of the XA21-mediated signaling pathway (Seo et al., 2011). OsMPK3 suppressing plants display enhanced resistance to *Xoo*, suggesting that it serves as negative regulator in the XA21-mediated response. In contrast, OsMPK17-1 knockouts displayed increased susceptibility to *Xoo*, suggesting a positive role in XA21-mediated immunity. OsMPK3/6 and OsMKK4 are activated by chitin (Kishi-Kaboshi et al., 2010; Kim et al., 2012).

MAPKs PHOSPHORYLATE TRANSCRIPTION FACTORS

Transcriptional reprogramming of immune responses in the nucleus is regulated by transcription factors including the WRKY and ethylene-responsive factor [ERF, also called ethylene-responsive element binding protein (EREBP)] families (Gutterson and Reuber, 2004; Ishihama and Yoshioka, 2012). In animals, MAPKs are activated and then often translocate to the nucleus where MAPKs will directly or indirectly phosphorylate transcription factors (Harding et al., 2005; Rodriguez et al., 2010). Examples of nuclear localization of MAPKs have been reported in *Arabidopsis* and rice (Cheong et al., 2003; Yoo et al., 2008; Koo et al., 2009). Therefore, WRKY proteins and EREBPs constitute an important link between pathogen-activated MAPK signaling pathways and downstream transcriptional reprogramming.

High-density protein microarrays, employed to identify downstream factors of MAPKs in *Arabidopsis*, revealed that many WRKYs are directly regulated by MAPKs (Popescu et al., 2009). For example, *Arabidopsis* WRKY33 (AtWRKY33) is induced by conserved microbial signatures, such as the oomycete-derived peptide Pep25 (Lippok et al., 2007). Subsequent experiments showed that AtWRKY33 is phosphorylated by MPK3/MPK6 *in vivo* in response to *Botrytis cinerea* infection and by MPK4 at least *in vitro* (Mao et al., 2011). Phosphorylation of AtWRKY33 inhibits the growth of pathogenic fungi and bacteria by promoting the production of camalexin, a major antimicrobial phytoalexin. Mutation of MPK3/MPK6 phosphorylation sites in AtWRKY33 compromises its ability to complement the camalexin induction in the AtWRKY mutant. Another transcription factor, ethylene response factor (ERF104), is directly associated and phosphorylated by MPK6 but not MPK3 (Bethke et al., 2009). Perception of flg22 via FLS2 induces disruption of the MPK6/ERF104 complex, releasing ERF104 to its target promoters including *PDF1.2* (plant defensin 1.2).

There is an increasing body of evidence that suggests MAPKs also regulate transcription factors indirectly. Two WRKY transcription factors AtWRKY25 and AtWRKY33 interact with MPK4 substrate 1 (MKS1) in yeast, suggesting that these WRKYs regulate gene expression downstream of MPK4 (Andreasson et al., 2005). It was later reported that AtWRKY33 also forms an *in vivo* complex with MPK4 and MKS1 (Qiu et al., 2008a). However, although MKS1 is directly associated with AtWRKY33 and is phosphorylated by MPK4, no interaction has been detected between AtWRKY33 and MPK4 (Andreasson et al., 2005; Qiu et al., 2008a). This suggests MPK4 and AtWRKY33 associate indirectly and require the adaptor protein MKS1 for their interaction (Qiu et al., 2008a). Following pathogen perception, the MKS1–AtWRKY33 complex binds the phytoalexin deficient 3 (PAD3) promoter, which promotes camalexin synthesis.

To date, there are only a few reports suggesting that MAPKs phosphorylate rice transcription factors in response to pathogen infection. For example, OsMPK17-1 phosphorylates OsWRKY33 *in vitro*, which binds to the W-box element in the *OsPR1* gene promoter (Koo et al., 2009). OsMPK17-1 also phosphorylates the transcription factor OsEREBP1 *in vitro* (Cheong et al., 2003). Transient co-expression of OsMPK17-1 and OsEREBP1 in *Arabidopsis* protoplasts elevates the expression of the β-glucuronidase reporter gene driven by the ethylene-responsive element GCC box

in several basic PR gene promoters. Thus, OsMPK17-1 is involved in rice defense signal transduction and is responsible for the direct phosphorylation of a transcription factor(s).

Although a role for MAPK-mediated phosphorylation of WRKYs has not been demonstrated for XA21-mediated immunity, several WRKYs interact directly with XA21 in yeast. For example, OsWRKY62, identified as XB10 in a yeast two-hybrid screen using the XA21 intracellular domain as bait, interacts with the XA21 kinase domain in rice protoplasts (Park and Ronald, 2012) and negatively regulates XA21-mediated immunity (Peng et al., 2008). Transgenic rice plants overexpressing OsWRKY62 are compromised in XA21-mediated immunity and are impaired in the activation the defense-related genes *OsPR1* and *OsPR10* (Peng et al., 2008). Additionally, OsWRKY76 was recently shown to negatively regulate XA21-mediated immunity when challenged with *Xoo* (Seo et al., 2011). Although these studies indicate a functional link between OsWRKYs and XA21-mediated immunity, XA21 has not been shown to directly phosphorylate the WRKYs, thus the role of phosphorylation is unknown.

CONCLUSION AND PERSPECTIVES

Recognition of conserved microbial signatures by PRRs is critical to plant survival. PRR activation induces rapid autophosphorylation, leading to phosphorylation of many other proteins. Despite the importance of phosphorylation in PRR-mediated immunity, only a few phosphorylation sites of PRRs have been identified. Those phosphorylation sites were initially found by targeted mutagenesis. Although recent advances in phosphoproteomic analyses using mass spectrometry have greatly expanded our capability to identify phosphopeptides (Benschop et al., 2007; Nuhse et al., 2007; Stulemeijer and Joosten, 2008; Kersten et al., 2009), this approach has not yet lead to the identification of additional *in vivo* PRR phosphosites. This lack of success may be due to the observed rapid endocytosis and/or degradation of PRRs following perception of conserved microbial signatures (Robatzek et al., 2006; Robatzek, 2007; Chen et al., 2010a), which likely serves as a barrier to identifying PRR phosphorylation sites using mass spectrometry. Progress in mass spectrometric technology to enhance sensitivity of detection of low abundance phosphopeptides is needed to overcome this limitation. Once identified, such sites can be confirmed using independent techniques such as immunoblotting with anti-phospho-specific antibodies and *in vivo* genetic studies.

In addition to slow progress in identifying residues phosphorylated on the PRR itself, other proteins that could serve as targets of PRR phosphorylation have not yet been identified. Therefore, there is still a gap in our understanding of how precisely PRRs are able to initiate early signaling events such as activation of MAPKs, a rapid calcium influx and an oxidative burst. To answer these fundamental questions, it will be essential to identify such target proteins and to determine how these proteins regulate downstream events. Phosphoproteomic comparison is one method that can identify proteins that become phosphorylated during PRR-mediated immunity. For example, quantitative phosphoproteomic analyses performed on flg22- or xylanase-treated *Arabidopsis* cells successfully revealed several differentially phosphorylated proteins such as auxin efflux carriers and respiratory burst oxidase protein D (Nuhse et al., 2007; Stulemeijer and Joosten, 2008).

Another important goal is to identify the substrates of MAPKs that are phosphorylated during PRR-mediated immunity. To date, only a few transcription factors have been shown to be phosphorylated by MAPKs during PRR-mediated immune responses. Studies utilizing protein microarrays, protein complex immunoprecipitations, and phosphoproteomic analyses will continue to uncover additional transcription factors and other potential MAPK targets, further contributing to our understanding of the role of phosphorylation in plant immune responses.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Health (NIH, GM55962) and the National Science Foundation (NSF, IOS-0817738).

REFERENCES

Aifa, S., Frikha, F., Miled, N., Johansen, K., Lundstrom, I., and Svensson, S. P. (2006). Phosphorylation of Thr654 but not Thr669 within the juxtamembrane domain of the EGF receptor inhibits calmodulin binding. *Biochem. Biophys. Res. Commun.* 347, 381–387.

Andaya, C. B., and Ronald, P. C. (2003). A catalytically impaired mutant of the rice Xa21 receptor kinase confers partial resistance to *Xanthomonas oryzae* pv. oryzae. *Physiol. Mol. Plant Pathol.* 62, 203–208.

Andreasson, E., Jenkins, T., Brodersen, P., Thorgrimsen, S., Petersen, N. H., Zhu, S., Qiu, J. L., Micheelsen, P., Rocher, A., Petersen, M., Newman, M. A., Bjorn Nielsen, H., Hirt, H., Somssich, I., Mattsson, O., and Mundy, J. (2005). The MAP kinase substrate MKS1 is a regulator of plant defense responses. *EMBO J.* 24, 2579–2589.

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W. L., Gomez-Gomez, L., Boller, T., Ausubel, F. M., and Sheen, J. (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* 415, 977–983.

Benschop, J. J., Mohammed, S., O'Flaherty, M., Heck, A. J., Slijper, M., and Menke, F. L. (2007). Quantitative phosphoproteomics of early elicitor signaling in *Arabidopsis*. *Mol. Cell. Proteomics* 6, 1198–1214.

Besant, P. G., and Attwood, P. V. (2005). Mammalian histidine kinases. *Biochim. Biophys. Acta* 1754, 281–290.

Bethke, G., Unthan, T., Uhrig, J. F., Poschl, Y., Gust, A. A., Scheel, D., and Lee, J. (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.

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.

Cardinale, F., Meskiene, I., Ouaked, F., and Hirt, H. (2002). Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. *Plant Cell* 14, 703–711.

Carrera, A. C., Alexandrov, K., and Roberts, T. M. (1993). The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. *Proc. Natl. Acad. Sci. U.S.A.* 90, 442–446.

Chen, F., Gao, M. J., Miao, Y. S., Yuan, Y. X., Wang, M. Y., Li, Q., Mao, B. Z., Jiang, L. W., and He, Z. H. (2010a). Plasma membrane localization and potential endocytosis of constitutively expressed XA21 proteins in transgenic rice. *Mol. Plant* 3, 917–926.

Chen, X., Chern, M., Canlas, P. E., Jiang, C., Ruan, D., Cao, P., and Ronald, P. C. (2010b). A conserved threonine residue in the juxtamembrane domain of the XA21 pattern recognition receptor is critical for kinase autophosphorylation and XA21-mediated immunity. *J. Biol. Chem.* 285, 10454–10463.

Chen, X., Chern, M., Canlas, P. E., Ruan, D., Jiang, C., and Ronald, P. C. (2010c). An ATPase promotes autophosphorylation of the

pattern recognition receptor XA21 and inhibits XA21-mediated immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8029–8034.

Cheong, Y. H., Moon, B. C., Kim, J. K., Kim, C. Y., Kim, M. C., Kim, I. H., Park, C. Y., Kim, J. C., Park, B. O., Koo, S. C., Yoon, H. W., Chung, W. S., Lim, C. O., Lee, S. Y., and Cho, M. J. (2003). BWMK1, a rice mitogen-activated protein kinase, locates in the nucleus and mediates pathogenesis-related gene expression by activation of a transcription factor. *Plant Physiol.* 132, 1961–1972.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 18, 465–476.

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.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J. D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448, 497–500.

Ciesla, J., Fraczyk, T., and Rode, W. (2011). Phosphorylation of basic amino acid residues in proteins: important but easily missed. *Acta Biochim. Pol.* 58, 137–148.

Dardick, C., and Ronald, P. (2006). Plant and animal pathogen recognition receptors signal through non-RD kinases. *PLoS Pathog.* 2, e2. doi: 10.1371/journal.ppat.0020002.

de la Fuente van Bentem, S., and Hirt, H. (2009). Protein tyrosine phosphorylation in plants: more abundant than expected? *Trends Plant Sci.* 14, 71–76.

del Pozo, O., Pedley, K. F., and Martin, G. B. (2004). MAPKKKalpha is a positive regulator of cell death associated with both plant immunity and disease. *EMBO J.* 23, 3072–3082.

Frei Dit Frey, N., Mbengue, M., Kwaaitaal, M., Nitsch, L., Altenbach, D., Haweker, H., Lozano-Duran, R., Njo, M. F., Beeckman, T., Huettel, B., Borst, J. W., Panstruga, R., and Robatzek, S. (2012). Plasma membrane calcium ATPases are important components of receptor-mediated signaling in plant immune responses and development. *Plant Physiol.* 159, 798–809.

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.

Gao, M., Liu, J., Bi, D., Zhang, Z., Cheng, F., Chen, S., and Zhang, Y. (2008). MEKK1, MKK1/MKK2 and MPK4 function together in a mitogen-activated protein kinase cascade to regulate innate immunity in plants. *Cell Res.* 18, 1190–1198.

Ghelis, T. (2011). Signal processing by protein tyrosine phosphorylation in plants. *Plant Signal. Behav.* 6, 942–951.

Gomez-Gomez, L., Bauer, Z., and Boller, T. (2001). Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in *Arabidopsis*. *Plant Cell* 13, 1155–1163.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5, 1003–1011.

Group, M. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* 7, 301–308.

Gutterson, N., and Reuber, T. L. (2004). Regulation of disease resistance pathways by AP2/ERF transcription factors. *Curr. Opin. Plant Biol.* 7, 465–471.

Harding, A., Tian, T., Westbury, E., Frische, E., and Hancock, J. F. (2005). Subcellular localization determines MAP kinase signal output. *Curr. Biol.* 15, 869–873.

He, C., Fong, S. H., Yang, D., and Wang, G. L. (1999). BWMK1, a novel MAP kinase induced by fungal infection and mechanical wounding in rice. *Mol. Plant Microbe Interact.* 12, 1064–1073.

Heese, A., Hann, D. R., Gimenez-Ibanez, S., Jones, A. M., He, K., Li, J., Schroeder, J. I., Peck, S. C., and Rathjen, J. P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12217–12222.

Ishihama, N., and Yoshioka, H. (2012). Post-translational regulation of WRKY transcription factors in plant immunity. *Curr. Opin. Plant Biol.* (in press). doi: 10.1016/j.pbi.2012.02.003.

Jensen, O. N. (2004). Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. *Curr. Opin. Chem. Biol.* 8, 33–41.

Jung, K. H., Cao, P., Seo, Y. S., Dardick, C., and Ronald, P. C. (2010). The rice kinase phylogenomics database: a guide for systematic analysis of the rice kinase super-family. *Trends Plant Sci.* 15, 595–599.

Kadota, Y., Goh, T., Tomatsu, H., Tamauchi, R., Higashi, K., Muto, S., and Kuchitsu, K. (2004). Cryptogein-induced initial events in tobacco BY-2 cells: pharmacological characterization of molecular relationship among cytosolic Ca(2+) transients, anion efflux and production of reactive oxygen species. *Plant Cell Physiol.* 45, 160–170.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11086–11091.

Kersten, B., Agrawal, G. K., Durek, P., Neigenfind, J., Schulze, W., Walther, D., and Rakwal, R. (2009). Plant phosphoproteomics: an update. *Proteomics* 9, 964–988.

Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M. H. (1998). Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell* 93, 605–615.

Kim, S. H., Oikawa, T., Kyozuka, J., Wong, H. L., Umemura, K., Kishi-Kaboshi, M., Takahashi, A., Kawano, Y., Kawasaki, T., and Shimamoto, K. (2012). The bHLH Rac immunity1 (RAI1) is activated by OsRac1 via OsMAPK3 and OsMAPK6 in rice immunity. *Plant Cell Physiol.* 53, 740–754.

Kishi-Kaboshi, M., Okada, K., Kurimoto, L., Murakami, S., Umezawa, T., Shibuya, N., Yamane, H., Miyao, A., Takatsuji, H., Takahashi, A., and Hirochika, H. (2010). A rice fungal MAMP-responsive MAPK cascade regulates metabolic flow to antimicrobial metabolite synthesis. *Plant J.* 63, 599–612.

Koo, S. C., Moon, B. C., Kim, J. K., Kim, C. Y., Sung, S. J., Kim, M. C., Cho, M. J., and Cheong, Y. H. (2009). OsBWMK1 mediates SA-dependent defense responses by activating the transcription factor OsWRKY33. *Biochem. Biophys. Res. Commun.* 387, 365–370.

Korasick, D. A., Mcmichael, C., Walker, K. A., Anderson, J. C., Bednarek, S. Y., and Heese, A. (2010). Novel functions of stomatal cytokinesis-defective 1 (SCD1) in innate immune responses against bacteria. *J. Biol. Chem.* 285, 23342–23350.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R., and Pugin, A. (2002). Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell* 14, 2627–2641.

Lee, I., Seo, Y. S., Coltrane, D., Hwang, S., Oh, T., Marcotte, E. M., and Ronald, P. C. (2011). Genetic dissection of the biotic stress response using a genome-scale gene network for rice. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18548–18553.

Lee, S. W., Han, S. W., Sririyanum, M., Park, C. J., Seo, Y. S., and Ronald, P. C. (2009). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. *Science* 326, 850–853.

Li, J., Wen, J., Lease, K. A., Doke, J. T., Tax, F. E., and Walker, J. C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* 110, 213–222.

Lippok, B., Birkenbihl, R. P., Rivory, G., Brummer, J., Schmelzer, E., Logemann, E., and Somssich, I. E. (2007). Expression of AtWRKY33 encoding a pathogen- or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements. *Mol. Plant Microbe Interact.* 20, 420–429.

Liu, G. Z., Pi, L. Y., Walker, J. C., Ronald, P. C., and Song, W. Y. (2002). Biochemical characterization of the kinase domain of the rice disease resistance receptor-like kinase XA21. *J. Biol. Chem.* 277, 20264–20269.

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., Heese, A., Devarenne, T. P., He, P., and Shan, L. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* 332, 1439–1442.

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.

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.

Meszaros, T., Helfer, A., Hatzimasoura, E., Magyar, Z., Serazetdinova, L., Rios, G., Bardoczy, V., Teige, M., Koncz, C., Peck, S., and Bogre, L. (2006). The *Arabidopsis* MAP kinase kinase MKK1 participates in defence responses to the bacterial elicitor flagellin. *Plant J.* 48, 485–498.

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15, 1–9.

Nakagami, H., Sugiyama, N., Mochida, K., Daudi, A., Yoshida, Y., Toyoda, T., Tomita, M., Ishihama, Y., and Shirasu, K. (2010). Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants. *Plant Physiol.* 153, 1161–1174.

Nam, K. H., and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* 110, 203–212.

Navazio, L., Moscatiello, R., Bellincampi, D., Baldan, B., Meggio, F., Brini, M., Bowler, C., and Mariani, P. (2002). The role of calcium in oligogalacturonide-activated signalling in soybean cells. *Planta* 215, 596–605.

Nicaise, V., Roux, M., and Zipfel, C. (2009). Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol.* 150, 1638–1647.

Nuhse, T. S., Bottrill, A. R., Jones, A. M., and Peck, S. C. (2007). Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. *Plant J.* 51, 931–940.

Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* 198, 249–266.

Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127, 635–648.

Park, C. J., Peng, Y., Chen, X., Dardick, C., Ruan, D., Bart, R., Canlas, P. E., and Ronald, P. C. (2008). Rice XB15, a protein phosphatase 2C, negatively regulates cell death and XA21-mediated innate immunity. *PLoS Biol.* 6, e231. doi: 10.1371/journal.pbio.0060231.

Park, C. J., and Ronald, P. C. (2012). Cleavage and nuclear localization of the rice XA21 immune receptor. *Nat. Commun.* 3, 920.

Peng, Y., Bartley, L. E., Chen, X., Dardick, C., Chern, M., Ruan, R., Canlas, P. E., and Ronald, P. C. (2008). OsWRKY62 is a negative regulator of basal and Xa21-mediated defense against *Xanthomonas oryzae* pv. oryzae in rice. *Mol. Plant* 1, 446–458.

Pitzschke, A., Schikora, A., and Hirt, H. (2009). MAPK cascade signalling networks in plant defence. *Curr. Opin. Plant Biol.* 12, 421–426.

Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., and Dinesh-Kumar, S. P. (2009). MAPK target networks in *Arabidopsis thaliana* revealed using functional protein microarrays. *Genes Dev.* 23, 80.

Qiu, J. L., Fiil, B. K., Petersen, K., Nielsen, H. B., Botanga, C. J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M. C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K. D., Mattsson, O., Glazebrook, J., Mundy, J., and Petersen, M. (2008a). *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO J.* 27, 2214–2221.

Qiu, J. L., Zhou, L., Yun, B. W., Nielsen, H. B., Fiil, B. K., Petersen, K., Mackinlay, J., Loake, G. J., Mundy, J., and Morris, P. C. (2008b). *Arabidopsis* mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. *Plant Physiol.* 148, 212–222.

Rao, K. P., Richa, T., Kumar, K., Raghuram, B., and Sinha, A. K. (2010). In silico analysis reveals 75 members of mitogen-activated protein kinase kinase kinase gene family in rice. *DNA Res.* 17, 139–153.

Reyna, N. S., and Yang, Y. (2006). Molecular analysis of the rice MAP kinase gene family in relation to *Magnaporthe grisea* infection. *Mol. Plant Microbe Interact.* 19, 530–540.

Robatzek, S. (2007). Vesicle trafficking in plant immune responses. *Cell. Microbiol.* 9, 1–8.

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis. Genes Dev.* 20, 537–542.

Rodriguez, M. C., Petersen, M., and Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. *Annu. Rev. Plant Biol.* 61, 621–649.

Ronald, P. C., and Beutler, B. (2010). Plant and animal sensors of conserved microbial signatures. *Science* 330, 1061–1064.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F. G., Tor, M., De Vries, S., and Zipfel, C. (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.

Schulze, B., Mentzel, T., Jehle, A. K., Mueller, K., Beeler, S., Boller, T., Felix, G., and Chinchilla, D. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *J. Biol. Chem.* 285, 9444–9451.

Schwessinger, B., and Ronald, P. C. (2012). Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* 63, 451–482.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A., and Zipfel, C. (2011). Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet.* 7, e1002046. doi: 10.1371/journal.pgen.1002046.

Segonzac, C., and Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria. *Curr. Opin. Microbiol.* 14, 54–61.

Seo, Y. S., Chern, M., Bartley, L. E., Han, M., Jung, K. H., Lee, I., Walia, H., Richter, T., Xu, X., Cao, P., Bai, W., Ramanan, R., Amonpant, F., Arul, L., Canlas, P. E., Ruan, R., Park, C. J., Chen, X., Hwang, S., Jeon, J. S., and Ronald, P. C. (2011). Towards establishment of a rice stress response interactome. *PLoS Genet.* 7, e1002020. doi: 10.1371/journal.pgen.1002020.

Shan, L., He, P., Li, J., Heese, A., Peck, S. C., Nurnberger, T., Martin, G. B., and Sheen, J. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host Microbe* 4, 17–27.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64, 204–214.

Song, F., and Goodman, R. M. (2002). OsBIMK1, a rice MAP kinase gene involved in disease resistance responses. *Planta* 215, 997–1005.

Stulemeijer, I. J., and Joosten, M. H. (2008). Post-translational modification of host proteins in pathogen-triggered defence signalling in plants. *Mol. Plant Pathol.* 9, 545–560.

Sugiyama, N., Nakagami, H., Mochida, K., Daudi, A., Tomita, M., Shirasu, K., and Ishihama, Y. (2008). Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in *Arabidopsis. Mol. Syst. Biol.* 4, 193.

Thiel, K. W., and Carpenter, G. (2007). Epidermal growth factor receptor juxtamembrane region regulates allosteric tyrosine kinase activation. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19238–19243.

Wang, Y. S., Pi, L. Y., Chen, X., Chakrabarty, P. K., Jiang, J., De Leon, A. L., Liu, G. Z., Li, L., Benny, U., Oard, J., Ronald, P. C., and Song, W. Y. (2006). Rice XA21 binding protein 3 is a ubiquitin ligase required for full Xa21-mediated disease resistance. *Plant Cell* 18, 3635–3646.

Weintz, G., Olsen, J. V., Fruhauf, K., Niedzielska, M., Amit, I., Jantsch, J., Mages, J., Frech, C., Dolken, L., Mann, M., and Lang, R. (2010). The phosphoproteome of toll-like receptor-activated macrophages. *Mol. Syst. Biol.* 6, 371.

Xu, W. H., Wang, Y. S., Liu, G. Z., Chen, X., Tinjuangjun, P., Pi, L. Y., and Song, W. Y. (2006). The autophosphorylated Ser686, Thr688, and Ser689 residues in the intracellular juxtamembrane domain of XA21 are implicated in stability control of rice receptor-like kinase. *Plant J.* 45, 740–751.

Yoo, S. D., Cho, Y. H., Tena, G., Xiong, Y., and Sheen, J. (2008). Dual control of nuclear EIN3 by bifurcate MAPK cascades in C2H4 signalling. *Nature* 451, 789–795.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y., and Zhou, J. M. (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.

Zhang, S., and Klessig, D. F. (2001). MAPK cascades in plant defense signaling. *Trends Plant Sci.* 6, 520–527.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125, 749–760.

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: 08 May 2012; paper pending published: 01 June 2012; accepted: 18 July 2012; published online: 03 August 2012.

*Citation: Park C-J, Caddell DF and Ronald PC (2012) Protein phosphorylation in plant immunity: insights into the regulation of pattern recognition receptor-mediated signaling. Front. Plant Sci. **3**:177. doi: 10.3389/fpls.2012.00177*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 24 July 2012
doi: 10.3389/fpls.2012.00169

MAP kinase cascades in *Arabidopsis* innate immunity

Magnus W. Rasmussen, Milena Roux, Morten Petersen and John Mundy*

Department of Biology, University of Copenhagen, Copenhagen, Denmark

Edited by:
Alex Jones, The Sainsbury Laboratory, UK

Reviewed by:
Birgit Kersten, Johann Heinrich von Thünen Institute, Germany
Jesus V. Jorrin Novo, University of Cordoba, Spain

***Correspondence:**
John Mundy, Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, 2200 Copenhagen, Denmark.
e-mail: mundy@bio.ku.dk

Plant mitogen-activated protein kinase (MAPK) cascades generally transduce extracellular stimuli into cellular responses. These stimuli include the perception of pathogen-associated molecular patterns (PAMPs) by host transmembrane pattern recognition receptors which trigger MAPK-dependent innate immune responses. In the model *Arabidopsis*, molecular genetic evidence implicates a number of MAPK cascade components in PAMP signaling, and in responses to immunity-related phytohormones such as ethylene, jasmonate, and salicylate. In a few cases, cascade components have been directly linked to the transcription of target genes or to the regulation of phytohormone synthesis. Thus MAPKs are obvious targets for bacterial effector proteins and are likely guardees of resistance proteins, which mediate defense signaling in response to the action of effectors, or effector-triggered immunity. This mini-review discusses recent progress in this field with a focus on the *Arabidopsis* MAPKs MPK3, MPK4, MPK6, and MPK11 in their apparent pathways.

Keywords: calcium signaling, hypersensitive response, MAP kinase cascade, MAP kinase substrates, pathogen effectors, pattern recognition receptors, reactive oxygen species, resistance proteins

INTRODUCTION

Plants have evolved an effective basal defense system to detect and limit the growth of pathogens. Pathogens may be recognized by the host via the perception of conserved microbial structures termed pathogen-associated molecular patterns (PAMPs). PAMPs are recognized via transmembrane pattern recognition receptors (PRRs) that bind specific PAMPs and initiate intracellular immune responses (Zipfel, 2008). These PAMP-triggered immunity (PTI) responses include the generation of reactive oxygen species (ROS), extracellular alkalinization, and protein phosphorylation with associated gene regulation that ultimately restricts the growth of the microbial intruder (Gimenez-Ibanez and Rathjen, 2010).

Mitogen-activated protein kinase (MAPK) signaling plays central roles in such intracellular immunity pathways. In general, MAP kinase signaling is initiated by the stimulus-triggered activation of a MAP kinase kinase kinase (MAP3K; also called MEKK). MAP3K activation, which may be directly or indirectly effected by a PRR, in turn leads to the phosphorylation and activation of downstream MAP kinase kinases (MAP2K; also called MKK or MEK). Subsequently, the MAP2K phosphorylates the downstream MAPK sequentially leading to changes in its subcellular localization and/or phosphorylation of downstream substrates including transcription factors which alter patterns of gene expression (see **Figure 1**). General functions of MAPK cascades in plant biology have recently been reviewed elsewhere (Fiil et al., 2009; Rodriguez et al., 2010; Komis et al., 2011).

MAPK CASCADES IN PTI

A few PRRs have been documented to stimulate MAPK signaling upon perception of PAMPs. These include the flagellin receptor FLS2 (Felix et al., 1999; Gómez-Gómez and Boller, 2000), the bacterial elongation factor EF-Tu receptor EFR (Zipfel et al., 2006), and the chitin receptor CERK1 (Miya et al., 2007).

The *Arabidopsis* genome encodes 60 MAP3Ks, 10 MAP2Ks, and 20 MAPKs (Ichimura et al., 2002). This indicates that MAPK cascades may not simply consist of single MAP3Ks, MAP2Ks, and MAPKs connected together. Instead, it suggests that there is some level of redundancy, and that the spatial and temporal activities of different components may be strictly regulated to minimize wanton cross-talk. The three MAPKs MPK3, MPK4, and MPK6 are the most intensively studied plant MAPKs, and all three were implicated in defense signaling a decade ago (Petersen et al., 2000; Asai et al., 2002). MPK11, a close homolog to MPK4, has also recently been shown to be activated by PAMP treatment (Bethke et al., 2012).

MPK3, MPK4, and MPK6 are all activated by PAMPs such as flg22 (a conserved 22 amino acid flagellin peptide) and elf18 (elongation factor-Tu peptide; Felix et al., 1999; Zipfel et al., 2006). However, these three MAPK cascades are differently regulated already at the PRR level. For example, the two receptor kinases BAK1 and BKK1 genetically regulate PAMP signaling through their interactions with cognate PRRs (Roux et al., 2011; Schwessinger et al., 2011). The BAK1 mutant allele *bak1-5* carries a Cys408Tyr substitution adjacent to its kinase catalytic loop. This impairs its flg22-regulated kinase activity and inhibits phosphorylation of MPK4. However, the catalytic complex formed between mutant BAK1 in *bak1-5* and FLS2 is still able to induce phosphorylation of MPK3/MPK6 (Roux et al., 2011; Schwessinger et al., 2011). Interestingly, MPK3/MPK6 phosphorylation was impaired in only the double *bak1-5 bkk1* background and not in the individual *bak1-5* and *bkk1* lines (Roux et al., 2011).

Asai et al. (2002) developed an elegant protoplast expression system in an attempt to identify signaling components downstream of FLS2. With this system they were able to show a complete MAPK cascade downstream of FLS2 consisting of the MAP3K MEKK1, two MAP2Ks (MKK4 and MKK5), and the MAPKs MPK3/MPK6. However, genetic evidence later showed

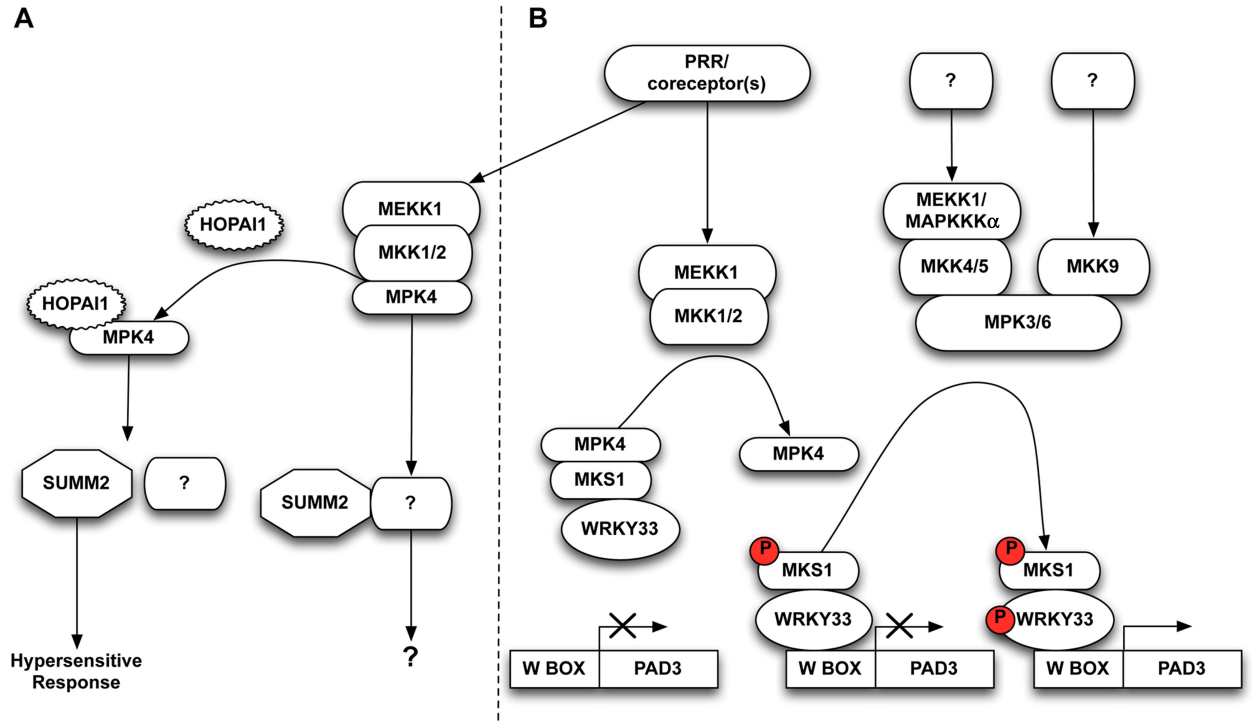


FIGURE 1 | (A) MAPK signaling cascades are attractive targets for bacterial effectors. The *P. syringae* HopAI1 effector irreversibly inactivates MPK4 to prevent immune responses. The R protein SUMM2 may guard processes downstream of MPK4 independent from MKS1, and triggers a hypersensitive response in the event of loss or inactivation of MPK4. **(B)** PAMP perception by PRRs instigates a signaling cascade, often via co-receptors, which causes activation of MAP3K MEKK1 and two MAP2Ks MKK1 and MKK2. These phosphorylate and activate MPK4 which then phosphorylates its substrate MKS1, releasing MKS1 in complex with WRKY33. MPK3/MPK6 sequentially phosphorylate WRKY33 allowing it to promote *PAD3* transcription, thus activating plant defense.

that MEKK1 kinase activity was dispensable for MPK3/MPK6 activation, although *mekk1* plants were impaired in MPK4 activation (Rodriguez et al., 2007). Interestingly, expressing a kinase dead version of MEKK1 in *mekk1* plants completely restored the activation of MPK4 upon treatment with flg22, suggesting that MEKK1 may "simply" act as a scaffold protein (Rodriguez et al., 2007). Biochemical and genetic studies further revealed that the two MAP2Ks MKK1 and MKK2 interact with both MEKK1 and with MPK4, and that flg22-induced MPK4 activation is impaired in the double *mkk1 mkk2* mutant. This indicates that MKK1 and MKK2 are partially redundant in MPK4 mediated downstream signaling (Gao et al., 2008; Qiu et al., 2008b).

MPK4 was originally reported as a negative regulator of plant immunity because the *mpk4* mutant accumulates high levels of salicylic acid, constitutively expresses pathogenesis-related (*PR*) genes, and has a severely dwarfed growth phenotype (Petersen et al., 2000). This phenotype is very similar to that of the *mekk1* single and *mkk1 mkk2* double mutants, further supporting their functional relationships (Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008b).

MAPK CASCADES IN EFFECTOR-TRIGGERED IMMUNITY

In addition to PTI, plants also employ resistance (R) proteins as cytoplasmic receptors to directly or indirectly recognize specific pathogenic effector proteins injected into host cells as virulence factors. Effector-triggered immunity (ETI) and PTI share a number of responses, although ETI also includes varying levels of rapid, localized cell death in what is called the hypersensitive response. R protein-dependent recognition initiates immune responses in ETI. R proteins may recognize effector proteins either directly or indirectly by monitoring changes in the effector's host target(s). This latter case gave rise to the guard hypothesis in which R proteins guard host guardees that are manipulated by pathogen effectors (Van Der Biezen and Jones, 1998).

The genetic characterization of the MEKK1/MKK1–MKK2/MPK4 cascade as a negative regulatory pathway of defense responses was at odds with the activation of the pathway by PAMPs. Instead, it was possible that the severe phenotypes of the kinase knockout mutants were caused by activation of one or more R protein(s) guarding this kinase pathway. Indeed, in an elegant screen for suppressors of the *mkk1 mkk2* double mutant, Zhang et al. (2012) identified the R protein SUMM2 (suppressor of *mkk1 mkk2*). The T-DNA insertion line *summ2-8* completely suppressed the severe *mkk1 mkk2* phenotype in respect to morphology, cell death, ROS levels and *PR* gene expression (Zhang et al., 2012). The analogous knockout phenotype of the upstream MAP3K *mekk1* is also completely suppressed in the *summ2-8* background. Interestingly, although the *mpk4* mutant shares a similar phenotype with the knockouts of its upstream kinase partners, the *mpk4* phenotype is not fully suppressed by the *summ2-8*

mutation, as double *mpk4 summ2-8* mutants still retain residual cell death and low levels of ROS. This suggests that MPK4 is involved in other pathways independent of SUMM2, and that MPK4 may be guarded by additional R proteins (Zhang et al., 2012; **Figure 1A**).

The importance of MAPK signaling in immunity is emphasized by studies reporting bacterial effector proteins targeting MAPK cascades for downregulation (Zhang et al., 2007a,b, 2012; Cui et al., 2010). For example, the *Pseudomonas syringae* effector protein HopAI1 targets and irreversibly inactivates MPK3, MPK4, and MPK6, thereby suppressing immune responses which would otherwise inhibit bacterial growth (Zhang et al., 2007a, 2012). In addition, the *P. syringae* effector protein AvrB has been shown to interact with and induce the phosphorylation of MPK4, although it has not been shown if this phosphorylation occurs as a direct effect of AvrB action or via recognition of AvrB by the plant immune system (Cui et al., 2010).

In plants carrying functional SUMM2 alleles, immune responses are activated by bacterial effector proteins targeting the MPK4 pathway (**Figure 1A**). For example, inducible expression of the bacterial HopAI1 effector in wild-type plants gives rise to a defense phenotype similar to that seen in *mekk1*, *mkk1 mkk2*, and *mpk4* mutants including elevated levels of ROS, *PR* gene expression, and cell death (Zhang et al., 2012). SUMM2 apparently does not interact directly with the kinase components of the MEKK1/MKK1–MKK2/MPK4 signaling cascade, suggesting that SUMM2 most likely guards a downstream target of MPK4 activity (Zhang et al., 2012). At present, the best studied *in vivo* substrate of MPK4 activity is MPK4 substrate 1 (MKS1) which forms a nuclear complex with MPK4 and the WRKY33 transcription factor (Andreasson et al., 2005; Qiu et al., 2008a). Phosphorylation of MKS1 follows MPK4 activation by flg22 perception and, once phosphorylated, MKS1 is released from complexes with MPK4, thereby releasing the WRKY33 transcription factor to bind to its cognate target genes (Qiu et al., 2008a). It has therefore been proposed that MPK4 and MKS1 sequester WRKY33 in the absence of pathogens, and free WRKY33 to induce resistance upon pathogen perception (**Figure 1B**, left).

As MKS1 is the only known direct target of MPK4, Zhang et al. (2012) tested whether MKS1 interacted with the R protein SUMM2 that seemingly guards MPK4 activity. However, no interaction between SUMM2 and MKS1 was detected. Since *mks1* mutants have a wild-type growth phenotype, and the *mpk4* phenotype is strongly suppressed in the *mks1* background, SUMM2 may guard a process downstream of MPK4 that is independent of MKS1 (Petersen et al., 2010).

WRKY TRANSCRIPTION FACTORS

The plant-specific WRKY family is a large group of transcription factors which bind a conserved W-box sequence in the promoters of numerous genes including those encoding PR proteins. WRKY33 was found to induce the transcription of *PHYTOALEXIN DEFICIENT 3* (*PAD3*) which encodes the cytochrome P450 monooxygenase 71B15 required for synthesis of the antimicrobial compound camalexin (Zhou et al., 1999; Qiu et al., 2008a; **Figure 1B**). The *wrky33* mutant exhibits enhanced susceptibility toward necrotrophic pathogens such as *Botrytis cinerea*, while WRKY33 overexpression results in increased resistance due to enhanced *PAD3* expression (Zheng et al., 2006).

MPK3 and MPK6 activities also induce the production of camalexin. Transient overexpression of the constitutively active, phospho-mimic mutant forms of MKK4/MKK5 ($MKK4^{DD}$ and $MKK5^{DD}$), which are the upstream MAP2Ks of MPK3/MPK6, has been reported to induce transcription of both *PAD2*, which encodes γ-glutamylcysteine synthetase functioning in glutathione biosynthesis, and *PAD3*. Both PAD2 and PAD3 are necessary for camalexin production (Parisy et al., 2007; Ren et al., 2008). Pathogen-induced camalexin accumulation is partially comprised in *mpk3* but not notably in *mpk6* mutants, yet camalexin accumulation in *mpk3 mpk6* double mutants is almost completely abolished (Ren et al., 2008). While this implicates MPK3/MPK6 in camalexin synthesis, caution should be applied in evaluating results obtained from the *mpk3 mpk6* double mutant as it is arrested at the cotyledon stage and is unable to initiate true leaves (Wang et al., 2007). Upstream of MPK3/MPK6 in camalexin induction, MKK4 and MKK5 are activated by the MAP3Ks MEKK1 and MAPKKKα in response to fungal pathogens (Ren et al., 2008). Yet another MAP2K, MKK9, whose upstream MAP3K(s) remains unidentified, is also involved in MPK3/MPK6 activation, as plants expressing phospho-mimic $MKK9^{DD}$ produce even more camalexin than plants expressing $MKK4^{DD}$ or $MKK5^{DD}$ (Xu et al., 2008).

To delineate the link between MPK3/MPK6 activation and camalexin accumulation, Mao et al. (2011) elegantly introduced the phospho-mimic mutant $NtMEK2^{DD}$, an MKK4 and/or MKK5 ortholog from *Nicotiana tabacum*, into an array of different *wrky* mutants in a search for essential transcription factors involved in MPK3/MPK6 mediated camalexin induction. Interestingly, $NtMEKK2^{DD}$ was able to induce camalexin accumulation in all tested mutant lines except *wrky33*. In addition, WRKY33 proved to be a substrate of MKP3/MPK6 activity, and overexpression of non-phosphorylatable forms of WRKY33 could not fully complement the inability of *wrky33* mutants to express *PAD3* and accumulate camalexin (Mao et al., 2011; **Figure 1B**, right).

WRKY33-induced *PAD3* expression therefore appears to involve both MPK4- and MPK3/MPK6-mediated signaling (Andreasson et al., 2005; Qiu et al., 2008a; Mao et al., 2011). Mao et al. (2011) proposed a model in which PAD3-mediated camalexin induction occurs differentially depending on the type of pathogen causing the immune response. In this model, bacterial pathogens induce an MPK4 mediated response while fungal pathogens initiate an MPK3/MPK6 mediated response. This hypothesis is based on overexpression of the constitutively active MKK4/MKK5 ortholog $NtMEKK2^{DD}$, rendering MPK3/MPK6 hyperactive and able to induce *PAD3* expression (Mao et al., 2011). In support of this hypothesis, the *mpk3 mpk6* double mutant is comprised in *B. cinerea*-induced *PAD3* induction (Ren et al., 2008). Nonetheless, and as noted above, some care should be taken with experiments based on *mpk3 mpk6* double mutants given their developmental lethality (Wang et al., 2007).

An alternative model may therefore be proposed which combines the MPK4 and MPK3/MPK6 pathways into a dual control of *PAD3* regulation in response to pathogen perception (**Figure 1B**). In such a model, WRKY33 is sequestered in a nuclear complex

comprising at least MPK4 and MKS1 in unchallenged plants, and is released following PAMP perception (Qiu et al., 2008a). Phosphorylation is dispensable for WRKY33 to bind its cognate W-box *cis*-elements, although it does promote transcriptional activation (Mao et al., 2011). This is illustrated by the fact that *PAD3* expression is induced in *mpk4* plants (Qiu et al., 2008a), perhaps due to the basal activity of free non-phosphorylated WRKY33 or by free WRKY33 activated by basal MPK3 and/or MPK6 activity. In this scenario, once WRKY33 is released from its nuclear complex with MPK4 and MKS1, it is phosphorylated and hence activated by MPK3/MPK6, thereby inducing camalexin levels through *PAD3* expression. The elevated *PAD3* expression induced from *NtMEKK2*^{DD} hyper-activated MPK3/MPK6 (Mao et al., 2011) is not in conflict with this model, as it is likely that hyperactive MPK3/MPK6 are able to phosphorylate residual free WRKY33, thus bypassing other possible feedback mechanisms in *PAD3* expression.

In this model, MPK4 and MPK3/MPK6 function together as a binary switch conferring dual level regulation. Clarification of the mode of action in which MPK4 and MPK3/MPK6 function clearly needs further elucidation and should include experiments using catalytically inactive and/or inactivatable MPK4 (Petersen et al., 2000; Brodersen et al., 2006). Application of fungal PAMPs to plants expressing catalytically inactive MPK4 might indicate whether phosphorylation of free WRKY33 by endogenous MPK3/MPK6 is enough to induce expression of *PAD3*.

MAPK IN GENERAL STRESS SIGNALING

The refined work of Popescu et al. (2009) identified a MAP2K–MAPK phosphorylation network covering 570 MAPK substrates by combinatorially pairing active MAP2Ks with MAPKs, and then subjecting them to a protein microarray phosphorylation assay. Interestingly, the substrates identified were enriched for transcription factors involved in stress responses. Notably, MPK6 phosphorylated 32% of the identified targets, of which 40% overlapped with MPK3 targets (Popescu et al., 2009). This is in agreement with earlier data, similarly obtained from a protein microarray study (Feilner et al., 2005). Equally noteworthy is the finding that MPK3 also shared 50% of its targets with MPK4, revealing intensive synergy in MAPK signaling (Popescu et al., 2009).

In addition to MAPK cascades, ROS also play a pivotal role in stress signaling (Rodriguez et al., 2010). OXI1, a serine/threonine kinase induced by general ROS-generating stimuli, is required for full activation of MPK3/MPK6 after treatment with H_2O_2 (Rentel et al., 2004). Although OXI1 is characterized as an upstream regulator of MPK3/MPK6 activation, MPK3/MPK6 have been shown to phosphorylate OXI1 *in vitro*. This suggests that there is a feedback loop, but *in vivo* data supporting such a loop has not been shown (Forzani et al., 2011).

In addition to MAPK cascade signaling, PAMP perception also induces Ca^{2+} dependent kinases (CDPKs) by regulating Ca^{2+} influx channels (Ma et al., 2009; Kwaaitaal et al., 2011). Recent findings indicate that Ca^{2+} ATPases regulate Ca^{2+} efflux and function to regulate innate immune defenses (Zhu et al., 2010). Of particular interest is the Ca^{2+} ATPase ACA8 which was shown to interact with FLS2, and which may well regulate CDPK signaling through flg22 perception (Frei dit Frey et al., 2012).

MPK8 activity has been shown to negatively regulate the expression of *OXI1* in order to maintain ROS homeostasis. Remarkably, activation of MPK8 is not limited to the upstream MAP2K MKK3, as the Ca^{2+} binding protein calmodulin (CaM) is able to bind and activate MPK8 in an Ca^{2+}-dependent manner (Takahashi et al., 2011). CaM-mediated MPK8 activation is interesting because it bypasses the traditional, sequential activation of MAPKs and also unequivocally links MAPK activation with the ROS burst and ion flux during stress signaling. In addition, CaM also mediates MAPK downregulation. MAP kinase phosphatase 1 (MKP1), which interacts with MPK3, MPK4, and MPK6 (Ulm et al., 2002), binds CaM in a Ca^{2+}-dependent manner and stimulates MKP1 phosphatase activity (Lee et al., 2008). The associations between CDPKs and MAPK cascades have recently been review elsewhere (Wurzinger et al., 2011).

Much progress has been made in understanding how MAPK signaling functions in plant immunity. In *Arabidopsis*, 3 of the 60 identified MAP3Ks are involved in defense, namely MEKK1 (Asai et al., 2002), EDR1 (Frye et al., 2001), and MEKKα (del Pozo et al., 2004; Ren et al., 2008). In addition, at least 6 of the 10 identified MAP2Ks (MKK1, MKK2, MKK4, MKK5, MKK7, and MKK9) are involved in defense signaling (Asai et al., 2002; Djamei et al., 2007; Dóczi et al., 2007; Zhang et al., 2007b; Yoo et al., 2008). This situation requires tight regulation of the spatial and temporal kinase activities in order to impose specificity upon downstream signaling. To shed light on this regulation, high-throughput methods such as those used by Popescu et al. (2009) are particularly valuable and help to outline MAPK signaling cascades. While this progress may be lauded, further work needs to focus on identifying direct, *in vivo* kinase substrates and their respective phosphorylation sites. This may bring us closer to bridging the apparent gap between PRRs and MAPK cascades, and to understanding how specificity is achieved among MAPK pathways both spatially and temporally.

ACKNOWLEDGMENTS

This work was supported by grants to John Mundy from the Danish Research Council for Technology and Production (23-03-0076) and the Strategic Research Council (09-067148) and to Milena Roux from the Natural Science Council (11-116368).

REFERENCES

Andreasson, E., Jenkins, T., Brodersen, P., Thorgrimsen, S., Petersen, N. H., Zhu, S., Qiu, J.-L., Micheelsen, P., Rocher, A., Petersen, M., Newman, M. A., Bjørn Nielsen, H., Hirt, H., Somssich, I., Mattsson, O., and Mundy, J. (2005). The MAP kinase substrate MKS1 is a regulator of plant defense responses. *EMBO J.* 24, 2579–2589.

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W. L., Gomez-Gomez, L., Boller, T., Ausubel, F. M., and Sheen, J. (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* 415, 977–983.

Bethke, G., Pecher, P., Eschen-Lippold, L., Tsuda, K., Katagiri, F., Glazebrook, J., Scheel, D., and Lee, J. (2012). Activation of the *Arabidopsis thaliana* mitogen-activated protein kinase MPK11 by the flagellin-derived elicitor peptide, flg22. *Mol. Plant Microbe Interact.* 25, 471–480.

Brodersen, P., Petersen, M., Nielsen, H. B., Zhu, S., Newman, M., Shokat, K. M., Rietz, S., Parker, J., and Mundy, J. (2006). *Arabidopsis* MAP kinase 4 regulates salicylic acid- and jasmonic

acid/ethylene-dependent responses via EDS1 and PAD4. *Plant J.* 47, 532–546.

Cui, H., Wang, Y., Xue, L., Chu, J., Yan, C., Fu, J., Chen, M., Innes, R. W., and Zhou, J.-M. (2010). Pseudomonas syringae effector protein AvrB perturbs *Arabidopsis* hormone signaling by activating MAP kinase 4. *Cell Host Microbe* 7, 164–175.

del Pozo, O., Pedley, K. F., and Martin, G. B. (2004). MAPKKK[alpha] is a positive regulator of cell death associated with both plant immunity and disease. *EMBO J.* 23, 3072–3082.

Djamei, A., Pitzschke, A., Nakagami, H., Rajh, I., and Hirt, H. (2007). trojan horse strategy in *Agrobacterium* transformation: abusing MAPK defense signaling. *Science* 318, 453–456.

Dóczi, R., Brader, G., Pettkó-Szandtner, A., Rajh, I., Djamei, A., Pitzschke, A., Teige, M., and Hirt, H. (2007). The *Arabidopsis* mitogen-activated protein kinase kinase MKK3 is upstream of group C mitogen-activated protein kinases and participates in pathogen signaling. *Plant Cell* 19, 3266–3279.

Feilner, T., Hultschig, C., Lee, J., Meyer, S., Immink, R. G. H., Koenig, A., Possling, A., Seitz, H., Beveridge, A., Scheel, D., Cahill, D. J., Lehrach, H., Kreutzberger, J., and Kersten, B. (2005). High throughput identification of potential *Arabidopsis* mitogen-activated protein kinases substrates. *Mol. Cell. Proteomics* 4, 1558–1568.

Felix, G., Duran, J. D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18, 265–276.

Fiil, B. K., Petersen, K., Petersen, M., and Mundy, J. (2009). Gene regulation by MAP kinase cascades. *Curr. Opin. Plant Biol.* 12, 615–621.

Forzani, C., Carreri, A., de la Fuente van Bentem, S., Lecourieux, D., Lecourieux, F., and Hirt, H. (2011). The *Arabidopsis* protein kinase Pto-interacting 1-4 is a common target of the oxidative signal-inducible 1 and mitogen-activated protein kinases. *FEBS J.* 278, 1126–1136.

Frei dit Frey, N., Mbengue, M., Kwaaitaal, M., Nitsch, L., Altenbach, D., Haweker, H., Lozano-Duran, R., Njo, M. F., Beeckman, T., Huettel, B., Borst, J. W., Panstruga, R., and Robatzek, S. (2012). Plasma membrane calcium ATPases are important components of receptor-mediated signaling in plant immune responses and development. *Plant Physiol.* 159, 798–809.

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.

Gao, M., Liu, J., Bi, D., Zhang, Z., Cheng, F., Chen, S., and Zhang, Y. (2008). MEKK1, MKK1/MKK2 and MPK4 function together in a mitogen-activated protein kinase cascade to regulate innate immunity in plants. *Cell Res.* 18, 1190–1198.

Gimenez-Ibanez, S., and Rathjen, J. P. (2010). The case for the defense: plants versus *Pseudomonas syringae*. *Microbes Infect.* 12, 428–437.

Gómez-Gómez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5, 1003–1011.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B. E., Morris, P. C., Innes, R. W., Ecker, J. R., Scheel, D., Klessig, D. F., Machida, Y., Mundy, J., Ohashi, Y., and Walker, J. C. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* 7, 301–308.

Komis, G., Illés, P., Beck, M., and Šamaj, J. (2011). Microtubules and mitogen-activated protein kinase signalling. *Curr. Opin. Plant Biol.* 14, 650–657.

Kwaaitaal, M., Huisman, R., Maintz, J., Reinstädler, A., and Panstruga, R. (2011). Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*. *Biochem. J.* 440, 355–365.

Lee, K., Song, E. H., Kim, H. S., Yoo, J. H., Han, H. J., Jung, M. S., Lee, S. M., Kim, K. E., Kim, M. C., Cho, M. J., and Chung, W. S. (2008). Regulation of MAPK phosphatase 1 (AtMKP1) by calmodulin in *Arabidopsis*. *J. Biol. Chem.* 283, 23581–23588.

Ma, W., Qi, Z., Smigel, A., Walker, R. K., Verma, R., and Berkowitz, G. A. (2009). Ca^{2+}, cAMP, and transduction of non-self perception during plant immune responses. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20995–21000.

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.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19613–19618.

Parisy, V., Poinssot, B., Owsianowski, L., Buchala, A., Glazebrook, J., and Mauch, F. (2007). Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. *Plant J.* 49, 159–172.

Petersen, K., Qiu, J.-L., Lütje, J., Fiil, B. K., Hansen, S., Mundy, J., and Petersen, M. (2010). *Arabidopsis* MKS1 is involved in basal immunity and requires an intact N-terminal domain for proper function. *PLoS ONE* 5, e14364. doi: 10.1371/journal.pone.0014364

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H. B., Lacy, M., Austin, M. J., Parker, J. E., Sharma, S. B., Klessig, D. F., Martienssen, R., Mattsson, O., Jensen, A. B., and Mundy, J. (2000). *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. *Cell* 103, 1111–1120.

Popescu, S. C., Popescu, G. V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., and Dinesh-Kumar, S. P. (2009). MAPK target networks in *Arabidopsis thaliana* revealed using functional protein microarrays. *Genes Dev.* 23, 80–92.

Qiu, J.-L., Fiil, B. K., Petersen, K., Nielsen, H. B., Botanga, C. J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M. C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K. D., Mattsson, O., Glazebrook, J., Mundy, J., and Petersen, M. (2008a). *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO J.* 27, 2214–2221.

Qiu, J.-L., Zhou, L., Yun, B.-W., Nielsen, H. B., Fiil, B. K., Petersen, K., MacKinlay, J., Loake, G. J., Mundy, J., and Morris, P. C. (2008b). *Arabidopsis* mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. *Plant Physiol.* 148, 212–222.

Ren, D., Liu, Y., Yang, K.-Y., Han, L., Mao, G., Glazebrook, J., and Zhang, S. (2008). A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5638–5643.

Rentel, M. C., Lecourieux, D., Ouaked, F., Usher, S. L., Petersen, L., Okamoto, H., Knight, H., Peck, S. C., Grierson, C. S., Hirt, H., and Knight, M. R. (2004). OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. *Nature* 427, 858.

Rodriguez, M. C., Adams-Phillips, L., Liu, Y., Wang, H., Su, S. H., Jester, P. J., Zhang, S., Bent, A. F., and Krysan, P. J. (2007). MEKK1 is required for flg22-induced MPK4 activation in *Arabidopsis* plants. *Plant Physiol.* 143, 661.

Rodriguez, M. C., Petersen, M., and Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. *Annu. Rev. Plant Biol.* 61, 621–649.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F. G., Tör, M., De Vries, S., and Zipfel, C. (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.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A., and Zipfel, C. (2011). Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet.* 7, e1002046. doi: 10.1371/journal.pgen.1002046

Takahashi, F., Mizoguchi, T., Yoshida, R., Ichimura, K., and Shinozaki, K. (2011). Calmodulin-dependent activation of MAP kinase for ROS homeostasis in *Arabidopsis*. *Mol. Cell* 41, 649–660.

Ulm, R., Ichimura, K., Mizoguchi, T., Peck, S. C., Zhu, T., Wang, X., Shinozaki, K., and Paszkowski, J. (2002). Distinct regulation of salinity and genotoxic stress responses by *Arabidopsis* MAP kinase phosphatase 1. *EMBO J.* 21, 6483–6493.

Van Der Biezen, E. A., and Jones, J. D. G. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* 23, 454–456.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J. C., and Zhang, S. (2007). Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis*. *Plant Cell* 19, 63–73.

Wurzinger, B., Mair, A., Pfister, B., and Teige, M. (2011). Cross-talk of calcium-dependent protein kinase and MAP kinase signaling. *Plant Signal. Behav.* 6, 8–12.

Xu, J., Li, Y., Wang, Y., Liu, H., Lei, L., Yang, H., Liu, G., and Ren, D. (2008). Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in *Arabidopsis*. *J. Biol. Chem.* 283, 26996–27006.

Yoo, S.-D., Cho, Y.-H., Tena, G., Xiong, Y., and Sheen, J. (2008). Dual control of nuclear EIN3 by bifurcate MAPK cascades in C_2H_4 signalling. *Nature* 451, 789–795.

Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., and Zhou, J. M. (2007a). A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe* 1, 175–185.

Zhang, X., Dai, Y., Xiong, Y., DeFraia, C., Li, J., Dong, X., and Mou, Z. (2007b). Overexpression of *Arabidopsis* MAP kinase kinase 7 leads to activation of plant basal and systemic acquired resistance. *Plant J.* 52, 1066–1079.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J., and Zhang, Y. (2012). 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.

Zheng, Z., Qamar, S. A., Chen, Z., and Mengiste, T. (2006). *Arabidopsis* WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *Plant J.* 48, 592–605.

Zhou, N., Tootle, T. L., and Glazebrook, J. (1999). *Arabidopsis* PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *Plant Cell* 11, 2419–2428.

Zhu, X., Caplan, J., Mamillapalli, P., Czymmek, K., and Dinesh-Kumar, S. P. (2010). Function of endoplasmic reticulum calcium ATPase in innate immunity-mediated programmed cell death. *EMBO J.* 29, 1007–1018.

Zipfel, C. (2008). Pattern-recognition receptors in plant innate immunity. *Curr. Opin. Immunol.* 20, 10–16.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D. G., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125, 749–760.

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: 02 May 2012; paper pending published: 24 May 2012; accepted: 09 July 2012; published online: 24 July 2012.

*Citation: Rasmussen MW, Roux M, Petersen M and Mundy J (2012) MAP kinase cascades in Arabidopsis innate immunity. Front. Plant Sci. **3**:169. doi: 10.3389/fpls.2012.00169*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 22 August 2012
doi: 10.3389/fpls.2012.00200

The endoplasmic reticulum in plant immunity and cell death

Ruth Eichmann and Patrick Schäfer*

The School of Life Sciences, University of Warwick, Coventry, UK

Edited by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

Reviewed by:
Yusuke Saijo, Max Planck Institute for Plant Breeding Research, Germany
Frederikke Gro Malinovsky, The Sainsbury Laboratory, UK

***Correspondence:**
Patrick Schäfer, The School of Life Sciences, University of Warwick, Gibbet Hill Campus, Coventry CV4 7AL, UK.
e-mail: p.schafer@warwick.ac.uk

The endoplasmic reticulum (ER) is a highly dynamic organelle in eukaryotic cells and a major production site of proteins destined for vacuoles, the plasma membrane, or apoplast in plants. At the ER, these secreted proteins undergo multiple processing steps, which are supervised and conducted by the ER quality control system. Notably, processing of secreted proteins can considerably elevate under stress conditions and exceed ER folding capacities. The resulting accumulation of unfolded proteins is defined as ER stress. The efficiency of cells to re-establish proper ER function is crucial for stress adaptation. Besides delivering proteins directly antagonizing and resolving stress conditions, the ER monitors synthesis of immune receptors. This indicates the significance of the ER for the establishment and function of the plant immune system. Recent studies point out the fragility of the entire system and highlight the ER as initiator of programed cell death (PCD) in plants as was reported for vertebrates. This review summarizes current knowledge on the impact of the ER on immune and PCD signaling. Understanding the integration of stress signals by the ER bears a considerable potential to optimize development and to enhance stress resistance of plants.

Keywords: programed cell death, plant immunity, unfolded protein response, stress, endoplasmic reticulum quality control

INTRODUCTION

The endoplasmic reticulum (ER) is an organelle with important functions in eukaryotic cells. It connects to other cellular compartments [e.g., nucleus, Golgi apparatus, mitochondria, peroxisomes, plasma membrane (PM)] and, as one of the largest Ca^{2+} stores, participates in intracellular Ca^{2+} signaling. It is further involved in lipid and hormone biosynthesis (Staehelin, 1997; Sparkes et al., 2009; Lynes and Simmen, 2011). Importantly, the ER quality control (ER-QC) system mediates and monitors the processing and folding of secretory proteins destined for transport to the PM, vacuole, or apoplast, identifies misfolded proteins and transfers them to the ER-associated degradation (ERAD) machinery (Vitale and Boston, 2008; Liu and Howell, 2010; Hüttner and Strasser, 2012). Among the proteins processed by the plant's ER-QC are important PM-resident proteins involved in adaptation to environmental stress, e.g., hormone or immune receptors (Saijo, 2010). ER integrity is central to proper function of cells and whole organisms. Especially under stress conditions, any impairment of ER function can result in disturbed plant development and plant immunity (Wang et al., 2005; Vitale and Boston, 2008; Saijo, 2010).

REGULATION OF ER INTEGRITY AND ER STRESS SIGNALING IN EUKARYOTES

Protein folding demand and capacities in the ER are usually in equilibrium. However, responses to environmental stresses create an increased requirement for secreted proteins. If this demand exceeds the ER-QC working capacity, unfolded proteins accumulate in the ER, which the cell senses as ER stress. Prolonged ER stress impairs ER function and thus threatens cellular integrity. Chemicals, such as the *N*-glycosylation inhibitor tunicamycin (TM) or the reducing agent dithiothreitol (DTT), which inhibits the formation of disulfide bonds, are widely used to induce and examine ER stress (Martínez and Chrispeels, 2003; Kamauchi et al., 2005; Vitale and Boston, 2008; Liu and Howell, 2010).

In animals, mainly three ER membrane proteins constitute the cell's ER stress surveillance system: the type I transmembrane protein kinase/endoribonuclease inositol-requiring enzyme 1 (IRE1 α and β), the type I transmembrane protein kinase RNA-like ER kinase (PERK), and the type II transmembrane basic leucine-zipper (bZIP) domain-containing activating transcription factor 6 (ATF6). In yeast cells, IRE1 is the only ER stress sensor (Mori, 2009). Under non-stressed conditions, luminal parts of these ER stress sensors bind to luminal binding proteins (BiPs), which keeps the sensors in an inactive state. If unfolded proteins accumulate, BiPs disconnect from ER stress sensors to mediate processing of unfolded proteins. Once liberated, ER stress sensors initiate different adaptive signaling cascades defined as unfolded protein response (UPR) to re-establish proper ER function. The UPR enhances the synthesis of antioxidants and ER-QC members, attenuates translation, suppresses expression of secretory genes, and elevates ERAD of unfolded proteins (Schröder, 2006, 2008; Liu and Howell, 2010; Hetz, 2012; Higa and Chevet, 2012; Jäger et al., 2012). **Figure 1A** summarizes processes involved in UPR activation by the three ER stress sensors in animals. BiP release allows ATF6 translocation to the Golgi apparatus, where its cytosolic part (cATF6), a functional bZIP transcription factor, is cleaved off by serine proteases S1P and S2P, a process called regulated intramembrane proteolysis (RIP). cATF6 then enters the nucleus and promotes transcription of *UPR* genes and the bZIP transcription factor *XBP1* (Yoshida et al., 2001). Upon BiP release, IRE1 oligomerizes and activates its endoribonuclease domain, leading

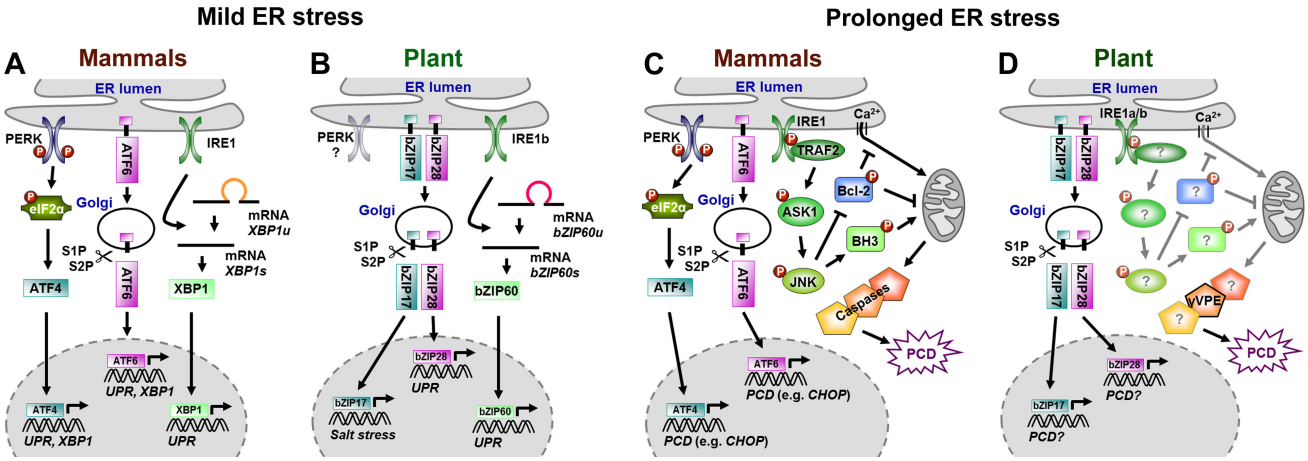


FIGURE 1 | Signaling in mammals and plants under mild (A,B) and prolonged ER stress (C,D). Models indicate overlaps and differences in ER stress signaling. Conservation in mammalian **(A)** and plant **(B)** UPR signaling in response to mild ER stress. Various components involved in mammalian ER-PCD signaling under prolonged ER stress have been identified **(C)**, whereas plant ER-PCD signaling is almost unknown **(D)**. Question marks (in **D**) indicate postulated orthologs or structural homologs of plant ER-PCD signaling. XBP1u/bZIP60u, unspliced mRNA; XBP1s/bZIP60s, spliced mRNA.

to the unconventional splicing of a 26 nucleotide intron out of *XBP1* or its yeast counterpart *HAC1*, which allows the resulting proteins to enter the nucleus (Mori, 2009; Walter and Ron, 2011; Hetz, 2012). Phosphorylation by the PERK kinase activates the eukaryotic translation initiation factor eIF2α, which attenuates translation but selectively promotes the translation of the transcription factor ATF4 (Harding et al., 2000). Eventually, ATF4, ATF6, and XBP1 (HAC1) elevate transcription of *UPR* genes (Mori, 2009; Walter and Ron, 2011; Hetz, 2012).

In plants, the ER-QC and ER stress responses are apparently conserved as suggested by sequence homologies found in *Arabidopsis* for members of the ER translocon and oligosaccharyltransferase complexes as well as for UPR and ERAD components (Liu and Howell, 2010). Further, transcripts of genes encoding proteins of the ER-QC machinery [e.g., chaperones BiPs, CALRETICULINs (CRTs), CALNEXINs (CNXs) or PROTEIN DISULFIDE ISOMERASEs (PDIs)], or the ERAD pathway are induced by ER stress (Jelitto-Van Dooren et al., 1999; Leborgne-Castel et al., 1999; Koizumi et al., 2001; Martínez and Chrispeels, 2003; Kamauchi et al., 2005; Lu and Christopher, 2008; Su et al., 2011; Hüttner and Strasser, 2012). Putative plant ER stress sensors and signaling components have been identified (**Figure 1B**), however, except for IRE, respective plant proteins do not show sequence but structural or functional homology (Koizumi et al., 2001; Liu and Howell, 2010). *Arabidopsis* possesses at least two IRE1-like proteins, while only one homolog is present in rice (*Oryza sativa*). AtIRE1a, AtIRE1b, and OsIRE1 harbor all structural features of yeast and mammalian IRE1. AtIRE1a and OsIRE1 are capable of autotransphosphorylation, and the putative ER stress sensor domain of AtIRE1a, AtIRE1b, and OsIRE1 can functionally replace that of yeast IRE1 (Koizumi et al., 2001; Noh et al., 2002; Okushima et al., 2002). There are at least three ER-resident transmembrane bZIP transcription factors in *Arabidopsis*, which are involved in ER stress responses, AtbZIP17, AtbZIP28, and AtbZIP60 (Urade, 2009; Liu and Howell, 2010). *Atbzip* mutants do not display morphological or developmental differences under non-stress conditions, but are more sensitive to salt stress (*Atbzip17*, Liu et al., 2007b), heat (*Atbzip28*, Gao et al., 2008), or DTT treatment (*Atbzip60*, Humbert et al., 2012). The expression of salt stress responsive genes is impaired in *Atbzip17* mutants (Liu et al., 2007b) as is the induction of canonical *UPR* genes in *Atbzip28* and *Atbzip60* mutants after TM treatment (Iwata and Koizumi, 2005a; Liu et al., 2007a; Iwata et al., 2008; Lu and Christopher, 2008; Tajima et al., 2008). Similar to ATF6 in mammals, AtbZIP17 and AtbZIP28 possess canonical S1P cleavage sites and are activated by a RIP-like process upon ER stress (Liu et al., 2007a,b, 2008a; Gao et al., 2008; Tajima et al., 2008; Che et al., 2010). RIP of AtbZIP17 and AtbZIP28 requires passage through the Golgi apparatus, where cleavage by the subtilisin-like serine protease AtS1P and subsequent processing by the metalloprotease AtS2P take place (Liu et al., 2007a,b; Che et al., 2010; Srivastava et al., 2012). How these bZIPs sense ER stress and how Golgi transition is mediated, is not clear. However, TM treatment apparently promotes the interaction of AtbZIP28 with the small GTPase SAR1b and the guanidine exchange factor SEC12, which are putatively involved in coat protein complex II (COPII) vesicle formation during ER-to-Golgi transport (Srivastava et al., 2012). AtbZIP60 lacks a canonical S1P cleavage site and its activation is independent of S1P and S2P (Iwata et al., 2008). Similar to mammalian XBP1 and yeast HAC1, recent studies in *Arabidopsis* and rice revealed unconventional splicing of a 23 nucleotide intron from the *AtbZIP60* mRNA by AtIRE1b or AtIRE1a, and a 20 nucleotide intron from its rice ortholog*OsbZIP50/OsbZIP74* mRNA by OsIRE1, e.g., after TM or salicylic acid (SA) treatment. This leads to a frame shift that removes the transmembrane domain of the new proteins and allows nuclear entrance (Deng et al., 2011; Nagashima et al., 2011; Hayashi et al., 2012; Humbert et al., 2012; Lu et al., 2012; Moreno et al., 2012). There are no obvious PERK homologs in *Arabidopsis* (Koizumi et al., 2001; Urade, 2009).

ER STRESS AS INITIATOR OF PROGRAMED CELL DEATH

The UPR is supposed to ensure cell survival. However, under prolonged or severe ER stress, mammalian cells activate an apoptosis-like programed cell death (ER-PCD) to eliminate damaged cells from stressed organisms (Schröder, 2006; Hetz, 2012; Jäger et al., 2012). The ER stress sensors ATF6, PERK, and IRE1 are central regulators of this process as well (**Figure 1C**), although it is unclear how they perceive and differentiate signals to switch from UPR to apoptosis. ER-PCD obviously merges with other apoptosis pathways, involving enhanced generation of reactive oxygen species (ROS), and apoptosis-promoting Ca^{2+} signaling at ER and mitochondria (Chakrabarti et al., 2011; Gorman et al., 2012; Hetz, 2012; Jäger et al., 2012). The induction of the pro-apoptotic bZIP transcription factor CHOP (C/EBP-homologs protein) by ATF6 and PERK/ATF4 during ER-PCD apparently is most relevant. CHOP down-regulates anti-apoptotic proteins (e.g., BCL-2), but induces members of the pro-apoptotic (BH3)-only protein family, e.g., BIM (BCL-2-INTERACTING MEDIATOR OF CELL DEATH) or GADD34 (GROWTH ARREST AND DNA DAMAGE-INDUCIBLE 34; Gorman et al., 2012; Hetz, 2012; Jäger et al., 2012). In addition, IRE1 activates ER-PCD by interacting with TRAF2 (TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR 2; Gorman et al., 2012; Jäger et al., 2012). This initiates consecutive phosphorylation of ASK1 (APOPTOSIS SIGNAL-REGULATING KINASE 1) and JNK (JUN N-TERMINAL KINASE). Phosphorylation by JNK inactivates anti-apoptotic regulators such as BCL-2, but activates pro-apoptotic BH3-only proteins such as BIM or BID (BH3-interacting domain death agonist). BH3-only proteins promote the cell death activation-related oligomerization and translocation of BAX and BAK to the mitochondrial membrane, followed by cytochrome *c* release and caspase activation for execution of apoptosis. BCL-2-dependent regulation of Ca^{2+} homeostasis of the ER also affects permeability transition and apoptosis signaling at mitochondria (Chakrabarti et al., 2011; Gorman et al., 2012; Hetz, 2012). BAX and BAK themselves can interact with IRE1 and promote its ability to activate ASK1 and JNK, processes that are apparently blocked by the cell survival protein BI-1 (BAX INHIBITOR-1; Bailly-Maitre et al., 2009; Lisbona et al., 2009). Dynamic differential interactions with pro- and anti-apoptotic proteins modulated by the intensity and duration of ER stress signals might regulate separate functions of IRE1, and timely coordinated on- and offset of ATF6, PERK, and IRE1 signaling may play a decisive role in determining cell fate. In such a scenario, ER stress would initially activate the adaptive UPR via IRE1-mediated splicing of XBP1. However, down-regulation of the IRE1/XBP1 branch upon prolonged ER stress may give rise to pro-apoptotic IRE1/TREF2/ASK1/JNK, RIDD, and/or PERK signaling (Gorman et al., 2012; Hetz, 2012). Autophagy is further suggested to abolish ER stress in yeast and mammals as it might support the removal of unfolded proteins (Bernales et al., 2006). Here, the PERK-elF2α-ATF4 and IRE/TRAF2/JNK pathways might connect autophagy to ER stress via the BECLIN1-BCL2 interaction and the induction of autophagy genes, respectively. Although ER stress-associated autophagy is thought to have a cytoprotective function, other studies suggest a role in ER-PCD. However, regulators of this cell death pathway and its link to ER stress are currently unknown (Verfaillie et al., 2010; Aronson and Davies, 2012).

As in animal cells, cell death follows induction of UPR in TM-treated plants (Zuppini et al., 2004; Iwata and Koizumi, 2005b; Watanabe and Lam, 2008; Ishikawa et al., 2011). The molecular basis of plant ER-PCD and the role of plant bZIPs therein are largely unknown (**Figure 1D**). However, regulation of ER-PCD seems to be partially conserved across kingdoms, as *Arabidopsis* BI-1 (AtBI-1) is involved in restriction of ER-PCD in *Arabidopsis* as well (Watanabe and Lam, 2008; Ishikawa et al., 2011). *AtBI-1* is AtbZIP60-dependently up-regulated in response to TM (Kamauchi et al., 2005; Iwata et al., 2008; Watanabe and Lam, 2008). AtBI-1-mediated inhibition of ER-PCD in *Arabidopsis* is likely un-related to UPR modification, but rather to the suppression of ER-dependent ROS production or regulation of cell death associated ER Ca^{2+} homeostasis (Watanabe and Lam, 2008, 2009). In *Arabidopsis*, a Gβ subunit of an ER-resident heterotrimeric GTP-binding protein, AGB1, might be involved in the promotion of ER-PCD (Wang et al., 2007; Chen and Brandizzi, 2012). Disturbed ER protein retention after silencing of *NbERD2a*/*NbERD2b* interferes with ER-QC and reduces ER stress alleviation, resulting in enhanced PCD in response to bacterial pathogens (Xu et al., 2012). New insights into the role of vacuolar processing enzymes with caspase1-like activities in the execution of ER-PCD come from Qiang et al. (2012). These studies demonstrate the dependence of the mutualistic fungus *Piriformospora indica* on ER-PCD for successful *Arabidopsis* root colonization. *P. indica* induces ER stress but suppresses the adaptive UPR pathway. Consequently, the *P. indica*-induced ER stress triggers a vacuolar cell death pathway whose execution depends on γ VACUOLAR PROCESSING ENZYME (γVPE). This ER-PCD can be phenocopied by the application of TM to *Arabidopsis* roots. The analyses further show that γVPE is responsible for enhanced VPE and caspase 1-like activities during TM- and *P. indica*-induced ER-PCD (Qiang et al., 2012).

ER – EXECUTOR OF PLANT IMMUNITY AND PUTATIVE TARGET OF PATHOGEN EFFECTORS

Plants ward off pathogens by a multi-layered immune system. PM localized pattern recognition receptors (PRRs) detect conserved molecules, so-called microbe-associated molecular patterns (MAMPs), of invading microbes. Well-characterized PRRs are FLAGELLIN-SENSING 2 (FLS2), which recognizes bacterial flagellin, the ELONGATION-FACTOR TU (EF-Tu) RECEPTOR (EFR), which detects bacterial EF-Tu, and the chitin receptors CHITIN ELICITOR BINDING PROTEIN (CEBiP) and CHITIN ELICITOR RECEPTOR KINASE (CERK; Monaghan and Zipfel, 2012). MAMP perception by these PRRs initiates immune signaling pathways, defined as MAMP-triggered immunity (MTI), which involve Ca^{2+} fluxes across the PM, a rapid production of ROS, the activation of mitogen-activated protein kinase cascades and WRKY transcription factors, eventually resulting in the induction of defense mechanisms including callose depositions and the synthesis of antimicrobial pathogenesis-related (PR) proteins (Jones and Dangl, 2006; Boller and Felix, 2009). Successful pathogens have evolved effector molecules to suppress MTI. Plant RESISTANCE (R) proteins specifically recognize pathogen effectors or their activities and initiate effector-triggered immunity

(ETI), typically involving hypersensitive response (HR)-related PCD (Chisholm et al., 2006; Jones and Dangl, 2006). The ER participates in plant innate immunity in several ways. Firstly, immunity depends on the secretory apparatus for the production of immune proteins (Wang et al., 2005; Nekrasov et al., 2009; Saijo et al., 2009). NONEXPRESSOR OF PR GENES 1 (NPR1), the master regulator of SA-dependent systemic acquired resistance (SAR), coordinately controls the up-regulation of *PR* genes and genes encoding proteins of the secretory pathway during SAR (Wang et al., 2005). Secondly, synthesis and proper function of PRRs (e.g., EFR) rely on *N*-glycosylation and the ER-QC system, which involves staurosporine and temperature sensitive-3a (STT3A), glucosidase II, the H/KDEL receptor ERD2b, the UDP-glucose:glycoprotein glucosyltransferase (UGGT)/CRT3 cycle and the stromal cell-derived factor-2 (SDF2)/ERdj3B/BiP complex (Li et al., 2009; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009; Saijo, 2010). Susceptibility of ER-QC mutants to pathogens differs qualitatively and quantitatively from that of *efr* mutants, suggesting the existence of EFR-independent but ER-QC-dependent immune response (Li et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). Meanwhile, a number of membrane-localized immune receptors have been identified, whose functions depend on ER-QC, among them the rice PRR XA21 involved in resistance to *Xanthomonas oryzae* pv. *oryzae* (Park et al., 2010a,b), an induced receptor kinase (IRK), which is involved in *N*-mediated resistance of tobacco to tobacco mosaic virus (Caplan et al., 2009), and glycosylated Cf proteins, which confer race-specific resistance to the fungal pathogen *Cladosporium fulvum* (Liebrand et al., 2012). Similar to FLS2, the ER-QC disturbance does not affect CERK1 function in *Arabidopsis* (Li et al., 2009; Nekrasov et al., 2009). However, the rice homolog OsCERK1 seems to interact with a Hop/Sti1-Hsp90 chaperone complex for maturation in the ER prior to transport to the PM (Chen et al., 2010). ER-QC also monitors glycosylation and proper folding of some immunity-related Toll-like receptors (TLRs) that recognize MAMPs in animals (Yang et al., 2007). Interestingly, PRRs TLR4 and TLR2 activate the IRE1α-XBP1 pathway to enhance secretion of certain proinflammatory cytokines in macrophages, and loss of XBP1 function impairs immunity against the bacterial pathogen *Francisella tularensis* (Martinon et al., 2010).

Induction of the ER-QC machinery accompanies synthesis of immunity-associated proteins in plants (Jelitto-Van Dooren et al., 1999; Wang et al., 2005). Consequently, ER-QC mutants are more susceptible to ER stress inducers and pathogens (Wang et al., 2005; Li et al., 2009; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). Similarly, proper execution of defense responses may rely on the induction of *UPR* genes. Recently, the heat-shock factor-like transcription factor TBF1 has been identified as important transcriptional regulator of *UPR* genes, and *Arabidopsis tbf1* mutants are impaired in the execution of SAR and EFR-mediated MTI (Pajerowska-Mukhtar et al., 2012). The *Nicotiana benthamiana* homolog of *AtbZIP60*, *NbbZIP60*, is induced in response to inoculation with avirulent *Pseudomonas cichorii* and required to arrest its growth (Tateda et al., 2008). Furthermore, *AtIRE1a* and *AtIRE1b* expression is pathogen-responsive, and both proteins are required for SA or pathogen-dependent splicing of *AtbZIP60*, expression of ER-QC genes, secretion of defense proteins and thus execution of SAR (Moreno et al., 2012).

Together, this underlines the functional importance of the ER in both MTI and ETI, and designates it as a potential effector target. Consistent with this, many viruses employ host UPR by targeting ER stress sensors to enhance folding of viral proteins or to modulate immune responses in mammals (Ke and Chen, 2011; Qian et al., 2012). In tobacco, infection with *Potato virus X* or overexpression of a viral movement protein induces *bZIP60* and *UPR* genes possibly to suppress host cell death responses (Ye et al., 2011). In addition, Yamamoto et al. (2011) showed that ATF6β is part of mice immunity against the protozoan parasite *Toxoplasma gondii*. ROP18, a serine/threonine kinase, which is secreted into the host cell during infection, interacts with ATF6β and mediates its proteasome-dependent degradation. Thus, ATF6β constitutes a target for the *T. gondii* ROP18 virulence factor possibly to suppress UPR-mediated host defense. Likewise, the *Salmonella enterica* leucine-rich repeat (LRR) effector protein SlrP targets the host ER-QC member ERdj3. This supports infection as it leads to the accumulation of unfolded proteins eventually promoting host cell death (Bernal-Bayard et al., 2010). In *Caenorhabditis elegans*, the increased requirement of secreted proteins during the activation of immune responses imposes ER stress to the organism itself, which requires XBP1-mediated UPR to avoid onset of ER-PCD (Richardson et al., 2010). Several bacterial toxins, e.g., Shiga toxin produced by enterohemorrhagic bacteria, can enter the ER and seem to initiate cell death through prolonged UPR signaling by activating ER stress sensors (Tesh, 2012).

CONCLUSIONS AND PERSPECTIVE

As production site of antimicrobial proteins and of immune signaling components, the ER functions as central regulator in the execution of immune responses in plants and animals. Therefore, the disturbance of ER integrity is certainly of primary relevance for pathogens to achieve host cell infection. Plants further rely on proper ER function and likely ER membrane localized stress sensors for adaptation to abiotic stress such as salt or heat stress (Liu et al., 2008a,b, 2011; Che et al., 2010; Cui et al., 2012). Taken together, the improvement of plant UPR in order to maintain ER homeostasis under unfavorable conditions may increase plant adaptability to biotic and abiotic stress, which bears a potential to enhance crop yield and yield stability.

ACKNOWLEDGMENTS

We kindly thank the German Research Council (Deutsche Forschungsgemeinschaft) for financial support through grants to Ruth Eichmann (EI835/1-1) and Patrick Schäfer (SCHA1444/3-3).

REFERENCES

Aronson, L. I., and Davies, F. E. (2012). DangER: protein ovERload – targeting protein degradation to treat myeloma. *Haematologica* 97, 1119–1130.

Bailly-Maitre, B., Belgardt, B. F., Jordan, S. D., Coornaert, B., von Freyend, M. J., Kleinridders, A., Mauer, J., Cuddy, M., Kress, C. L., Willmes, D., Essig, M., Hampel, B., Protzer, U., Reed, J. C., and Brüning, J. C. (2009). Hepatic Bax inhibitor-1 inhibits IRE1α and protects from obesity-associated insulin resistance and glucose intolerance. *J. Biol. Chem.* 285, 6198–6207.

Bernal-Bayard, J., Cardenal-Muñoz, E., and Ramos-Morales, F. (2010). The *Salmonella* type III secretion effector, *Salmonella* leucine-rich repeat protein (SlrP), targets the human chaperone ERdj3. *J. Biol. Chem.* 285, 16360–16368.

Bernales, S., McDonald, K. L., and Walter, P. (2006). Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. *PLoS Biol.* 4, e423. doi: 10.1371/journal.pbio.0040423

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.

Caplan, J. L., Zhu, X., Mamillapalli, P., Marathe, R., Anandalakshmi, R., and Dinesh-Kumar, S. P. (2009). Induced ER chaperones regulate a receptor-like kinase to mediate antiviral innate immune response in plants. *Cell Host Microbe* 6, 457–469.

Chakrabarti, A., Chen, A. W., and Varner, J. D. (2011). A review of the mammalian unfolded protein response. *Biotechnol. Bioeng.* 108, 2777–2793.

Che, P., Bussell, J. D., Zhou, W., Estavillo, G. M., Pogson, B. J., and Smith, S. M. (2010). Signaling from the endoplasmic reticulum activates brassinosteroid signaling and promotes acclimation to stress in *Arabidopsis*. *Sci. Signal.* 3, ra69.

Chen, L., Hamada, S., Fujiwara, M., Zhu, T., Thao, N. P., Wong, H. L., Krishna, P., Ueda, T., Kaku, H., Shibuya, N., Kawasaki, T., and Shimamoto, K. (2010). The Hop/Sti1-Hsp90 chaperone complex facilitates the maturation and transport of a PAMP receptor in rice innate immunity. *Cell Host Microbe* 7, 185–196.

Chen, Y., and Brandizzi, F. (2012). AtIRE1A/AtIRE1B and AGB1 independently control two essential unfolded protein response pathways in *Arabidopsis*. *Plant J.* 69, 266–277.

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803–814.

Cui, F., Liu, L., Zhao, Q., Zhang, Z., Li, Q., Lin, B., Wu, Y., Tang, S., and Xie, Q. (2012). *Arabidopsis* ubiquitin conjugase UBC32 is an ERAD component that functions in brassinosteroid-mediated salt stress tolerance. *Plant Cell* 24, 233–244.

Deng, Y., Humbert, S., Liu, J. X., Srivastava, R., Rothstein, S. J., and Howell, S. H. (2011). Heat induces the splicing by IRE1 of a mRNA encoding a transcription factor involved in the unfolded protein response in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7247–7252.

Gao, H., Brandizzi, F., Benning, C., and Larkin, R. M. (2008). A membrane-tethered transcription factor defines a branch of the heat stress response in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16398–16403.

Gorman, A. M., Healy, S. J., Jäger, R., and Samali, A. (2012). Stress management at the ER: regulators of ER stress-induced apoptosis. *Pharmacol. Ther.* 134, 306–316.

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* 6, 1099–1108.

Hayashi, S., Wakasa, Y., Takahashi, H., Kawakatsu, T., and Takaiwa, F. (2012). Signal transduction by IRE1-mediated splicing of bZIP50 and other stress sensors in the endoplasmic reticulum stress response of rice. *Plant J.* 69, 946–956.

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.* 13, 89–102.

Higa, A., and Chevet, E. (2012). Redox signaling loops in the unfolded protein response. *Cell. Signal.* 24, 1548–1555.

Humbert, S., Zhong, S., Deng, Y., Howell, S. H., and Rothstein, S. J. (2012). Alteration of the bZIP60/IRE1 pathway affects plant response to ER stress in *Arabidopsis thaliana*. *PLoS ONE* 7, e39023. doi: 10.1371/journal.pone.0039023

Hüttner, S., and Strasser, R. (2012). Endoplasmic reticulum-associated degradation of glycoproteins in plants. *Front. Plant Sci.* 3: 67. doi: 10.3389/fpls.2012.00067

Ishikawa, T., Watanabe, N., Nagano, M., Kawai-Yamada, M., and Lam, E. (2011). Bax inhibitor-1: a highly conserved endoplasmic reticulum-resident cell death suppressor. *Cell Death Differ.* 18, 1271–1278.

Iwata, Y., Fedoroff, N. V., and Koizumi, N. (2008). *Arabidopsis* bZIP60 is a proteolysis-activated transcription factor involved in the endoplasmic reticulum stress response. *Plant Cell* 20, 3107–3121.

Iwata, Y., and Koizumi, N. (2005a). An *Arabidopsis* transcription factor, AtbZIP60, regulates the endoplasmic reticulum stress response in a manner unique to plants. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5280–5285.

Iwata, Y., and Koizumi, N. (2005b). Unfolded protein response followed by induction of cell death in cultured tobacco cells treated with tunicamycin. *Planta* 220, 804–807.

Jäger, R., Bertrand, M. J., Gorman, A. M., Vandenabeele, P., and Samali, A. (2012). The unfolded protein response at the crossroads of cellular life and death during endoplasmic reticulum stress. *Biol. Cell* 104, 259–270.

Jelitto-Van Dooren, E. P., Vidal, S., and Denecke, J. (1999). Anticipating endoplasmic reticulum stress. A novel early response before pathogenesis-related gene induction. *Plant Cell* 11, 1935–1944.

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

Kamauchi, S., Nakatani, H., Nakano, C., and Urade, R. (2005). Gene expression in response to endoplasmic reticulum stress in *Arabidopsis thaliana*. *FEBS J.* 272, 3461–3476.

Ke, P. Y., and Chen, S. S. (2011). Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro. *J. Clin. Invest.* 121, 37–56.

Koizumi, N., Martinez, I. M., Kimata, Y., Kohno, K., Sano, H., and Chrispeels, M. J. (2001). Molecular characterization of two *Arabidopsis* Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. *Plant Physiol.* 127, 949–962.

Leborgne-Castel, N., Jelitto-Van Dooren, E. P. W. M., Crofts, A. J., and Denecke, J. (1999). Overexpression of BiP in tobacco alleviates endoplasmic reticulum stress. *Plant Cell* 11, 459–469.

Li, J., Zhao-Hui, C., Batoux, M., Nekrasov, V., Roux, M., Chinchilla, D., Zipfel, C., and Jones, J. D. (2009). Specific ER quality control components required for biogenesis of the plant innate immune receptor EFR. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15973–15978.

Liebrand, T. W., Smit, P., Abd-El-Haliem, A., de Jonge, R., Cordewener, J. H., America, A. H., Sklenar, J., Jones, A. M., Robatzek, S., Thomma, B. P., Tameling, W. I., and Joosten, M. H. (2012). ER-quality control chaperones facilitate the biogenesis of Cf receptor-like proteins involved in pathogen resistance of tomato. *Plant Physiol.* 159, 1819–1833.

Lisbona, F., Rojas-Rivera, D., Thielen, P., Zamorano, S., Todd, D., Martinon, F., Glavic, A., Kress, C., Lin, J. H., Walter, P., Reed, J. C., Glimcher, L. H., and Hetz, C. (2009). BAX inhibitor-1 is a negative regulator of the ER stress sensor IRE1α. *Mol. Cell* 33, 679–691.

Liu, J. X., and Howell, S. H. (2010). Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *Plant Cell* 22, 2930–2942.

Liu, J. X., Srivastava, R., Che, P., and Howell, S. H. (2007a). An endoplasmic reticulum stress response in *Arabidopsis* is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28. *Plant Cell* 19, 4111–4119.

Liu, J. X., Srivastava, R., Che, P., and Howell, S. H. (2007b). Salt stress responses in *Arabidopsis* utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. *Plant J.* 51, 897–909.

Liu, J. X., Srivastava, R., Che, P., and Howell, S. H. (2008a). Salt stress signaling in *Arabidopsis thaliana* involves a membrane-bound transcription factor AtbZIP17 as a signal transducer. *Plant Signal. Behav.*3, 56–57.

Liu, J. X., Srivastava, R., and Howell, S. H. (2008b). Stress-induced expression of an activated form of AtbZIP17 provides protection from salt stress in *Arabidopsis*. *Plant Cell Environ.* 31, 1735–1743.

Liu, L., Cui, F., Li, Q., Yin, B., Zhang, H., Lin, B., Wu, Y., Xia, R., Tang, S., and Xie, Q. (2011). The endoplasmic reticulum-associated degradation is necessary for plant salt tolerance. *Cell Res.* 21, 957–969.

Lu, D. P., and Christopher, D. A. (2008). Endoplasmic reticulum stress activates the expression of a subgroup of protein disulfideisomerase genes and AtbZIP60 modulates the response in *Arabidopsis thaliana*. *Mol. Genet. Genomics* 280, 199–210.

Lu, S. J., Yang, Z. T., Sun, L., Sun, L., Song, Z. T., and Liu, J. X. (2012). Conservation of IRE1-regulated bZIP74 mRNA unconventional splicing in rice (*Oryza sativa* L.) involved in ER stress responses. *Mol. Plant* 5, 504–514.

Lu, X., Tintor, N., Mentzel, T., Kombrink, E., Boller, T., Robatzek, S., Schulze-Lefert, P., and Saijo, Y. (2009). Uncoupling of sustained MAMP receptor signaling from early outputs in an *Arabidopsis* endoplasmic reticulum glucosidase II allele. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22522–22527.

Lynes, E. M., and Simmen, T. (2011). Urban planning of the endoplasmic

reticulum (ER): how diverse mechanisms segregate the many functions of the ER. *Biochim. Biophys. Acta* 1813, 1893–1905.

Martínez, I. M., and Chrispeels, M. J. (2003). Genomic analysis of the unfolded protein response in *Arabidopsis* shows its connection to important cellular processes. *Plant Cell* 15, 561–576.

Martinon, F., Chen, X., Lee, A. H., and Glimcher, L. H. (2010). TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. *Nat. Immunol.* 11, 411–418.

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15, 349–357.

Moreno, A. A., Mukhtar, M. S., Blanco, F., Boatwright, J. L., Moreno, I., Jordan, M. R., Chen, Y., Brandizzi, F., Dong, X., Orellana, A., and Pajerowska-Mukhtar, K. M. (2012). IRE1/bZIP60-mediated unfolded protein response plays distinct roles in plant immunity and abiotic stress responses. *PLoS ONE* 7, e31944. doi: 10.1371/journal.pone.0031944

Mori, K. (2009). Signalling pathways in the unfolded protein response: development from yeast to mammals. *J. Biochem.* 146, 743–750.

Nagashima, Y., Mishiba, K., Suzuki, E., Shimada, Y., Iwata, Y., and Koizumi, N. (2011). *Arabidopsis* IRE1 catalyses unconventional splicing of bZIP60 mRNA to produce the active transcription factor. *Sci. Rep.* 1, 29.

Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z. H., Lacombe, S., Rougon, A., Bittel, P., Kiss-Papp, M., Chinchilla, D., van Esse, H. P., Jorda, L., Schwessinger, B., Nicaise, V., Thomma, B. P., Molina, A., Jones, J. D., and Zipfel, C. (2009). Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO J.* 28, 3428–3438.

Noh, S. J., Kwon, C. S., and Chung, W. I. (2002). Characterization of two homologs of Ire1p, a kinase/endoribonuclease in yeast, in *Arabidopsis thaliana. Biochim. Biophys. Acta* 1575, 130–134.

Okushima, Y., Koizumi, N., Yamaguchi, Y., Kimata, Y., Kohno, K., and Sano, H. (2002). Isolation and characterization of a putative transducer of endoplasmic reticulum stress in *Oryza sativa. Plant Cell Physiol.* 43, 532–539.

Pajerowska-Mukhtar, K. M., Wang, W., Tada, Y., Oka, N., Tucker, C. L., Fonseca, J. P., and Dong, X. (2012). The HSF-like transcription factor TBF1 is a major molecular switch for plant growth-to-defense transition. *Curr. Biol.* 22, 103–112.

Park, C. J., Bart, R., Chern, M., Canlas, P. E., Bai, W., and Ronald, P. C. (2010a). Overexpression of the endoplasmic reticulum chaperone BiP3 regulates XA21-mediated innate immunity in rice. *PLoS ONE* 5, e9262. doi: 10.1371/journal.pone.0009262

Park, C. J., Han, S. W., Chen, X., and Ronald, P. C. (2010b). Elucidation of XA21-mediated innate immunity. *Cell. Microbiol.* 12, 1017–1025.

Qian, Z., Xuan, B., Chapa, T. J., Gualberto, N., and Yu, D. (2012). Murine cytomegalovirus targets transcription factor ATF4 to exploit the unfolded-protein response. *J. Virol.* 86, 6712–6723.

Qiang, X., Zechmann, B., Reitz, M. U., Kogel, K. H., and Schäfer, P. (2012). The mutualistic fungus *Piriformospora indica* colonizes *Arabidopsis* roots by inducing an endoplasmic reticulum stress-triggered caspase-dependent cell death. *Plant Cell* 24, 794–809.

Richardson, C. E., T. Kooistra, and Kim, D. H. (2010). An essential role for XBP-1 in host protection against immune activation in *C. elegans. Nature* 463, 1092–1095.

Saijo, Y. (2010). ER quality control of immune receptors and regulators in plants. *Cell. Microbiol.* 12, 716–724.

Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajerowska-Mukhtar, K., Häweker, H., Dong, X., Robatzek, S., and Schulze-Lefert, P. (2009). Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J.* 28, 3439–3449.

Schröder, M. (2006). The unfolded protein response. *Mol. Biotechnol.* 34, 279–290.

Schröder, M. (2008). Endoplasmic reticulum stress responses. *Cell. Mol. Life. Sci.* 65, 862–894.

Sparkes, I. A., Frigerio, L., Tolley, N., and Hawes, C. (2009). The plant endoplasmic reticulum: a cell-wide web. *Biochem. J.* 423,145–155.

Srivastava, R., Chen, Y., Deng, Y., Brandizzi, F., and Howell, S. H. (2012). Elements proximal to and within the transmembrane domain mediate the organelle-to-organelle movement of bZIP28 under ER stress conditions. *Plant J.* 70, 1033–1042.

Staehelin, L. A. (1997). The plant ER: a dynamic organelle composed of a large number of discrete functional domains. *Plant J.* 11, 1151–1165.

Su, W., Liu, Y., Xia, Y., Hong, Z., and Li, J. (2011). Conserved endoplasmic reticulum-associated degradation system to eliminate mutated receptor-like kinases in *Arabidopsis. Proc. Natl. Acad. Sci. U.S.A.* 108, 870–875.

Tajima, H., Iwata, Y., Iwano, M., Takayama, S., and Koizumi, N. (2008). Identification of an *Arabidopsis* transmembrane bZIP transcription factor involved in the endoplasmic reticulum stress response. *Biochem. Biophys. Res. Commun.* 374, 242–247.

Tateda, C., Ozaki, R., Onodera, Y., Takahashi, Y., Yamaguchi, K., Berberich, T., Koizumi, N., and Kusano, T. (2008). NtbZIP60, an endoplasmic reticulum-localized transcription factor, plays a role in the defense response against bacterial pathogens in *Nicotiana tabacum. J. Plant Res.* 121, 603–611.

Tesh, V. L. (2012). Activation of cell stress response pathways by Shiga toxins. *Cell. Microbiol.* 14, 1–9.

Urade, R. (2009). The endoplasmic reticulum stress signaling pathways in plants. *Biofactors* 35, 326–331.

Vitale, A., and Boston, R. S. (2008). Endoplasmic reticulum quality control and the unfolded protein response: insights from plants. *Traffic* 9, 1581–1588.

Verfaillie, T., Salazar, M., Velasco, G., and Agostinis, P. (2010). Linking ER stress to autophagy: potential implications for cancer therapy. *Int. J. Cell Biol.* 2010, 930509.

Walter, P., and Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334, 1081–1086.

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.

Wang, S., Narendra, S., and Fedoroff, N. (2007). Heterotrimeric G protein signaling in the *Arabidopsis* unfolded protein response. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3817–3822.

Watanabe, N., and Lam, E. (2008). BAX inhibitor-1 modulates endoplasmic reticulum stress-mediated programmed cell death in *Arabidopsis. J. Biol. Chem.* 283, 3200–3210.

Watanabe, N., and Lam, E. (2009). Bax inhibitor-1, a conserved cell death suppressor, is a key molecular switch downstream from a variety of biotic and abiotic stress signals in plants. *Int. J. Mol. Sci.* 10, 3149–3167.

Xu, G., Li, S., Xie, K., Zhang, Q., Wang, Y., Tang, Y., Liu, D., Hong, Y., He, C., and Liu, Y. (2012). Plant ERD2-like proteins function as ER luminal protein receptors and participate in programmed cell death during innate immunity. *Plant J.* doi: 10.1111/j.1365-313X.2012.05053.x [Epub ahead of print].

Yamamoto, M., Ma, J. S., Mueller, C., Kamiyama, N., Saiga, H., Kubo, E., Kimura, T., Okamoto, T., Okuyama, M., Kayama, H., Nagamune, K., Takashima, S., Matsuura, Y., Soldati-Favre, D., and Takeda, K. (2011). ATF6β is a host cellular target of the *Toxoplasma gondii* virulence factor ROP18. *J. Exp. Med.* 208, 1533–1546.

Yang, Y., Liu, B., Dai, J., Srivastava, P. K., Zammit, D. J., Lefrancois, L., and Li, Z. (2007). Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. *Immunity* 26, 215–226.

Ye, C., Dickman, M. B., Whitham, S. A., Payton, M., and Verchot, J. (2011). The unfolded protein response is triggered by a plant viral movement protein. *Plant Physiol.* 156, 741–755.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107, 881–891.

Zuppini, A., Navazio, L., and Mariani, P. (2004). Endoplasmic reticulum stress-induced programmed cell death in soybean cells. *J. Cell Sci.* 117, 2591–2598.

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: 02 July 2012; paper pending published: 16 July 2012; accepted: 09 August 2012; published online: 22 August 2012.

*Citation: Eichmann R and Schäfer P (2012) The endoplasmic reticulum in plant immunity and cell death. Front. Plant Sci. **3**:200. doi: 10.3389/fpls.2012.00200*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 24 October 2012
doi: 10.3389/fpls.2012.00238

Regulation of plant immune receptors by ubiquitination

Giulia Furlan†, Jörn Klinkenberg† and Marco Trujillo*

Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany

Edited by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

Reviewed by:
Zhi-Yong Wang, Carnegie Institution for Science, USA
Sandra Goritschnig, University of California at Berkeley, USA

***Correspondence:**
Marco Trujillo, Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany.
e-mail: mtrujillo@ipb-halle.de

† Giulia Furlan and Jörn Klinkenberg have contributed equally to this work.

From pathogen perception and the activation of signal transduction cascades to the deployment of defense responses, protein ubiquitination plays a key role in the modulation of plant immunity. Ubiquitination is mediated by three enzymes, of which the E3 ubiquitin ligases, the substrate determinants, have been the major focus of attention. Accumulating evidence suggests that ubiquitination modulates signaling mediated by pattern recognition receptors and is important for the accumulation of nucleotide-binding leucine-rich repeat type intracellular immune sensors. Recent studies also indicate that ubiquitination directs vesicle trafficking, a function that has been clearly established for immune signaling in animals. In this mini review, we discuss these and other recent advances and highlight important open questions.

Keywords: E3 ubiquitin ligases, vesicle trafficking, receptor-like kinases, effectors, protein degradation, ubiquitination, PTI

INTRODUCTION

Ubiquitin is a highly conserved protein found in all eukaryotes and is involved in almost all aspects of plant physiology, including immunity. Ubiquitination is the reversible attachment of ubiquitin moieties to specific target proteins and it is mediated by three enzymes (Vierstra, 2009). In the initial step, ubiquitin is activated by an ubiquitin-activating enzyme (E1). The activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme. A ubiquitin ligase (E3) then binds the E2 and the target protein. The ligase generally acts as a scaffold bringing the E2 and the target into close proximity to mediate the linkage of ubiquitin via its C-terminal glycine to an ε-Lysine (Lys) residue of the target. Because E3 ligases determine the specificity of the reaction they have attracted by far the most attention. Target proteins can be modified by the attachment of single ubiquitin molecules (monoubiquitination) or of ubiquitin polymers linked internally through one of seven Lys residues present in ubiquitin (polyubiquitination). Generally, conjugated ubiquitin monomers or polymers act as portable recognition modules that facilitate protein–protein interaction.

Lys48-linked ubiquitin chains promote protein breakdown by the 26S proteasome, a proteolytic complex that degrades the target with the concomitant release of the ubiquitin moieties for reuse. Alternatively linked ubiquitin chains can direct non-proteolytic events that participate in the regulation of vesicular trafficking, chromatin structure, and transcription (Ikeda and Dikic, 2008).

Post-translational modifications, such as ubiquitination, play key roles in signal transduction cascades. Understanding how such modifications translate into signal modulation has become a major research focus in recent years. This mini review focuses on recent reports implicating ubiquitination in the regulation of plant immune sensors and vesicle trafficking.

UBIQUITINATION AND PATHOGEN PERCEPTION

The plant immune system can be conceptually divided into two branches characterized by different types of receptors (Jones and Dangl, 2006). The first branch is mediated by plasma membrane (PM) located pattern recognition receptors (PRRs), which recognize conserved pathogen molecules, so called pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by PRRs ultimately results in PAMP-triggered immunity (PTI). The second branch is activated by intracellular nucleotide-binding leucine-rich repeat (NB-LRR) immune sensors, which directly or indirectly perceive virulence factors, known as effectors, and results in the activation of effector-triggered immunity (ETI; Bent and Mackey, 2007).

A connection between ubiquitination and plant immunity was first suggested by a study showing that *suppressor of G2 allele of skp1* (*sgt1*) mutants were compromised in ETI (Azevedo et al., 2002). SGT1 is a component of the RAR1 (required for MLA12 resistance 1)-SGT1-HSP90 (heat shock protein 90) chaperone complex and association with components involved in protein ubiquitination has been shown for members of this complex. For example, S-phase kinase-associated protein 1 (SKP1) and its associated protein Cullin1, which are subunits of SKP1-Cullin1-F-box (SCF) ubiquitin ligases, were found to interact with SGT1 in plants (Azevedo et al., 2002; Liu et al., 2002). Subsequent studies have demonstrated that this chaperone complex plays a central role in the accumulation of NB-LRR proteins (Shirasu, 2009).

More recent findings have shown the direct regulation of NB-LRR accumulation through ubiquitin-mediated degradation via the 26S proteasome. Loss-of-function mutation of *constitutive expressor of PR genes 1* (*CPR1*, also named *CPR30*), which encodes an F-box motif protein, leads to the accumulation of the Toll–interleukin-receptor-like (TIR) type NB-LRR protein SNC1 (suppressor of *npr1-1*, constitutive 1) and the coiled-coil (CC) type NB-LRR protein RPS2 (resistance to *Pseudomonas syringae* 2) resulting in autoimmune responses (Cheng et al., 2011; Gou et al., 2012). Accordingly, *CPR1* overexpression reduced SNC1 and RPS2 levels and immune response intensity. CPR1 was shown to interact with the ASK1 (*Arabidopsis* SKP1) and ASK2 subunits of SCF

complexes. Furthermore, CPR1 interacts with SNC1 and RPS2, suggesting that they are its ubiquitination substrates and therefore mediate their stability (Gou et al., 2009; Cheng et al., 2011). Degradation mediated by CPR1 may reflect fine tuning mechanisms by which the plant is able to mount an immune response of appropriate intensity.

Another example of NB-LRR regulation by ubiquitination comes from a study conducted by Jeong et al. (2010), who uncovered an interesting link between light perception and immunity mediated by hypersensitive response to TCV (HRT), a CC-NB-LRR which mediates resistance against the *turnip crinkle virus* (TCV). HRT protein levels decreased in the dark or after blue-light induction, resulting in enhanced susceptibility. Application of proteasome inhibitor prevented blue-light-dependent degradation of HRT and consequently, plants were more resistant to TCV (Cooley et al., 2000; Jeong et al., 2010). HRT accumulation was reduced in mutants of the blue-light receptors cryptochrome 2 (CRY2) and phototropin 2 (PHO2). Importantly, HRT interacted with the ubiquitin ligase constitutively photomorphogenic 1 (COP1), but not with CRY2 or PHO2 (Jeong et al., 2010). Because CRY2 and PHO2 do interact with COP1 and they are required for HRT accumulation, it was proposed that they negatively regulate HRT degradation via COP1. However, the exact function of COP1 still remains to be determined.

In contrast to the intracellular NB-LRR immune sensors, surface-localized PRR receptor-like kinases (RLKs), relay external cues into the cell. PRRs recognize PAMPs such as flagellin, a component of the bacterial flagella, or chitin, a component of the fungal cell wall (Monaghan and Zipfel, 2012). Indication for the involvement of ubiquitination in the regulation of PRR signaling was first provided by the bacterial effector protein AvrPtoB, which is an active E3 ligase with a C-terminal U-box/RING-like domain (Janjusevic et al., 2006). AvrPtoB physically interacts with and ubiquitinates the flagellin receptor flagellin-sensitive 2 (FLS2; **Figure 1A**). Expression of AvrPtoB resulted in a reduction of FLS2 levels, indicating that AvrPtoB facilitates its degradation (Göhre et al., 2008). AvrPtoB is also able to ubiquitinate and mediate the degradation of at least one more PRR, namely the chitin receptor chitin elicitor receptor kinase 1 (CERK1; Gimenez-Ibanez et al., 2009).

Recently, the plant U-box (PUB) ligases PUB12 and PUB13 were shown to mediate the endogenous ubiquitination of FLS2 (Lu et al., 2011; **Figure 1B**). PUB12 and PUB13 interact constitutively with brassinosteroid-insensitive 1-associated receptor kinase 1 (BAK1), while treatment with flg22, a conserved N-terminal peptide of flagellin, is required to induce their interaction with FLS2 (Chinchilla et al., 2007). Although phosphorylation of PUB12 and PUB13 by BAK1 was needed for the interaction with FLS2, it

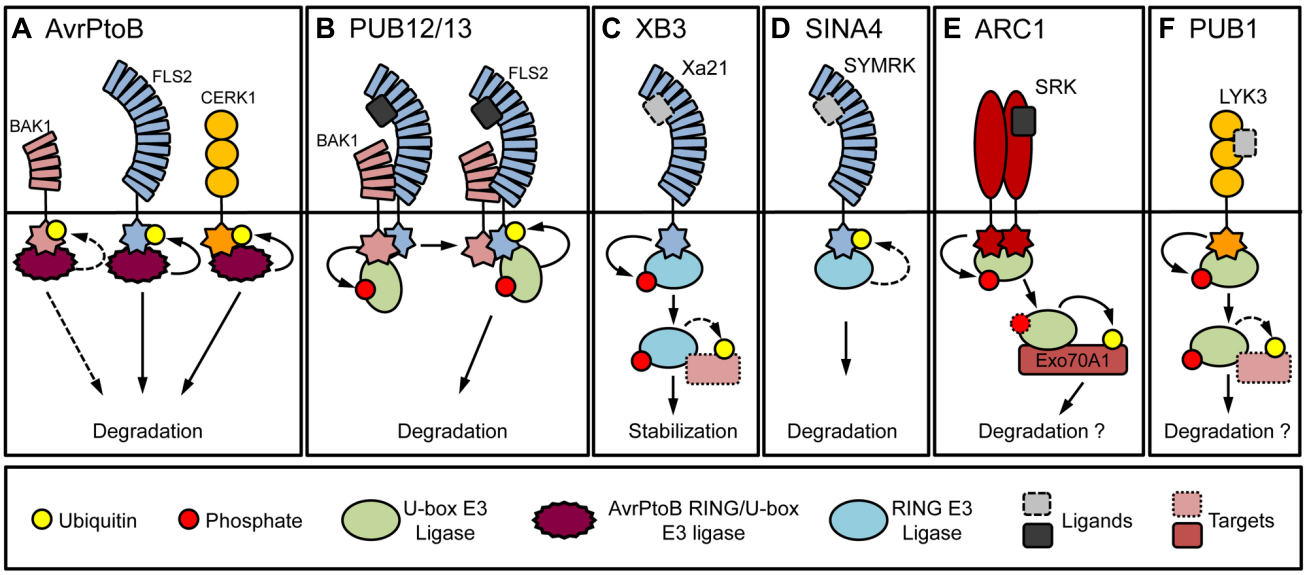


FIGURE 1 | Ubiquitin ligases that interact with receptor kinases. (A) The effector protein AvrPtoB from *P. syringae* pv. *tomato* binds to the co-receptor BAK1, the LRR-RLK FLS2 and the LysM RLK CERK1. AvrPtoB is able to ubiquitinate FLS2 and CERK1 and mediate their degradation. AvrPtoB can ubiquitinate BAK1 weakly *in vitro*. The mechanism leading to reduced RLK levels by AvrPtoB activity *in vivo* requires further clarification. **(B)** PUB12 and PUB13 constitutively interact with the co-receptor kinase BAK1. Constitutive phosphorylation of PUB12 and PUB13 by BAK1 is enhanced by flg22 which induces the interaction with FLS2. PUB12 and PUB13 ubiquitinate FLS2 and mediate its degradation. **(C)** The rice XB3 ligase interacts with the LRR-RLK XA21. XA21 phosphorylates XB3 *in vitro*. Whether ligand binding is required for the phosphorylation is not known. XB3 contributes to XA21 accumulation and is therefore unlikely to ubiquitinate XA21. XB3 conceivably targets a protein that affects XA21 accumulation. **(D)** The *L. japonicus* SINA4 was shown to interact with and negatively regulate the levels of the LRR-RLK SYMRK, which mediates symbiotic signaling. **(E)** The *B. napus* ARC1 interacts and is phosphorylated by the S-domain SRK, which mediates SI reaction. ARC1 was proposed to regulate SI through the degradation of Exo70A1. Further experimental clarification is needed to determine whether ARC1 affects SRK levels. **(F)** The *M. truncatula* PUB1 interacts with and is phosphorylated by LYK3, a LysM type RLK involved in nodulation. PUB1, a negative regulator of nodulation, does not ubiquitinate LYK3 *in vitro*. PUB1 might therefore target an alternate protein required for symbiosis. Shapes with dotted lines denote potential involvement (e.g., ligand) or a hypothetical target.

was dispensable for FLS2 ubiquitination. Interestingly, functional analysis of *pub12* and *pub13* mutants showed a phenotype reminiscent of *pub22*, *pub23*, and *pub24* mutants, which included enhanced responses to PAMPs and resistance to pathogens (Trujillo et al., 2008; Lu et al., 2011). Importantly, *pub12/pub13* double mutants displayed impaired reduction of FLS2 protein levels after flg22 treatment, indicating that they participate in the attenuation of signaling by regulating FLS2 turn-over. Of note, neither PUB12 or PUB13, nor AvrPtoB are able to effectively ubiquitinate BAK1 *in vitro* or affect BAK1 levels *in vivo*. Also, *in vitro* ubiquitination of FLS2 by PUB12, PUB13, and AvrPtoB is independent of its putative PEST domain although its mutation impairs endocytosis (Robatzek et al., 2006; Göhre et al., 2008; Lu et al., 2011). This suggests that FLS2 endocytosis and PUB12, PUB13, and AvrPtoB mediated degradation could be uncoupled.

PUB13 may have additional functions as suggested by studies which show that it negatively regulates cell death and influences flowering time (Li et al., 2012). *pub13* plants showed enhanced resistance against hemibiotrophic bacterial pathogens, in line with the results shown by Lu et al. (2011). Additionally, *pub13* mutants also displayed enhanced susceptibility to a necrotrophic pathogen (Li et al., 2012). Similarly, mutants of a gene encoding the putative PUB13 ortholog in rice, *spotted leaf 11* (*SPL11*), were also reported to show spontaneous cell death and altered defense responses (Zeng et al., 2004). Interestingly, both orthologs additionally affect flowering time regulation, although they display opposing phenotypes. Whereas flowering starts earlier in *pub13* plants, it is delayed in rice *spl11* mutants grown under long day conditions (Vega-Sanchez et al., 2008; Li et al., 2012). Both resistance and flowering time phenotypes were shown to be largely dependent on constitutively increased SA levels in *pub13*, as introgression of *phytoalexin deficient 4* (*pad4*) or *salicylic acid induction deficient 2* (*sid2*) mutations suppressed both phenotypes.

In rice, the ubiquitin ligase XB3 (XA21-binding protein 3) interacts *in vivo* with the PRR XA21 (*Xanthomonas oryzae* pv. *oryzae* resistance 21), which is also able to phosphorylate XB3 (Wang et al., 2006). Reduced expression of *XB3* results in lower protein levels of XA21 and decreased resistance to the avirulent *X. oryzae* pv. *oryzae*, suggesting that XB3 is required for the accumulation of XA21 (**Figure 1C**). In *Lotus japonicus*, the RING-type ligase seven in absentia 4 (SINA4), was shown to interact with symbiosis RLK (SYMRK) and to negatively influence infection thread development during rhizobia infection (Den Herder et al., 2012; **Figure 1D**). Expression of SINA4 reduced SYMRK levels indicating a regulatory function of SINA4.

Because PRRs are integral membrane proteins, regulation of protein levels requires different cellular processes than in the case of NB-LRRs. Transport of RLKs to and from the PM is mediated by vesicle trafficking. Ubiquitination is closely involved in many steps of vesicle trafficking; it directs trafficking decisions related to both the biosynthetic secretory pathway and the removal of PM proteins via the endocytic pathway. Cell signaling and endocytic trafficking of membrane proteins have traditionally been regarded as two independent processes. However, recent studies, mainly from non-plant systems, have demonstrated that these two processes are intimately intertwined (Scita and Di Fiore, 2010).

UBIQUITINATION AND IMMUNE RECEPTOR TRAFFICKING

Remodeling of the PM protein composition is emerging as a key aspect regulating receptor signaling and mediating signal resolution in space and time (Scita and Di Fiore, 2010). Endocytosis can regulate cell signaling by controlling the number of available receptors. This paradigm has been demonstrated for several receptors in animal cells including receptor tyrosine kinases, G protein-coupled receptors, and others (for review, see Sorkin and von Zastrow, 2009).

Recent studies suggest that a similar paradigm could be valid in plants. The receptor FLS2 is internalized and degraded in response to binding to flg22 (Robatzek et al., 2006). Internalization and concomitant degradation have been suggested to mediate signal attenuation.

The mechanism by which AvrPtoB-, PUB12-, or PUB13-mediated PRR ubiquitination modulates protein levels, still remains to be clarified. PRR ubiquitination can lead to one of many fates which can include endocytosis, changes in PRR sorting after endocytosis or in protein secretion. In humans, toll-like receptor (TLR)-mediated signaling is regulated by the RING-type ligase Triad3A. Both the TLR4 and TLR9 are ubiquitinated by Triad3A leading to their degradation upon activation with lipopolysaccharide (LPS) and cytosine-guanosine dinucleotide motifs (CpG), respectively (Chuang and Ulevitch, 2004). However, initial endocytosis of the LPS receptor complex can also take place in a ubiquitination-independent manner (Husebye et al., 2006). This suggests that receptor ubiquitination may regulate protein levels by modulating PRR traffic at different stages after endocytosis.

Following internalization, cargoes go through a sorting process which decides whether they will be recycled and returned to the PM, or transported to the vacuole for degradation via multi-vesicular bodies (MVBs). This additional level of regulation is mediated by the endosomal sorting complex required for transport (ESCRT). Several studies showed that monoubiquitination of integral PM proteins is required for sorting into MVBs in yeast and animal cells (Hicke, 2001; Haglund et al., 2003; Raiborg et al., 2003).

In plants, one of the first studies to show the involvement of ubiquitination in vacuolar sorting was provided by Kasai et al. (2011). They demonstrated that the substitution of the Lys590 residue, which is mono- or diubiquitinated *in vivo*, blocked the degradation of the borate transporter BOR1. Furthermore, the Lys590Ala mutation impaired translocation from the early endosome (EE) and transport to the vacuole without affecting localization to the PM. A recent study suggested a potential role of monoubiquitination in the degradation of the iron-regulated transporter 1 (IRT1), an integral PM protein, via the lytic vacuole (Barberon et al., 2011). IRT1 was shown to cycle between the PM, *trans*-Golgi network (TGN)/EE, and the vacuole to maintain optimal metal uptake. However, mutation of putative ubiquitin-conjugation residues led to IRT1 stabilization at the PM. In addition, artificial monoubiquitination of the PM ATPase was sufficient to cause its endocytosis and targeting to the vacuole, supporting monoubiquitination as signal for vacuolar targeting (Herberth et al., 2012). Because RLKs are integral membrane proteins, it is likely that they are also subject to similar

processes in which ubiquitination orchestrates sorting into the vacuole.

Various components of the ESCRT bind ubiquitin and the deubiquitinating enzyme AMSH3 (associated molecule with the SH3 domain of STAM3) has been proposed to promote recycling of endocytosed proteins in animal cells. The *Arabidopsis* AMSH3 homolog is involved in vacuole biogenesis and vesicular traffic in general, including endocytosis (Isono et al., 2010). Interestingly, AMSH3 interacts with the ESCRT-III subunits vacuolar protein sorting 2.1 (VPS2.1) and VPS24.1 and regulates their localization (Katsiarimpa et al., 2011).

In the secretory pathway, components of the endoplasmic reticulum (ER)-quality control ensure the proper accumulation of PRRs. ER-quality control was shown to be required for the accumulation and proper function of elongation factor-Tu receptor (EFR) and FLS2 receptors (Nekrasov et al., 2009; Saijo et al., 2009). Mutant plants of the *stromal-derived factor-2* (*SDF2*) and the *luminal binding protein* (*BiP*), a member of the Hsp70 family of chaperones, were impaired in PAMP-triggered responses and resistance against the pathogens *P. syringae* and *Alternaria brassicicola* (Nekrasov et al., 2009). The ER-quality control machinery is largely dependent on ubiquitination of defective proteins to mediate their degradation (Smith et al., 2011).

In addition, components of the ER-associated protein degradation (ERAD), such as the stress-induced ubiquitin-conjugating enzyme 32 (UBC32), participate in the secretion control of integral PM proteins. Transient expression of UBC32 in tobacco resulted in the reduced accumulation of the barley powdery mildew resistance locus O-12 (MLO12), a known substrate of ERAD (Müller et al., 2005; Cui et al., 2012). The *bri1-9* and *bri1-5* mutant alleles of the brassinosteroid-insensitive 1 (BRI1) receptor cause the ER-retention of the functional receptors and the typical brassinosteroid-insensitive dwarf phenotype (Jin et al., 2007; Hong et al., 2008). The double mutant *ubc32/bri1-9* partially rescues the *bri1-9* dwarf phenotype by allowing the functional bri1-9 mutant form to bypass ERAD and accumulate. Because UBC32 is induced by various ER stressors, it is conceivable that it participates in the regulation of ERAD stress responses (Cui et al., 2012). In line with these observations, two homologs of the ER membrane-localized RING-type ubiquitin ligase of the yeast and mammalian Hrd1, were shown to function redundantly in *bri1-9* ERAD (Su et al., 2010). Double mutants of the two *Arabidopsis Hrd1* homologs suppressed the *bri1-9* phenotype. The former observations are also interesting in light of recent data that show the antagonism between brassinosteroid and immune signaling (Albrecht et al., 2011; Belkhadir et al., 2011).

REGULATION OF UBIQUITIN LIGASES

In many cases ubiquitin ligases are phosphorylated by interacting RLKs. It is therefore tempting to speculate that ligase phosphorylation regulates their activity or the interaction with target proteins.

One of the first examples showing such an interaction was the U-box type ubiquitin ligase from *Brassica napus* arm repeat containing 1 (ARC1) and S receptor kinase (SRK) which regulates self-incompatibility (SI; **Figure 1E**; Stone et al., 1999). ARC1 was shown to be phosphorylated by SRK (Gu et al., 1998). Interestingly, phosphorylation was required for the relocalization of ARC1 from the cytosol to the ER (Stone et al., 2003). Yeast two-hybrid analysis with different S-domain RLKs and several *Arabidopsis* PUBs suggested the conservation of the SI signaling pathway in *Arabidopsis* (Samuel et al., 2008). The *Arabidopsis* S-domain RLKs *Arabidopsis* receptor kinase 1 (ARK1) and ARK2 were also able to phosphorylate PUB9 and PUB13 *in vitro*. In addition, the *Nicotiana benthamiana* RLK CHRK1 had previously been reported to interact with *Nt*PUB4, the homolog of *Bn*ARC1 (Kim et al., 2003).

The *Medicago truncatula* PUB1 was shown to interact with lysin motif RLK 3 (LYK3), a putative RLK of *Sinorhizobium meliloti* Nod factors (Mbengue et al., 2010). PUB1 was also phosphorylated by LYK3, but was unable to ubiquitinate it *in vitro* (**Figure 1F**). Overexpression and knock-down experiments suggested that PUB1 is a negative regulator of infection and nodulation by *S. meliloti*.

In the case of PUB12 and PUB13, BAK1-mediated phosphorylation induced their association with FLS2 (Lu et al., 2011), suggesting that phosphorylation modulates ligase affinity and thus mediates the association to FLS2. However, PUB12 and PUB13 phosphorylation does not seem to be required for target ubiquitination, since they readily ubiquitinated FLS2 *in vitro*. Furthermore, most RING and PUB ligases display *in vitro* autoubiquitination, suggesting that additional factors are dispensable for their activity. Nevertheless, it still remains unknown whether PUB1, SINA4, or XB3, as well as other mentioned ubiquitin ligases, can ubiquitinate the corresponding RLKs. Instead, it is conceivable that phosphorylation triggers the interaction with alternative targets.

The relocalization of proteins prompted by interaction with ubiquitin ligases is a reoccurring theme. Intracellular relocalization of ubiquitin ligases may represent a mechanism by which their activity is restricted to a specific cellular context. The RING-type ligase keep on going (KEG) functions in abscisic acid signaling and its mutation suppresses the enhanced resistance against powdery mildew in *enhanced disease resistance 1* (*edr1*) plants (Wawrzynska et al., 2008). KEG interacts with EDR1, which was shown to localize to the ER. EDR1 is recruited by KEG to the TGN/EE when coexpressed (Gu and Innes, 2011). Another example is the previously mentioned ARC1, shown to interact with Exo70A1, a subunit of the exocyst complex. Coexpression of Exo70A1 with ARC1 resulted in their relocalization from the cytosol to punctate structures (Samuel et al., 2009). Similarly, SYMRK relocalizes from the PM to punctate structures in the cytosol in the presence of SINA4 (Den Herder et al., 2012). However, whether target ubiquitination is required for the relocalization, still needs to be shown. Further work is necessary to resolve the dynamic interactions and modifications occurring between regulatory ligases and immune receptor kinases.

CONCLUSIONS AND PERSPECTIVES

Surfacing data showing the manifold and central functions of ubiquitination in vesicle trafficking represent a preliminary confirmation in plants of long standing paradigms in yeast and animal cells. However, the general scarcity of ubiquitination

targets still obstructs insight into the cellular processes that are being regulated. Furthermore, it is necessary to discriminate between the different types of ubiquitination, since these mediate distinct fates of the tagged proteins. The importance of this aspect becomes apparent if one considers that ubiquitin is a common denominator involved in targeting of substrates to all three major protein degradation pathways in mammalian cells: the proteasome, the lysosome, and the autophagosome. In plants, most attention has been focused on the role of ubiquitination in mediating the turn-over of modified proteins by the proteasome, while relatively little is known about its role in directing proteins into the vacuole or autophagocytosis. However, at this point, the major challenge continues to be the identification of ligase targets.

ACKNOWLEDGMENTS

Work in the authors' laboratory is supported by the Leibniz Society and the Priority Program 1212 from the German Research Foundation (DFG).

REFERENCES

Albrecht, C., Boutrot, F., Segonzac, C., Schwessinger, B., Gimenez-Ibanez, S., Chinchilla, D., et al. (2011). Brassinosteroids inhibit pathogen-associated molecular pattern-triggered immune signaling independent of the receptor kinase BAK1. *Proc. Natl. Acad. Sci. U.S.A.* 109, 303–308.

Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K., and Schulze-Lefert, P. (2002). The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. *Science* 295, 2073–2076.

Barberon, M., Zelazny, E., Robert, S., Conejero, G., Curie, C., Friml, J., et al. (2011). Monoubiquitin-dependent endocytosis of the iron-regulated transporter 1 (IRT1) transporter controls iron uptake in plants. *Proc. Natl. Acad. Sci. U.S.A.* 108, E450–E458.

Belkhadir, Y., Jaillais, Y., Epple, P., Balsemao-Pires, E., Dangl, J. L., and Chory, J. (2011). Brassinosteroids modulate the efficiency of plant immune responses to microbe-associated molecular patterns. *Proc. Natl. Acad. Sci. U.S.A.* 109, 297–302.

Bent, A. F., and Mackey, D. (2007). Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* 45, 399–436.

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.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J. D., et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448, 497–500.

Chuang, T. H., and Ulevitch, R. J. (2004). Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nat. Immunol.* 5, 495–502.

Cooley, M. B., Pathirana, S., Wu, H. J., Kachroo, P., and Klessig, D. F. (2000). Members of the *Arabidopsis* HRT/RPP8 family of resistance genes confer resistance to both viral and oomycete pathogens. *Plant Cell* 12, 663–676.

Cui, F., Liu, L., Zhao, Q., Zhang, Z., Li, Q., Lin, B., et al. (2012). *Arabidopsis* ubiquitin conjugase UBC32 is an ERAD component that functions in brassinosteroid-mediated salt stress tolerance. *Plant Cell* 24, 233–244.

Den Herder, G., Yoshida, S., Antolin-Llovera, M., Ried, M. K., and Parniske, M. (2012). *Lotus japonicus* E3 Ligase SEVEN IN ABSENTIA4 destabilizes the symbiosis receptor-like kinase SYMRK and negatively regulates rhizobial infection. *Plant Cell* 24, 1691–1707.

Gimenez-Ibanez, S., Hann, D. R., Ntoukakis, V., Petutschnig, E., Lipka, V., and Rathjen, J. P. (2009). AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. *Curr. Biol.* 19, 423–429.

Göhre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., et al. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr. Biol.* 18, 1824–1832.

Gou, M., Su, N., Zheng, J., Huai, J., Wu, G., Zhao, J., et al. (2009). An F-box gene, CPR30, functions as a negative regulator of the defense response in *Arabidopsis*. *Plant J.* 60, 757–770.

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.

Gu, T., Mazzurco, M., Sulaman, W., Matias, D. D., and Goring, D. R. (1998). Binding of an arm repeat protein to the kinase domain of the S-locus receptor kinase. *Proc. Natl. Acad. Sci. U.S.A.* 95, 382–387.

Gu, Y., and Innes, R. W. (2011). The KEEP ON GOING protein of *Arabidopsis* recruits the ENHANCED DISEASE RESISTANCE1 protein to *trans*-Golgi network/early endosome vesicles. *Plant Physiol.* 155, 1827–1838.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat. Cell. Biol.* 5, 461–466.

Herberth, S., Shahriari, M., Bruderek, M., Hessner, F., Muller, B., Hulskamp, M., et al. (2012). Artificial ubiquitylation is sufficient for sorting of a plasma membrane ATPase to the vacuolar lumen of *Arabidopsis* cells. *Planta* 236, 63–77.

Hicke, L. (2001). Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell. Biol.* 2, 195–201.

Hong, Z., Jin, H., Tzfira, T., and Li, J. (2008). Multiple mechanism-mediated retention of a defective brassinosteroid receptor in the endoplasmic reticulum of *Arabidopsis*. *Plant Cell* 20, 3418–3429.

Husebye, H., Halaas, O., Stenmark, H., Tunheim, G., Sandanger, O., Bogen, B., et al. (2006). Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity. *EMBO J.* 25, 683–692.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep.* 9, 536–542.

Isono, E., Katsiarimpa, A., Muller, I. K., Anzenberger, F., Stierhof, Y. D., Geldner, N., et al. (2010). The deubiquitinating enzyme AMSH3 is required for intracellular trafficking and vacuole biogenesis in *Arabidopsis thaliana*. *Plant Cell* 22, 1826–1837.

Janjusevic, R., Abramovitch, R. B., Martin, G. B., and Stebbins, C. E. (2006). A bacterial inhibitor of host programmed cell death defenses is an E3 ubiquitin ligase. *Science* 311, 222–226.

Jeong, R. D., Chandra-Shekara, A. C., Barman, S. R., Navarre, D., Klessig, D. F., Kachroo, A., et al. (2010). Cryptochrome 2 and phototropin 2 regulate resistance protein-mediated viral defense by negatively regulating an E3 ubiquitin ligase. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13538–13543.

Jin, H., Yan, Z., Nam, K. H., and Li, J. (2007). Allele-specific suppression of a defective brassinosteroid receptor reveals a physiological role of UGGT in ER quality control. *Mol. Cell* 26, 821–830.

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

Kasai, K., Takano, J., Miwa, K., Toyoda, A., and Fujiwara, T. (2011). High boron-induced ubiquitination regulates vacuolar sorting of the BOR1 borate transporter in *Arabidopsis thaliana*. *J. Biol. Chem.* 286, 6175–6183.

Katsiarimpa, A., Anzenberger, F., Schlager, N., Neubert, S., Hauser, M. T., Schwechheimer, C., et al. (2011). The *Arabidopsis* deubiquitinating enzyme AMSH3 interacts with ESCRT-III subunits and regulates their localization. *Plant Cell* 23, 3026–3040.

Kim, M., Cho, H. S., Kim, D. M., Lee, J. H., and Pai, H. S. (2003). CHRK1, a chitinase-related receptor-like kinase, interacts with NtPUB4, an armadillo repeat protein, in tobacco. *Biochim. Biophys. Acta* 1651, 50–59.

Li, W., Ahn, I. P., Ning, Y., Park, C. H., Zeng, L., Whitehill, J., et al. (2012). The U-box/ARM E3 ligase PUB13 regulates cell death, defense and flowering time in *Arabidopsis*. *Plant Physiol.* 159, 239–250.

Liu, Y., Schiff, M., Serino, G., Deng, X. W., and Dinesh-Kumar, S. P. (2002). Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to Tobacco mosaic virus. *Plant Cell* 14, 1483–1496.

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.

Mbengue, M., Camut, S., de Carvalho-Niebel, F., Deslandes, L., Froidure, S., Klaus-Heisen, D., et al. (2010). The *Medicago truncatula* E3 ubiquitin ligase PUB1 interacts with the LYK3 symbiotic receptor and negatively regulates infection and nodulation. *Plant Cell* 22, 3474–3488.

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15, 349–357.

Müller, J., Piffanelli, P., Devoto, A., Miklis, M., Elliott, C., Ortmann, B., et al. (2005). Conserved ERAD-like quality control of a plant polytopic membrane protein. *Plant Cell* 17, 149–163.

Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z. H., Lacombe, S., et al. (2009). Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO J.* 28, 3428–3438.

Raiborg, C., Rusten, T. E., and Stenmark, H. (2003). Protein sorting into multivesicular endosomes. *Curr. Opin. Cell Biol.* 15, 446–455.

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Genes Dev.* 20, 537–542.

Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajerowska-Mukhtar, K., Haweker, H., et al. (2009). Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J.* 28, 3439–3449.

Samuel, M. A., Mudgil, Y., Salt, J. N., Delmas, F., Ramachandran, S., Chilelli, A., et al. (2008). Interactions between the S-domain receptor kinases and AtPUB-ARM E3 ubiquitin ligases suggest a conserved signaling pathway in *Arabidopsis*. *Plant Physiol.* 147, 2084–2095.

Samuel, M. A., Chong, Y. T., Haasen, K. E., Aldea-Brydges, M. G., Stone, S. L., and Goring, D. R. (2009). Cellular pathways regulating responses to compatible and self-incompatible pollen in *Brassica* and *Arabidopsis* stigmas intersect at Exo70A1, a putative component of the exocyst complex. *Plant Cell* 21, 2655–2671.

Scita, G., and Di Fiore, P. P. (2010). The endocytic matrix. *Nature* 463, 464–473.

Shirasu, K. (2009). The HSP90-SGT1 chaperone complex for NLR immune sensors. *Annu. Rev. Plant Biol.* 60, 139–164.

Smith, M. H., Ploegh, H. L., and Weissman, J. S. (2011). Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. *Science* 334, 1086–1090.

Sorkin, A., and von Zastrow, M. (2009). Endocytosis and signalling: intertwining molecular networks. *Nat. Rev. Mol. Cell Biol.* 10, 609–622.

Stone, S. L., Anderson, E. M., Mullen, R. T., and Goring, D. R. (2003). ARC1 is an E3 ubiquitin ligase and promotes the ubiquitination of proteins during the rejection of self-incompatible *Brassica* pollen. *Plant Cell* 15, 885–898.

Stone, S. L., Arnoldo, M., and Goring, D. R. (1999). A breakdown of *Brassica* self-incompatibility in ARC1 antisense transgenic plants. *Science* 286, 1729–1731.

Su, W., Liu, Y., Xia, Y., Hong, Z., and Li, J. (2010). Conserved endoplasmic reticulum-associated degradation system to eliminate mutated receptor-like kinases in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 870–875.

Trujillo, M., Ichimura, K., Casais, C., and Shirasu, K. (2008). Negative regulation of PAMP-triggered immunity by an E3 ubiquitin ligase triplet in *Arabidopsis*. *Curr. Biol.* 18, 1396–1401.

Vega-Sanchez, M. E., Zeng, L., Chen, S., Leung, H., and Wang, G. L. (2008). SPIN1, a K homology domain protein negatively regulated and ubiquitinated by the E3 ubiquitin ligase SPL11, is involved in flowering time control in rice. *Plant Cell* 20, 1456–1469.

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

Wang, Y. S., Pi, L. Y., Chen, X., Chakrabarty, P. K., Jiang, J., De Leon, A. L., et al. (2006). Rice XA21 binding protein 3 is a ubiquitin ligase required for full Xa21-mediated disease resistance. *Plant Cell* 18, 3635–3646.

Wawrzynska, A., Christiansen, K. M., Lan, Y., Rodibaugh, N. L., and Innes, R. W. (2008). Powdery mildew resistance conferred by loss of the ENHANCED DISEASE RESISTANCE1 protein kinase is suppressed by a missense mutation in KEEP ON GOING, a regulator of abscisic acid signaling. *Plant Physiol.* 148, 1510–1522.

Zeng, L. R., Qu, S., Bordeos, A., Yang, C., Baraoidan, M., Yan, H., et al. (2004). Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. *Plant Cell* 16, 2795–2808.

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: 28 June 2012; paper pending published: 30 July 2012; accepted: 09 October 2012; published online: 24 October 2012.

Citation: Furlan G, Klinkenberg J and Trujillo M (2012) Regulation of plant immune receptors by ubiquitination. Front. Plant Sci. ***3****:238. doi: 10.3389/fpls.2012.00238*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 17 September 2012
doi: 10.3389/fpls.2012.00215

Global SUMO proteome responses guide gene regulation, mRNA biogenesis, and plant stress responses

Magdalena J. Mazur and Harrold A. van den Burg*

Molecular Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, Netherlands

Edited by:
Alex Jones, The Sainsbury Laboratory, UK

Reviewed by:
Ning Li, University of Washington, USA
Sixue Chen, University of Florida, USA

****Correspondence:***
Harrold A. van den Burg, Molecular Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, P.O. box 94215, 1090 GE Amsterdam, Netherlands.
e-mail: h.a.vandenburg@uva.nl

Small Ubiquitin-like MOdifier (SUMO) is a key regulator of abiotic stress, disease resistance, and development in plants. The identification of >350 plant SUMO targets has revealed many processes modulated by SUMO and potential consequences of SUMO on its targets. Importantly, highly related proteins are SUMO-modified in plants, yeast, and metazoans. Overlapping SUMO targets include heat-shock proteins (HSPs), transcription regulators, histones, histone-modifying enzymes, proteins involved in DNA damage repair, but also proteins involved in mRNA biogenesis and nucleo-cytoplasmic transport. Proteomics studies indicate key roles for SUMO in gene repression by controlling histone (de)acetylation activity at genomic loci. The responsible heavily sumoylated transcriptional repressor complexes are recruited by plant transcription factors (TFs) containing an (ERF)-associated Amphiphilic Repression (EAR) motif. These TFs are not necessarily themselves a SUMO target. Conversely, SUMO acetylation (Ac) prevents binding of downstream partners by blocking binding of their SUMO-interaction peptide motifs to Ac-SUMO. In addition, SUMO acetylation has emerged as a mechanism to recruit specifically bromodomains. Bromodomains are generally linked with gene activation. These findings strengthen the idea of a bi-directional sumo-acetylation switch in gene regulation. Quantitative proteomics has highlighted that global sumoylation provides a dynamic response to protein damage involving SUMO chain-mediated protein degradation, but also SUMO E3 ligase-dependent transcription of HSP genes. With these insights in SUMO function and novel technical advancements, we can now study SUMO dynamics in responses to (a)biotic stress in plants.

Keywords: SUMO, chromatin, stress, heat shock, acetylation, histones

INTRODUCTION

Over the last decade much has been learned on Small Ubiquitin like MOdifier (SUMO). SUMO is a ∼100 amino-acid polypeptide that is covalently attached to target proteins in a process closely resembling conjugation of the well-studied tag ubiquitin (Wilkinson and Henley, 2010; Park et al., 2011b). SUMO conjugation involves formation of an isopeptide bond between the C-terminal diglycine (diGly) residues of SUMO and the ε-amino group of lysines in target proteins. The machinery responsible for SUMO conjugation, including SUMO itself, is highly conserved and essential in many eukaryotes (Nacerddine et al., 2005; Saracco et al., 2007; Kaminsky et al., 2009). Hundreds of proteins have been identified as SUMO targets (e.g., Miller et al., 2010). SUMO conjugation affects these targets in different ways, such as (i) stability, (ii) sub-cellular localization (including recruitment to various nuclear foci), (iii) protein–protein interactions, and (iv) protein activity. Remarkably, the level of sumoylation detected on SUMO targets is often low with less than 10–20% modified. Yet, SUMO attachment appears to affect the function of the entire pool of a target protein; a phenomena termed the "SUMO enigma" (Wilkinson and Henley, 2010). Although the mechanisms are not fully understood, the notion is that sumoylation is sufficient to change target function by altering protein localization and protein–protein interactions, which apparently persist after SUMO deconjugation. For example, recruitment of histone deacetylases (HDAC) to promoters due to sumoylation of transcription factors (TFs) leads to promoter-specific histone deacetylation causing chromatin compacting, which favors transcriptional repression (Garcia-Dominguez and Reyes, 2009). Importantly, this compact chromatin structure apparently requires SUMO conjugation, but is largely independent of SUMO deconjugation.

Critical for SUMO function is a binding pocket on SUMO that acts as a docking site for SUMO-interaction motifs (SIMs). This short peptide motif is found in partner proteins and comprises three hydrophobic residues that surround one additional residue (x), i.e., [VIL]x[VIL][VIL] or [VIL][VIL]x[VIL] (Kerscher, 2007; **Figure 1**). The SIM core aligns as an additional β-strand in the β-sheet of SUMO. In many cases, the SIM hydrophobic core is flanked by acidic residues (Asp/Glu) that provide additional electrostatic interactions with a basic interface on SUMO that surrounds the SIM-binding pocket. As SIM-containing partners are involved in a wide range of biological processes, it has proven to be difficult to predict the consequence of SUMO attachment for SUMO targets.

SUMO is commonly attached to Lys residues located in the consensus motif ΨKxE, where Ψ denotes a large hydrophobic residue

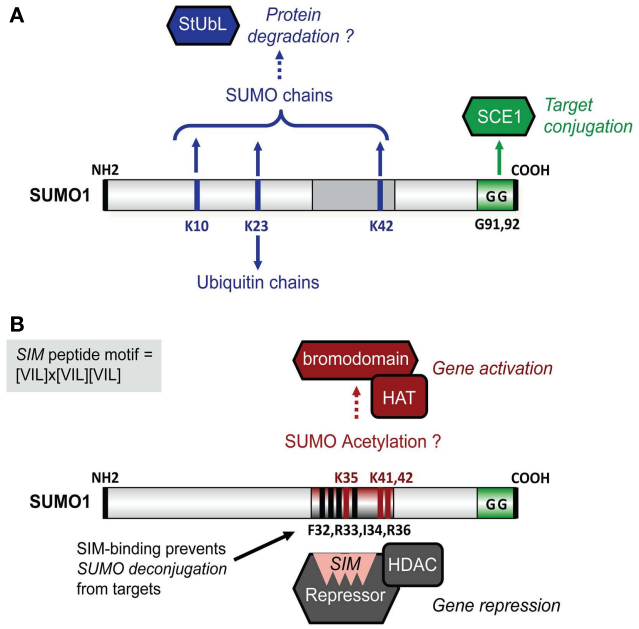


FIGURE 1 | Schematic structure of Arabidops SUMO1 and its potential interplay with other post-translational modifications. (A) SUMO is conjugated to targets via SCE1 that forms an isopeptide with diGly residues (GG) of SUMO and Lys residues in target proteins. SUMO1 and SUMO2 (*not shown*) contain also internal acceptor sites for SUMO chain editing (Lys10, Lys23, and Lys42; *top*) and mixed ubiquitin-SUMO chains (Lys23; *bottom*). SUMO chains are recognized by StUbLs that conjugate ubiquitin on internal lysines in SUMO chains. This leads to 26S proteasome-mediated protein degradation of SUMO conjugates. **(B)** A SUMO-acetylation switch controls gene regulation by SUMO-modified targets. For example, SIM-dependent recruitment of co-repressor complexes is linked with (HDAC-mediated) gene repression (*bottom*). These SUMO-SIM interactions are disrupted by SUMO acetylation (*top*), which likely involves HAT activity. On the other hand, SUMO acetylation (on possibly Lys35, Lys41, and/or Lys42) allows SUMO instead to interact with bromodomains; a domain found in transcriptional co-activators. In addition, SUMO-SIM interactions appear to prevent SUMO deconjugation by SUMO proteases.

(VILMFPC; Matic et al., 2010) and x represents any residue. This ΨKxE motif is recognized by the E2 SUMO conjugating enzyme SCE1 and this recognition is often sufficient for sumoylation (Bernier-Villamor et al., 2002). In fact, *in vitro* sumoylation reactions require usually only the E1 SUMO activating enzyme (SAE1/SAE2 dimer), SCE1, SUMO, and ATP. Proteomics studies have also identified divergent sumoylation motifs, such as the inverted consensus motif, the hydrophobic cluster sumoylation motif (HCSM), and extended versions like the phosphorylation-dependent sumoylation motifs (PDSM; Anckar and Sistonen, 2007; Blomster et al., 2010; Matic et al., 2010). The different motifs are frequently found in non-sumoylated proteins and are, therefore, not sufficient to predict SUMO targets. Conversely, sumoylation is also known to occur at non-consensus sites (between 20 and 40%). Together, this signifies that motif-based sequence searches with "known" sumoylation consensus motifs are not sufficient to unequivocally identify SUMO acceptor sites. To identify these sites, SUMO proteomics studies are needed.

APPROACHES AND OPPORTUNITIES FOR NEXT GENERATION SUMO PROTEOMICS

To perform SUMO proteomics, SUMO conjugates are now routinely purified using affinity-purification of His-tagged SUMO variants. While identification of the purified SUMO targets with mass spectrometry provides little problems, the identification of SUMO acceptor lysines in these targets remains difficult, as the MS/MS spectra corresponding to the modified isopeptides are often too complex to detect diGly-remnants or worse large SUMO tags left after tryptic digestion. In most cases, SUMO acceptor lysines are identified for each target separately using often MS/MS data obtained from *in vitro* sumoylated proteins. Such relatively simple MS/MS spectra are then analyzed with specific algorithms such as SUMmOn and ChopNSpice to facilitate annotation of both *in vitro* and biological data (Pedrioli et al., 2006; Hsiao et al., 2009; Jeram et al., 2010). A second problem is that tryptic digestion of SUMO leaves a large signature tag; this is now routinely circumvented by introducing an additional tryptic cleavage site (Arg residue) in SUMO directly adjacent to the diGly motif (+RGG C-terminus), which only leaves a diGly remnant on modified lysines after trypsin cleavage (Wohlschlegel et al., 2006; Miller et al., 2010; Vertegaal, 2011). Importantly, these His-tagged SUMO-RGG variants are fully functional in yeast, mammalian cells, and Arabidopsis.

A major development in SUMO proteomics is selective enrichment of diGly-modified peptides when isolating SUMO conjugates. This method is based on a His-tagged SUMO (RGG) variant in which all internal lysines are replaced for arginines allowing tailored protease digestion of SUMO conjugates (Matic et al., 2010). These Lys-deficient SUMO proteins are sensitive to trypsin but insensitive to Lys-C protease, which only cleaves after Lys residues. Lys-C digestion will, therefore, harness intact His-tagged SUMO proteins conjugated to Lys-C-generated peptides. These SUMO-modified isopeptides can effectively be purified using the His-tag. Trypsin digestion will subsequently yield diGly-modified signature peptides of the original SUMO conjugates. This approach identified 103 SUMO acceptor sites using HeLa cell cultures (Matic et al., 2010). However, one should be careful about substituting all lysines in SUMO, considering their importance for SIM docking, SUMO chain editing, and SUMO acetylation (see below).

Another key improvement is the development of monoclonal antibodies that recognize diGly-remnants left on isopeptides after trypsin digestion (Xu et al., 2010; Xu and Jaffrey, 2011). Immunoprecipitation with these antibodies followed by mass spectrometry-based diGly-remnant profiling provided 11,054 (Wagner et al., 2011), 9,957 (Emanuele et al., 2011), and >19,000 Ubiquitin-modified sites (Kim et al., 2011). Application of this antibody for SUMO proteomics in Arabidopsis is now feasible, i.e., one can perform diGly-remnant profiling in the *sumo1;sumo2* double mutant by complementing it with a His-tagged SUMO1-RGG variant. In combination with differential labeling techniques such as iTRAQ or 15N-isotope labeling, diGly-remnant profiling should provide a robust tool for quantitative SUMO proteomics under different stress conditions. One remaining complication is that trypsin digestion will also create diGly-remnants that originate from Ubiquitin and NEDD8 (RUB1/Related to ubiquitin 1 in

Arabidopsis) modifications. In order to distinguish SUMO from these other modifications, Miller et al. (2010) introduced a four-residue footprint (+QTGG) in Arabidopsis SUMO1 expressing a HIS-tagged H89R variant, which proved to be fully functional.

The first decade of SUMO research revealed extensive roles for SUMO in plant development including meristem differentiation and floral induction, defense signaling via the hormone salicylic acid, and adaptation to diverse abiotic stresses such as heat stress, drought, and cold (Kurepa et al., 2003; Catala et al., 2007; Miura et al., 2007; Ishida et al., 2009; Castro et al., 2012). For the coming era, we see three major challenges for research on sumoylation in plants. First, data on the spatio-temporal dynamics of target sumoylation remains missing for SUMO-controlled processes in plants. This requires (relative) quantification of SUMO conjugates in different cell types and conditions. Such approaches have come within reach due to the development of *in vivo*-biotin labeling of specific nuclei combined with a purification method to obtain these labeled intact nuclei (Deal and Henikoff, 2011). A second challenge is to perform SUMO target profiling for SUMO E3 ligases like SIZ1 (SAP and MIZ-finger domain-containing protein 1) and SUMO proteases. For example, only several SIZ1-specific SUMO targets have been identified so far, such as Inducer of CBF expression 1 (ICE1) and Global TF group E3 (GTE3; Miura et al., 2007; Garcia-Dominguez et al., 2008), while (de-)sumoylation of hundreds of SUMO targets must happen in a controlled manner in cells. Last-but-not-least, genome sequencing has revealed that several plant species contain additional SUMO paralogs other than the canonical SUMO isoforms. Evolution of divergent non-canonical SUMO genes has repeatedly occurred, e.g., grasses (Poaceae) have a unique diSUMO-like SUMO paralog (Srilunchang et al., 2010), while in Brassicaceae four additional SUMO paralogs have emerged (Kurepa et al., 2003). These paralogs have possibly unique roles in plant development and signaling, as seen for Arabidopsis SUMO3 (van den Burg et al., 2010). *In planta* expression of mature variants (with their diGly C-terminus exposed) of non-canonical Arabidopsis SUMO paralogs indicated that these paralogs possibly have preferred SUMO targets (Budhiraja et al., 2009). However, the extent to which conjugation of these non-canonical paralogs occurs remains unresolved, as biochemical data suggested that Arabidopsis SUMO3 and -5 are poor substrates for SUMO protease maturation and the E1 enzyme (Castano-Miquel et al., 2011). In agreement, overexpression of both mature and conjugation-deficient variants of SUMO3 did not affect global SUMO1/2 conjugation levels, while overexpression of SUMO1 or -2 variants caused global sumoylation (van den Burg et al., 2010). Studies on SUMO paralogs in mammals established that they have their own preferred SIM partners (Zhu et al., 2009). This provokes the idea that the non-canonical paralogs might act to control interactions between the canonical SUMOs and their partners. Hence, identification of both paralog-specific targets and interactors is needed to fully comprehend SUMO gene evolution in plants.

RELATED PROTEINS ARE SUMOYLATED IN ARABIDOPSIS AND OTHER EUKARYOTES

SUMO proteomics studies have identified in total >2,000 substrates in various organisms (Li et al., 2004; Panse et al., 2004; Vertegaal et al., 2004, 2006; Wohlschlegel et al., 2004; Denison et al., 2005; Hannich et al., 2005; Wykoff and O'shea, 2005; Ganesan et al., 2007; Golebiowski et al., 2009; Matafora et al., 2009; Westman et al., 2010; Galisson et al., 2011; Tatham et al., 2011). The consensus motif (ΨKxE) is significantly overrepresented in these different proteomics sets, e.g., on average more than 2 consensus motifs are found per human SUMO-2 target while the same motif is found only 0.6 times on average per protein in the entire human proteome (Golebiowski et al., 2009). In yeast alone, >500 SUMO (ScSmt3) conjugates were identified (Makhnevych et al., 2009). Furthermore, SUMO-affinity purifications and two-hybrid (Y2H) protein–protein interaction studies in yeast revealed another >250 SUMO-interacting proteins. A related study with *Drosophila* (*D. melanogaster*) cells also identified hundreds of SUMO targets and interactors (Nie et al., 2009), while for *Caenorhabditis elegans* ∼250 candidate SUMO targets have been identified (Kaminsky et al., 2009).

Miller et al. (2010) reported >350 SUMO1 targets in Arabidopsis. They used a strategy that restored sumoylation to endogenous levels complementing a lethal *sumo1;sumo2* mutant with a His-tagged genomic *SUMO1* clone fused to its own promoter. Using a stringent purification protocol, they obtained a high-confidence list of plant SUMO targets from plant extracts of this complemented line. Another plant study identified 238 candidate targets using the Arabidopsis SCE1 (148 interactors) and the SUMO protease ESD4 (Early in short days 4; 154 interactors) as Y2H baits (Elrouby and Coupland, 2010). Interestingly, a substantial set of these interactors was identified using ESD4 as bait. This appears to contradict with the fact that ESD4-like SUMO proteases preferentially recognize SUMO. Structural studies with Ulp1, a yeast homolog of ESD4, revealed that these proteases bind SUMO via two independent sites: (i) a catalytic site that forms a narrow tunnel trapping the diGly tail and (ii) an exosite that binds a distant epitope on the SUMO surface (Mossessova and Lima, 2000). Based on this, the "ESD4-interactome" most likely reflects Arabidopsis proteins that are efficiently sumoylated by the yeast SUMO machinery and this allows their interaction with ESD4. In support of this, 65 of the interactors identified were found with both ESD4 and SCE1 as Y2H bait. In a related study, SUMO from yeast (ScSmt3) was used as bait to identify SUMO targets (Hannich et al., 2005). In this case, the putative SUMO targets were confirmed by co-expressing Ubiquitin-like specific protease 1 (Ulp1) in yeast, which prevented reporter gene activation for SUMO targets but not for non-covalent interactors. In contrast, ESD4 interactors were still able to activate the reporter gene. This suggests that removal of ScSmt3 from SUMO conjugates by ESD4 is possibly less efficient than by Ulp1 and this could then allow reporter gene activation.

In addition, we searched the compiled Arabidopsis SUMOylome with the prevalent consensus peptide motif [VILMFPC]KxE (Matic et al., 2010). Seventy-one percent of the Arabidopsis SUMO targets identified by mass spectrometry contained this motif (Miller et al., 2010) and it was on average 2.15 times represented in these proteins. In contrast, only 52% of the SCE1/ESD4 interactors contained the motif, but those with the motif still had on average 1.95 motifs per protein (Elrouby and Coupland, 2010). This could signify that the Y2H set contains a considerable number of SUMO interactors rather than SUMO targets.

The large-scale studies in Arabidopsis have provided a list of SUMO targets for which in many cases related proteins were previously identified as SUMO target in metazoans, yeast, *Drosophila*, and/or *C. elegans* (Budhiraja et al., 2009; Elrouby and Coupland, 2010; Miller et al., 2010; Park et al., 2011a). The list of plant targets includes heat-shock proteins (HSPs), chromatin-associated proteins, and proteins involved in mRNA biogenesis. Based on this, a conserved role for SUMO is seen in chromatin-modifying complexes, histone acetylation, mRNA biogenesis, and possibly also in global sumoylation induced by cellular stress. Below, we further discuss this overlap in SUMO-controlled processes.

SUMOYLATION OF CONSERVED SUBUNITS OF CHROMATIN-MODIFYING PROTEIN COMPLEXES

A comprehensive analysis of SUMO targets in yeast and mammals established that many conserved histone-modifying enzymes, their co-regulators, ATP-dependent nucleosome-remodeling proteins, and histone chaperones are SUMO-modified (Garcia-Dominguez and Reyes, 2009). These proteins are integral subunits of chromatin-modifying complexes that are largely conserved between eukaryotes. Upon recruitment of these complexes by specific DNA-binding TFs, they control the accessibility of DNA and concomitantly gene expression. Examples include the SWI-independent 3 (SIN3)-HDAC complex, the Nucleosome-Remodeling and histone Deacetylation (NuRD) complex, and the HDAC-containing CoRest/LSD1 (Lysine-specific demethylase 1) repressor complex. Subunits of these complexes are also sumoylated in Arabidopsis including multiple members of various protein families. For example, different subunits of the Arabidopsis the SIN3 complex are sumoylated including SIN3-like homolog 2 (SNL2), SNL4, SNL5, and the class I histone deacetylase HDA19 (**Figure 2A**; Song et al., 2005; Song and Galbraith, 2006; Miller et al., 2010). Sumoylation of class I HDACs other than HDA19 has not yet been shown. Another putative SUMO target in the SIN3 complex is the subunit AtSAP18 (SIn3-associated protein, 18kDA), as sumoylation of HsSAP18 was established in HeLa cells (Golebiowski et al., 2009).

Besides the SIN3 complex, the NuRD complex also appears to be heavily sumoylated (McDonel et al., 2009; **Figure 2B**). The core of this complex is formed by the aforementioned class I HDACs and the ATPase nucleosome-remodeling factor Mi-2, which is also a SUMO target in HeLa cells (Golebiowski et al., 2009). The Arabidopsis homologs of Mi-2 are PKL (Pickle) and Pickle related 1 (PKR1, Chromatin Remodeling 4), which both were SUMO-modified *in planta* (Aichinger et al., 2009; Miller et al., 2010; Zhang et al., 2012). In support of a function of PKL/PKR1 in transcriptional repression, PKL appears to associate physically with histone H3K27 trimethylation (H3K27me3) enriched regions, a mark for gene silencing.

Studies on PKL/PKR1 homologs indicate that they might also be directly recruited to sumoylated TFs and this is possibly independent of the NuRD complex or HDAC activity. For example, Mi-2 is also part of the HDAC1-independent repressor complex *Drosophila* MEP-1-containing complex (dMec), as shown by studies on the *Drosophila* TFs Sp3 and Dorsal (Kunert and Brehm, 2009; Kunert et al., 2009). Recruitment of dMi-2 to Sp3 and Dorsal requires sumoylation of these TFs (Stielow et al., 2008a,b, 2010). However, silencing of NuRD subunits other than Mi-2 did not affect gene repression, while silencing of dMec components lifted sumoylation-dependent transcriptional repression. Also treatment with HDAC inhibitors did not influence Sp3-SUMO-mediated gene repression. Clearly, *Drosophila* dMi-2 can also trigger gene repression in an HDAC-independent manner, which involves apparently prior sumoylation of DNA-bound TFs.

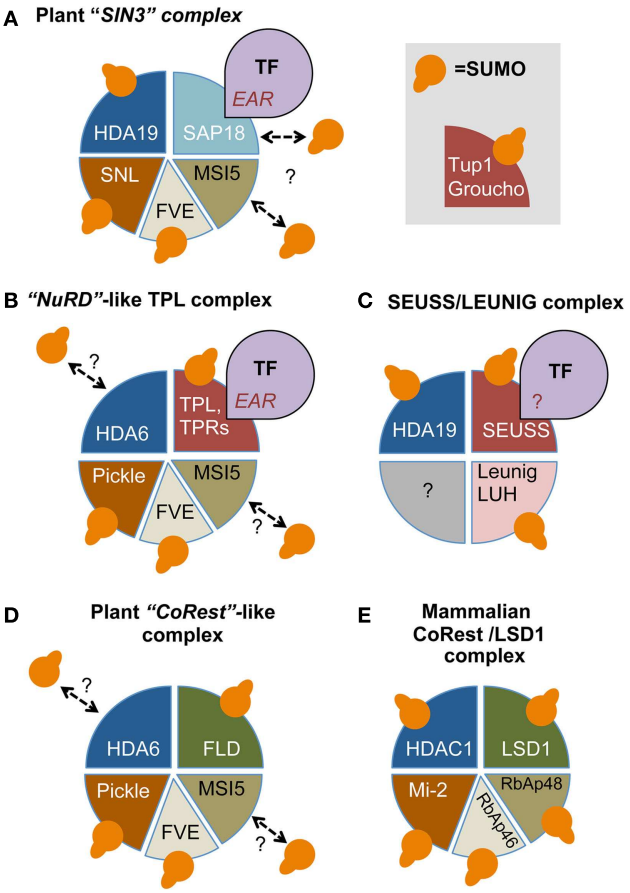


FIGURE 2 | Sumoylation impacts transcription repressor complexes conserved between mammals, yeast, and plants. (A) The heavily sumoylated SIN3 co-repressor complex contains next to SNLs, AtSAP18, a class I histone deacetylase, e.g., HDA6/HDA19, and the histone-chaperones FVE and MSI5. Subunits for which sumoylation was established are indicated. **(B)** Likewise, the NuRD-like co-repressor complex contains – instead of SIN3/SAP18 – the SUMO targets Pickle (PKL; the plant homolog of the mammalian Mi-2) and the co-adaptor Topless (TPL) or its homologs (TPRs). The TPL/TPRs and SAP18 are both recruited to DNA-bound transcription factors (TFs) by EAR motifs present in these TFs. The homologs of TPL in other eukaryotes (Groucho/TUP1-like) are also sumoylated. **(C)** Another sumoylated repressor complex consists of the co-adaptors SEUSS and LEUNIG (LUG). The peptide motif involved in recruiting this complex is unknown ("?"). **(D)** Finally, the CoRest/LSD1-like complex that contains in addition the lysine demethylase Flowering locus D (FLD) is also sumoylated at several subunits. The mammalian homologs of the CoRest subunits are indicated in **(E).** For further details on the role of SUMO in the different complexes we refer to the main text.

In addition to HDACs, the NuRd and SIN3 repressor complexes also share the SUMO targets RbAp46 and RbAp48 that act as histone chaperones and bind the histone dimer H3–H4 (Murzina et al., 2008). In Arabidopsis, these chaperones are represented by five homologs, including FVE/Multicopy Suppressor of IRA1 4 (MSI4) and MSI5 (Ausin et al., 2004; Jeon and Kim, 2011). So far, sumoylation has only been shown for FVE (Miller et al., 2010). These histone chaperones are in fact part of many chromatin-modifying complexes including a complex with histone acetyltransferase (HAT1), the Chromatin Assembly Factor-1 (CAF-1) that deposits nucleosomes, the Polycomb Repressive-like Complex 2 (PRC2) that catalyzes histone H3K27me3 (Loyola and Almouzni, 2004), and the CoRest/LSD1 repressor complex (Baron and Vellore, 2012; **Figures 2D,E**). LSD1 is, however, also reported to be part of the NuRD complex (Wang et al., 2009). Clearly, the NuRD and the CoRest/LSD1 complexes share many subunits. For example, physical interactions have been shown between Arabidopsis FVE, MSI5, HDA6, and Flowering locus D (FLD), which is an Arabidopsis homolog of LSD1 (Gu et al., 2011; Jeon and Kim, 2011; Yu et al., 2011). In addition, genetic data indicated that both FVE and FLD are required for HDA6-mediated deacetylation of the target locus Flowering locus C (FLC; He et al., 2003; Kim et al., 2004). Importantly, FLD acts as a SUMO target and sumoylation of FLD appears to suppress its repressor function, since expression of a sumoylation-deficient mutant of FLD in *fld* protoplasts strongly reduced *FLC* expression in comparison to wild type FLD (Jin et al., 2008).

It is too early to draw general conclusions on the effect of SUMO on HDAC recruitment and its activity when recruited to plant transcriptional repressor complexes like NuRD or SIN3. For example, sumoylation of human HDAC1 enhanced its transcriptional repression (David et al., 2002), while recruitment of *Drosophila* HDAC1 to sumoylated TFs depends on a SIM in HDAC1 itself, as shown for the *Drosophila* co-repressor Gro (Groucho; Ahn et al., 2009). Conversely, sumoylation of certain targets leads to displacement of HDACs from these targets, like for CoRest (Gocke and Yu, 2008).

SUMO not only controls HDAC activity in plants, but also histone demethylase, HAT, and histone methyltransferase (HMT) activity. For example, the HAT GCN5 and its two adaptor proteins Ada2a (transcriptional ADAptor 2a) and Ada2b are SUMO targets (Miller et al., 2010; Servet et al., 2010). The Ada2 adaptors enhance HAT activity of General control of amino-acid synthesis 5 (GCN5) and recruit GCN5 to TFs (Mao et al., 2006; Samara and Wolberger, 2011). Together they are part of the larger SAGA-type HAT complex, which is largely conserved between yeast, *Drosophila*, and mammals. Proteomics studies in the latter two organisms showed that the SAGA complex is also sumoylated at various subunits (Golebiowski et al., 2009; Makhnevych et al., 2009). In yeast, Gcn5 sumoylation appears to inhibit SAGA-mediated gene expression (Sterner et al., 2006), which agrees with the notion that sumoylation generally causes gene repression. In Arabidopsis, GCN5 is associated with about one third of 20,000 promoter regions analyzed (Benhamed et al., 2008). This means that regulation of histone (de)acetylation by SUMO could be widespread in Arabidopsis, involving many transcriptional programs at various genomic loci.

SUMO CONTROLS TOPLESS AND OTHER PLANT CO-ADAPTORS INVOLVED IN GENE REPRESSION

An important co-adaptor family is formed by the Groucho/Transducin 1-like (Gro/Tup1-like) family that mediates gene repression by acting with HDACs (Jennings and Ish-Horowicz, 2008; Ahn et al., 2009). The Arabidopsis genome encodes 14 Gro/Tup1-like co-adaptors (Liu and Karmarkar, 2008; Lee and Golz, 2012). Many of these Gro/Tup1-like homologs are sumoylated *in planta* including Leunig (LUG), LUG homolog (LUH), Topless (TPL), and TPL-related proteins 1 to 4 (TPR1, -2, -3, -4; Miller et al., 2010; **Figures 2B,C**). The TPL/TPRs appear to be part of a NuRD-like protein complex (**Figure 2B**). At least TPL interacts with FVE and PKR1, while FVE and MSI5 associate with HDA6 (Gu et al., 2011; Causier et al., 2012). Based on their domain organization, LUG/LUH form a different class than TPL/TPRs. LUG and LUH act redundantly and both interact physically with HDA19 and another co-adaptor SEUSS that recruits them to DNA-binding TFs (Sridhar et al., 2004; Gonzalez et al., 2007; **Figure 2C**). Also SEUSS is a SUMO target *in planta* and its SUMO acceptor site has been identified ($VK^{200}xE$; Miller et al., 2010). This site is conserved in three of the four Arabidopsis SEUSS homologs (SEU, SLK2, and SLK3). SUMO acceptor sites have not yet been identified for TPL or the other TPRs.

The consequence of SUMO attachment is unknown for both TPL/TPRs and LUG/SEU, but it has been studied for their *Drosophila* homolog Gro. SUMO conjugation of Gro promotes its transcriptional repressor activity via enhanced recruitment of HDAC1 (Ahn et al., 2009). An alternative mechanism emerged from studies of the *Drosophila* SUMO-targeted Ubiquitin ligase (StUbL) Degringolade (Dgrn). StUbLs bind poly-SUMO chains and target poly-SUMO-modified conjugates for proteasomal degradation by attaching ubiquitin to lysines in the SUMO chain (Perry et al., 2008; Denuc and Marfany, 2010). Dgrn binds to poly-sumoylated Gro and lifts Gro-mediated transcriptional repression. Dgrn does not bind to Gro itself but rather to the associated TF Hairy, which recruits Gro and the SUMO chains attached to Gro (Abed et al., 2011). These authors proposed that sumoylation sequestered Gro in larger oligomers. This antagonism between Drgn and Gro was not Hairy-specific, but affected Hairy-independent loci as well. In a similar manner, plant StUbLs might lift TPL/TPRs- or LUG/SEU-based gene repression sequestering them in larger oligomers.

EAR-CONTAINING TFs ARE NOT *PER SE* SUMO TARGETS DESPITE THEIR ROLE IN GENE REPRESSION

The TPL/TPRs interactome has recently been exposed using Y2H approaches, revealing >200 partners including many known interactors (Arabidopsis Interactome Mapping Consortium, 2011; Causier et al., 2012). This interactome included a wide range of TFs, many of which were previously implicated in transcriptional repression. Importantly, TPL/TPRs lack a clear DNA-binding domain. Instead, they are recruited to specific TFs that contain an Ethylene-responsive element binding factor (ERF)-associated Amphiphilic Repression (EAR) motif (Kagale and Rozwadowski, 2011; Causier et al., 2012). Considering the close ties between SUMO and HDAC repressor complexes, we examined the extent to which EAR-containing TFs are also subject to sumoylation, as

their sumoylation might influence recruitment of co-adaptors or other proteins to repressor complexes. We noted only a small overlap (27 proteins) between the TPL/TPRs interactome and the list of Arabidopsis SUMO substrates identified (**Table A1** in Appendix). Hence, EAR- and sumoylation-mediated recruitment of chromatin-modifying complexes to TFs likely involves different sets of TFs. Consequently, EAR-dependent recruitment of co-adaptors like TPL and AtSAP18 likely does not require sumoylation of the TFs involved. A similar situation was reported for *Drosophila*; the SUMO consensus motif was enriched in *Drosophila* TFs with a dual function in gene regulation (both induction and gene repression), while it was not in TFs that were predicted to have a single activity (Bauer et al., 2010).

SUMO ACETYLATION BLOCKS SUMO-SIM INTERACTIONS AND PROMOTES BROMODOMAIN-DEPENDENT GENE ACTIVATION

While SUMO modifies HDAC- and HAT-containing complexes, SUMO itself is a substrate for acetylation. SUMO acetylation (Ac) has been reported to mimic acetylation of the TF modified by Ac-SUMO (Cheema et al., 2010). SUMO acetylation neutralizes the basic charges that surround the SIM docking site and, most remarkably, prevents SUMO-SIM interactions (Ullmann et al., 2012; **Figure 1B**). Possibly, SUMO acetylation acts as a first step to resolve SUMO-mediated protein interactions, because when bound to SIMs certain SUMO conjugates are protected from deconjugation (Zhu et al., 2009). SUMO acetylation does not only attenuate "SIM-SUMO"-dependent gene silencing, but it also promotes SUMO-bromodomain interactions (Ullmann et al., 2012). Bromodomains are typically found in transcriptional co-activators and are unique in that they bind acetylated histones (Mujtaba et al., 2007). Conversely, the plant homeodomain (PHD) domain present in the Arabidopsis SUMO E3 ligase SIZ1 is required for sumoylation of two bromodomain-containing TFs, GTE3, and GTE5; sumoylation of GTE3 suppressed its binding to acetylated histone H3 (Garcia-Dominguez et al., 2008). Similarly, the PHD domain in the mammalian co-repressor Krüppel-associated protein (KAP1) controls intramolecular sumoylation of the adjacent bromodomain required for KAP1-mediated gene silencing (Ivanov et al., 2007). In this case, the PHD domain acts as a specific SUMO E3 ligase for bromodomains. Moreover, class II HDACs have been reported to promote sumoylation of specific substrates, suggesting that they also act as SUMO E3 ligases (Garcia-Dominguez and Reyes, 2009), while HATs likely promote SUMO acetylation. These findings support a model in which "PHD domain-mediated bromodomain sumoylation" and "HDAC/HAT-mediated SUMO (de)acetylation" provide the cell with a bi-directional transcriptional switch involving "SUMO-SIM" dependent gene silencing and "AcetylSUMO-bromodomain" dependent gene activation, respectively (**Figure 1B**).

SUMOYLATION CONTROLS mRNA BIOGENESIS AND NUCLEAR EXPORT

Small-Ubiquitin-like MOdifier proteomics studies have also revealed a major role for SUMO in mRNA biogenesis including mRNA processing, editing, and nuclear export in different eukaryotes including plants, as recently reviewed (Vethantham and Manley, 2009; Meier, 2012). The notion is that transient sumoylation events in the nucleus form a critical step in mRNA surveillance to retain unspliced pre-mRNAs in the nucleus. Currently, 39 Arabidopsis SUMO targets have been identified with a confirmed or predicted role in mRNA biogenesis. Studies in yeast and mammalian cells also revealed SUMO targets involved in 5′ pre-mRNA capping, splicing, 3′ processing, and mRNA export (Vethantham and Manley, 2009). For example, small nuclear ribonucleoproteins (snRNPs) involved in splicing of pre-mRNA in the spliceosome are SUMO targets. Moreover, several heterogeneous nuclear ribonucleoproteins (hnRNPs) that bind pre-mRNA are SUMO targets (Li et al., 2004; Blomster et al., 2009). Sumoylation of hnRNP C and M decreases their binding to nucleic acids (Vassileva and Matunis, 2004).

Notably, components of the plant nuclear pore complex (NPC) such as Importin-6 (IMP-6), IMPα1, and WPP domain interacting protein 1 (WIP1) are SUMO targets (Miller et al., 2010). Arabidopsis Nuclear pore anchor (NUA) is also a SUMO target, at least *in vitro* (Elrouby and Coupland, 2010). NUA interacts physically with the SUMO protease ESD4 at the nuclear rim (Xu et al., 2007). Loss of function mutations in ESD4, SIZ1, but also of two Arabidopsis genes involved in mRNA trafficking, NUA, and the scaffold nucleoporin Nup160, resulted in nuclear retention of both SUMO conjugates and mRNA (Xu et al., 2007; Muthuswamy and Meier, 2011). In addition, ESD4, its yeast homolog Ulp1, and its mammalian homologs SENP1 (Sentrin-specific protease 1) and SENP2 all localize to the inner side of the nuclear envelope through association with NPCs (Murtas et al., 2003; Mukhopadhyay and Dasso, 2007; Xu et al., 2007). Hence, Arabidopsis NUA and Nup160 connect SUMO conjugation directly with general nuclear import and export via NPCs in plants. Overall, the emerging picture is that SUMO controls many steps in mRNA biogenesis and nucleo-cytoplasmic trafficking.

GLOBAL (POLY-)SUMOYLATION AS RESPONSE TO STRESS-INDUCED PROTEIN DAMAGE

When exposed to abiotic stresses, such as heat shock, drought, or freezing, plants respond with global protein sumoylation (Kurepa et al., 2003). Similarly, protein-damaging agents like ethanol or the non-protein amino acid L-canavanine trigger global SUMO conjugation in Arabidopsis (Kurepa et al., 2003). This is clearly a general response, which is also seen in yeast and mammalian cells in response to heat stress and protein damage and it is essential for cell survival of HeLa cells after heat stress (Saitoh and Hinchey, 2000; Golebiowski et al., 2009; Tatham et al., 2011). Quantitative proteomics studies on HsSUMO-2 conjugation in HeLa cells have revealed that heat stress triggers differential sumoylation of hundreds of proteins (Golebiowski et al., 2009). Interestingly, many subunits of repressor complexes including SIN3, NuRd, and SetDB1 (that methylates histones which in turn promotes binding of HP1 proteins to maintain chromatin silencing) complexes showed enhanced sumoylation upon heat stress in HeLa cells. In contrast, sumoylation of the histones H2A, H2B, and H4, but also HDACs and HATs was reduced upon heat shock in these cells. Hence, many subunits of chromatin remodeling complexes become sumoylated, while the responsible enzymes and histones are deSUMOylated. Similar changes in Arabidopsis

SUMO1 conjugation levels were reported for specific groups of proteins when seedlings were exposed to heat stress (Miller and Vierstra, 2011). For example, the co-adaptors TPL, SEU, PKL, SWI3C, and CHR11 were more sumoylated upon heat stress. On the other hand, Arabidopsis histone H2B was less sumoylated after heat stress, as seen for HeLa cells.

One consequence of heat stress is that RNA splicing is generally inhibited (Yost and Lindquist, 1986). Concomitantly, snRNPS involved in RNA splicing showed less sumoylation upon heat stress in mammalian cells. Splicing inhibition promotes production and export of mRNAs coding for HSPs, as the corresponding genes generally lack introns, thereby allowing HSP-mediated cellular recovery after heat stress (Golebiowski et al., 2009). A follow-up proteomics study revealed that sumoylation levels changed for 564 out of 1355 HsSUMO-2 targets when the 26S proteasome was inhibited with MG132 (Tatham et al., 2011). Interestingly, the global sumoylation response triggered by heat stress is positively correlated with the response triggered by proteasome inhibition involving largely overlapping sets of targets. However, inhibition of protein synthesis blocked the global sumoylation response induced by proteasome inhibition, but not by heat stress. This implies that newly synthesized misfolded SUMO targets are destined for protein degradation, while heat stress enhances sumoylation of existing proteins possibly to aid their refolding (also in this case the responses are correlated, i.e., largely the same proteins are sumoylated; Tatham et al., 2011).

The picture is even more complex, as heat stress also triggered poly-SUMO chain editing on >900 SUMO targets in HeLa cells (Bruderer et al., 2011). SUMO targets implicated in gene regulation and chromatin structure were almost exclusively modified with five or more SUMO molecules, while SUMO targets involved in DNA replication, DNA repair, and mRNA biogenesis had variable SUMO chain lengths starting from three. Other studies indicated earlier that ubiquitin only co-purifies with SUMO isoforms that contain an internal acceptor site utilized for poly-SUMO chain formation, but not with isoforms that lack such sites like HsSUMO-1 (Schimmel et al., 2008). These poly-SUMO chains serve as docking site for StUbLs (Perry et al., 2008; Denuc and Marfany, 2010). These findings demonstrate an unexpected large regulatory role for StUbL-dependent protein degradation of SUMO conjugates after heat stress. Also in plants poly-SUMO chains and mixed ubiquitin-SUMO chains have been found (Miller et al., 2010). This means that poly-SUMO chain-mediated protein degradation likely occurs in plants. In support, *in vitro* sumoylation assays indicated that the canonical Arabidopsis SUMO isoforms SUMO1 and -2 contain an internal SUMO acceptor site (Lys10) used for SUMO chain formation (Colby et al., 2006; **Figure 1A**). Interestingly, *in vivo* studies revealed SUMO chain editing on other SUMO1 residues (Lys23 and Lys41), while Lys23 was also subject to ubiquitination (Miller et al., 2010). StUbLs contain tandem arrayed SIMs in their N-termini that recognize poly-SUMO chain-modified proteins and RING-finger domains in their C-termini involved in ubiquitination. Based on this, a putative StUbL protein family was recently proposed for plants based on protein sequence homology and sequence conservation across different plant species (Novatchkova et al., 2012). However, data on the function of this potential Arabidopsis StUbL is missing.

Within 5 min after heat stress, the mammalian SUMO machinery, including the SIZ1 homolog PIASy and the SUMO conjugating enzyme Ubc9, are transiently recruited to the *HSP70.1* promoter and induce PIASy-dependent sumoylation of PARP-1 [Poly(ADP-ribose) polymerase 1; Martin et al., 2009; Messner et al., 2009]. Poly(ADP-ribose) is associated with chromatin decompacting and nucleosome loss. PIASy-mediated sumoylation of PARP-1 is necessary for full activation of the *HSP70.1* gene. Martin et al. proposed that heat shock induces rapid sumoylation of PARP-1 at the *HSP70.1* promoter, followed by ubiquitylation and degradation. The latter requires the StUbL RNF4 (RING-finger protein 4) suggesting that PARP-1 is modified with poly-SUMO chains. In Arabidopsis, AtPARP1 becomes also sumoylated upon heat stress (Miller et al., 2010). Therefore, the effect of SUMO on PARP-1 function in heat stress is likely conserved in Arabidopsis. Moreover, overexpression of cytosolic HSP70 enhanced heat tolerance in Arabidopsis seedlings, while suppressing the global SUMOylation responses triggered by heat stress (Kurepa et al., 2003). These data, thus, suggest that heat shock-dependent PARP-1 sumoylation and degradation increases *HSP70* mRNA levels and correspondingly its protein levels, while a 4–5 fold increase in HSP70 protein levels suppresses the global sumoylation response in Arabidopsis.

Overall, these findings imply that sumoylation controls heat-shock responses at (i) the level of *HSP* transcripts, (ii) pre-mRNA processing level favoring nuclear export of *HSP* transcripts, and (iii) at the level of protein folding and degradation of misfolded proteins, until the levels of HSPs have increased to sufficient levels to deal with protein damage caused by heat stress.

ACKNOWLEDGMENTS

We are grateful to Frank Takken, Martin Rep, and the anonymous reviewers for providing critical review and helpful comments. This work was supported by the NWO (Netherland Scientific Organization; ALW-VIDI grant 864.10.004).

REFERENCES

Abed, M., Barry, K. C., Kenyagin, D., Koltun, B., Phippen, T. M., Delrow, J. J., Parkhurst, S. M., and Orian, A. (2011). Degringolade, a SUMO-targeted ubiquitin ligase, inhibits hairy/groucho-mediated repression. *EMBO J.* 30, 1289–1301.

Ahn, J. W., Lee, Y. A., Ahn, J. H., and Choi, C. Y. (2009). Covalent conjugation of groucho with SUMO-1 modulates its corepressor activity. *Biochem. Biophys. Res. Commun.* 379, 160–165.

Aichinger, E., Villar, C. B. R., Farrona, S., Reyes, J. C., Hennig, L., and Kohler, C. (2009). CHD3 proteins and polycomb group proteins antagonistically determine cell identity in Arabidopsis. *PLoS Genet.* 5, e1000605. doi: 10.1371/journal.pgen.1000605

Anckar, J., and Sistonen, L. (2007). SUMO: getting it on. *Biochem. Soc. Trans.* 35, 1409–1413.

Arabidopsis Interactome Mapping Consortium. (2011). Evidence for network evolution in an Arabidopsis interactome map. *Science* 333, 601–607.

Ausin, I., Alonso-Blanco, C., Jarillo, J. A., Ruiz-Garcia, L., and Martinez-Zapater, J. M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. *Nat. Genet.* 36, 162–166.

Baron, R., and Vellore, N. A. (2012). LSD1/CoREST Reversible opening-closing dynamics: discovery of a nanoscale clamp for chromatin

and protein binding. *Biochemistry* 51, 3151–3153.

Bauer, D. C., Buske, F. A., and Bailey, T. L. (2010). Dual-functioning transcription factors in the developmental gene network of Drosophila melanogaster. *BMC Bioinformatics* 11, 366. doi:10.1186/1471-2105-11-366

Benhamed, M., Martin-Magniette, M. L., Taconnat, L., Bitton, F., Servet, C., De Clercq, R., De Meyer, B., Buysschaert, C., Rombauts, S., Villarroel, R., Aubourg, S., Beynon, J., Bhalerao, R. P., Coupland, G., Gruissem, W., Menke, F. L. H., Weisshaar, B., Renou, J. P., Zhou, D. X., and Hilson, P. (2008). Genome-scale Arabidopsis promoter array identifies targets of the histone acetyltransferase GCN5. *Plant J.* 56, 493–504.

Bernier-Villamor, V., Sampson, D. A., Matunis, M. J., and Lima, C. D. (2002). Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. *Cell* 108, 345–356.

Blomster, H. A., Hietakangas, V., Wu, J., Kouvonen, P., Hautaniemi, S., and Sistonen, L. (2009). Novel proteomics strategy brings insight into the prevalence of SUMO-2 target sites. *Mol. Cell. Proteomics* 8, 1382–1390.

Blomster, H. A., Imanishi, S. Y., Siimes, J., Kastu, J., Morrice, N. A., Eriksson, J. E., and Sistonen, L. (2010). In vivo identification of sumoylation sites by a signature tag and cysteine-targeted affinity purification. *J. Biol. Chem.* 285, 19324–19329.

Bruderer, R., Tatham, M. H., Plechanovova, A., Matic, I., Garg, A. K., and Hay, R. T. (2011). Purification and identification of endogenous poly SUMO conjugates. *EMBO Rep.* 12, 142–148.

Budhiraja, R., Hermkes, R., Muller, S., Schmidt, J., Colby, T., Panigrahi, K., Coupland, G., and Bachmair, A. (2009). Substrates related to chromatin and to RNA-dependent processes are modified by Arabidopsis SUMO isoforms that differ in a conserved residue with influence on desumoylation. *Plant Physiol.* 149, 1529–1540.

Castano-Miquel, L., Segui, J., and Lois, L. M. (2011). Distinctive properties of Arabidopsis SUMO paralogues support the in vivo predominant role of AtSUMO1/2 isoforms. *Biochem. J.* 436, 581–590.

Castro, P. H., Tavares, R. M., Bejarano, E. R., and Azevedo, H. (2012). SUMO, a heavyweight player in plant abiotic stress responses. *Cell. Mol. Life Sci.* 10.1007/s00018-00012-01094–1012.

Catala, R., Ouyang, J., Abreu, I. A., Hu, Y., Seo, H., Zhang, X., and Chua, N. H. (2007). The Arabidopsis E3 SUMO ligase SIZ1 regulates plant growth and drought responses. *Plant Cell* 19, 2952–2966.

Causier, B., Ashworth, M., Guo, W., and Davies, B. (2012). The TOPLESS interactome: a framework for gene repression in Arabidopsis. *Plant Physiol.* 158, 423–438.

Cheema, A., Knights, C. D., Rao, M., Catania, J., Perez, R., Simons, B., Dakshanamurthy, S., Kolukula, V. K., Tilli, M., Furth, P. A., Albanese, C., and Avantaggiati, M. L. (2010). Functional mimicry of the acetylated C-terminal tail of p53 by a SUMO-1 acetylated domain, SAD. *J. Cell. Physiol.* 225, 371–384.

Colby, T., Matthai, A., Boeckelmann, A., and Stuible, H. P. (2006). SUMO-conjugating and SUMO-deconjugating enzymes from Arabidopsis. *Plant Physiol.* 142, 318–332.

David, G., Neptune, M. A., and Depinho, R. A. (2002). SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities. *J. Biol. Chem.* 277, 23658–23663.

Deal, R. B., and Henikoff, S. (2011). The INTACT method for cell type-specific gene expression and chromatin profiling in Arabidopsis thaliana. *Nat. Protoc.* 6, 56–68.

Denison, C., Rudner, A. D., Gerber, S. A., Bakalarski, C. E., Moazed, D., and Gygi, S. P. (2005). A proteomic strategy for gaining insights into protein sumoylation in yeast. *Mol. Cell. Proteomics* 4, 246–254.

Denuc, A., and Marfany, G. (2010). SUMO and ubiquitin paths converge. *Biochem. Soc. Trans.* 38, 34–39.

Elrouby, N., and Coupland, G. (2010). Proteome-wide screens for small ubiquitin-like modifier (SUMO) substrates identify Arabidopsis proteins implicated in diverse biological processes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17415–17420.

Emanuele, M. J., Elia, A. E. H., Xu, Q., Thoma, C. R., Izhar, L., Leng, Y., Guo, A., Chen, Y. N., Rush, J., Hsu, P. W. C., Yen, H. C. S., and Elledge, S. J. (2011). Global identification of modular cullin-RING ligase substrates. *Cell* 147, 459–474.

Galisson, F., Mahrouche, L., Courcelles, M., Bonneil, E., Meloche, S., Chelbi-Alix, M. K., and Thibault, P. (2011). A novel proteomics approach to identify SUMOylated proteins and their modification sites in human cells. *Mol. Cell. Proteomics* 10, M110 004796.

Ganesan, A. K., Kho, Y., Kim, S. C., Chen, Y., Zhao, Y., and White, M. A. (2007). Broad spectrum identification of SUMO substrates in melanoma cells. *Proteomics* 7, 2216–2221.

Garcia-Dominguez, M., March-Diaz, R., and Reyes, J. C. (2008). The PHD domain of plant PIAS proteins mediates sumoylation of bromodomain GTE proteins. *J. Biol. Chem.* 283, 21469–21477.

Garcia-Dominguez, M., and Reyes, J. C. (2009). SUMO association with repressor complexes, emerging routes for transcriptional control. *Biochim. Biophys. Acta* 1789, 451–459.

Gocke, C. B., and Yu, H. (2008). ZNF198 stabilizes the LSD1-CoREST-HDAC1 complex on chromatin through its MYM-type zinc fingers. *PLoS ONE* 3, e3255. doi:10.1371/journal.pone.0003255

Golebiowski, F., Matic, I., Tatham, M. H., Cole, C., Yin, Y., Nakamura, A., Cox, J., Barton, G. J., Mann, M., and Hay, R. T. (2009). System-wide changes to SUMO modifications in response to heat shock. *Sci. Signal.* 2, ra24.

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.

Gu, X., Jiang, D., Yang, W., Jacob, Y., Michaels, S. D., and He, Y. (2011). Arabidopsis homologs of retinoblastoma-associated protein 46/48 associate with a histone deacetylase to act redundantly in chromatin silencing. *PLoS Genet.* 7, e1002366. doi:10.1371/journal.pgen.1002366

Hannich, J. T., Lewis, A., Kroetz, M. B., Li, S. J., Heide, H., Emili, A., and Hochstrasser, M. (2005). Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. *J. Biol. Chem.* 280, 4102–4110.

He, Y., Michaels, S. D., and Amasino, R. M. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. *Science* 302, 1751–1754.

Hsiao, H. H., Meulmeester, E., Frank, B. T. C., Melchior, F., and Urlaub, H. (2009). "ChopNspice," a mass-spectrometric approach that allows identification of endogenous SUMO-conjugated peptides. *Mol. Cell. Proteomics* 2664–2675.

Ishida, T., Fujiwara, S., Miura, K., Stacey, N., Yoshimura, M., Schneider, K., Adachi, S., Minamisawa, K., Umeda, M., and Sugimoto, K. (2009). SUMO E3 Ligase HIGH PLOIDY2 Regulates Endocycle Onset and Meristem Maintenance in Arabidopsis. *Plant Cell* 21, 2284–2297.

Ivanov, A. V., Peng, H., Yurchenko, V., Yap, K. L., Negorev, D. G., Schultz, D. C., Psulkowski, E., Fredericks, W. J., White, D. E., Maul, G. G., Sadofsky, M. J., Zhou, M. M., and Rauscher, F. J. III. (2007). PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. *Mol. Cell* 28, 823–837.

Jennings, B. H., and Ish-Horowicz, D. (2008). The groucho/TLE/Grg family of transcriptional co-repressors. *Genome Biol.* 9, 205.

Jeon, J., and Kim, J. (2011). FVE, an Arabidopsis homologue of the retinoblastoma-associated protein that regulates flowering time and cold response, binds to chromatin as a large multiprotein complex. *Mol. Cells* 32, 227–234.

Jeram, S. M., Srikumar, T., Zhang, X. D., Anne Eisenhauer, H., Rogers, R., Pedrioli, P. G. A., Matunis, M., and Raught, B. (2010). An improved SUMmOn-based methodology for the identification of ubiquitin and ubiquitin-like protein conjugation sites identifies novel ubiquitin-like protein chain linkages. *Proteomics* 10, 254–265.

Jin, J. B., Jin, Y. H., Lee, J., Miura, K., Yoo, C. Y., Kim, W. Y., Van Oosten, M., Hyun, Y., Somers, D. E., Lee, I., Yun, D. J., Bressan, R. A., and Hasegawa, P. M. (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.

Kagale, S., and Rozwadowski, K. (2011). EAR motif-mediated transcriptional repression in plants: an underlying mechanism for epigenetic regulation of gene expression. *Epigenetics* 6, 141–146.

Kaminsky, R., Denison, C., Bening-Abu-Shach, U., Chisholm, A. D., Gygi, S. P., and Broday, L. (2009). SUMO regulates the assembly and function of a cytoplasmic intermediate filament protein in C. *elegans. Dev. Cell* 17, 724–735.

Kerscher, O. (2007). SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep.* 8, 550–555.

Kim, H. J., Hyun, Y., Park, J. Y., Park, M. J., Park, M. K., Kim, M. D., Lee, M. H., Moon, J., Lee, I., and Kim, J. (2004). A genetic link between cold responses and flowering time through FVE in Arabidopsis thaliana. *Nat. Genet.* 36, 167–171.

Kim, W., Bennett, E. J., Huttlin, E. L., Guo, A., Li, J., Possemato, A., Sowa, M. E., Rad, R., Rush, J., Comb, M. J., Harper, J. W., and Gygi, S. P. (2011). Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* 44, 325–340.

Kunert, N., and Brehm, A. (2009). Novel Mi-2 related ATP-dependent chromatin remodelers. *Epigenetics* 4, 209–211.

Kunert, N., Wagner, E., Murawska, M., Klinker, H., Kremmer, E., and Brehm, A. (2009). dMec: a novel Mi-2 chromatin remodelling complex involved in transcriptional repression. *EMBO J.* 28, 533–544.

Kurepa, J., Walker, J. M., Smalle, J., Gosink, M. M., Davis, S. J., Durham, T. L., Sung, D. Y., and Vierstra, R. D. (2003). The small ubiquitin-like modifier (SUMO) protein modification system in Arabidopsis. Accumulation of SUMO1 and -2 conjugates is increased by stress. *J. Biol. Chem.* 278, 6862–6872.

Lee, J. E., and Golz, J. F. (2012). Diverse roles of Groucho/Tup1 co-repressors in plant growth and development. *Plant Signal. Behav.* 7, 86–92.

Li, T., Evdokimov, E., Shen, R. F., Chao, C. C., Tekle, E., Wang, T., Stadtman, E. R., Yang, D. C. H., and Chock, P. B. (2004). Sumoylation of heterogeneous nuclear ribonucleoproteins, zinc finger proteins, and nuclear pore complex proteins: a proteomic analysis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8551–8556.

Liu, Z., and Karmarkar, V. (2008). Groucho/Tup1 family co-repressors in plant development. *Trends Plant Sci.* 13, 137–144.

Loyola, A., and Almouzni, G. (2004). Histone chaperones, a supporting role in the limelight. *Biochim. Biophys. Acta* 1677, 3–11.

Makhnevych, T., Sydorskyy, Y., Xin, X., Srikumar, T., Vizeacoumar, F. J., Jeram, S. M., Li, Z., Bahr, S., Andrews, B. J., Boone, C., and Raught, B. (2009). Global map of SUMO function revealed by protein-protein interaction and genetic networks. *Mol. Cell* 33, 124–135.

Mao, Y., Pavangadkar, K. A., Thomashow, M. F., and Triezenberg, S. J. (2006). Physical and functional interactions of Arabidopsis ADA2 transcriptional coactivator proteins with the acetyltransferase GCN5 and with the cold-induced transcription factor CBF1. *Biochim. Biophys. Acta* 1759, 69–79.

Martin, N., Schwamborn, K., Schreiber, V., Werner, A., Guillier, C., Zhang, X. D., Bischof, O., Seeler, J. S., and Dejean, A. (2009). PARP-1 transcriptional activity is regulated by sumoylation upon heat shock. *EMBO J.* 28, 3534–3548.

Matafora, V., D'amato, A., Mori, S., Blasi, F., and Bachi, A. (2009). Proteomics analysis of nucleolar SUMO-1 target proteins upon proteasome inhibition. *Mol. Cell. Proteomics* 8, 2243–2255.

Matic, I., Schimmel, J., Hendriks, I. A., Van Santen, M. A., Van De Rijke, F., Van Dam, H., Gnad, F., Mann, M., and Vertegaal, A. C. O. (2010). Site-specific identification of SUMO-2 targets in cells reveals an inverted SUMOylation motif and a hydrophobic cluster SUMOylation motif. *Mol. Cell* 39, 641–652.

McDonel, P., Costello, I., and Hendrich, B. (2009). Keeping things quiet: roles of NuRD and Sin3 co-repressor complexes during mammalian development. *Int. J. Biochem. Cell Biol.* 41, 108–116.

Meier, I. (2012). mRNA export and sumoylation-lessons from plants. *Biochim. Biophys. Acta,* 531–537.

Messner, S., Schuermann, D., Altmeyer, M., Kassner, I., Schmidt, D., Schar, P., Muller, S., and Hottiger, M. O. (2009). Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function. *FASEB J.* 23, 3978–3989.

Miller, M. J., Barrett-Wilt, G. A., Hua, Z., and Vierstra, R. D. (2010). Proteomic analyses identify a diverse array of nuclear processes affected by small ubiquitin-like modifier conjugation in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16512–16517.

Miller, M. J., and Vierstra, R. D. (2011). Mass spectrometric identification of SUMO substrates provides insights into heat stress-induced SUMOylation in plants. *Plant Signal. Behav.* 6, 130–133.

Miura, K., Jin, J. B., Lee, J., Yoo, C. Y., Stirm, V., Miura, T., Ashworth, E. N., Bressan, R. A., Yun, D. J., and Hasegawa, P. M. (2007). SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in Arabidopsis. *Plant Cell* 19, 1403–1414.

Mossessova, E., and Lima, C. D. (2000). Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Mol. Cell* 5, 865–876.

Mujtaba, S., Zeng, L., and Zhou, M. M. (2007). Structure and acetyl-lysine recognition of the bromodomain. *Oncogene* 26, 5521–5527.

Mukhopadhyay, D., and Dasso, M. (2007). Modification in reverse: the SUMO proteases. *Trends Biochem. Sci.* 32, 286–295.

Murtas, G., Reeves, P. H., Fu, Y. F., Bancroft, I., Dean, C., and Coupland, G. (2003). A nuclear protease required for flowering-time regulation in Arabidopsis reduces the abundance of small ubiquitin-related modifier conjugates. *Plant Cell* 15, 2308–2319.

Murzina, N. V., Pei, X. Y., Zhang, W., Sparkes, M., Vicente-Garcia, J., Pratap, J. V., Mclaughlin, S. H., Ben-Shahar, T. R., Verreault, A., Luisi, B. F., and Laue, E. D. (2008). Structural basis for the recognition of histone H4 by the histone-chaperone RbAp46. *Structure* 16, 1077–1085.

Muthuswamy, S., and Meier, I. (2011). Genetic and environmental changes in SUMO homeostasis lead to nuclear mRNA retention in plants. *Planta* 233, 201–208.

Nacerddine, K., Lehembre, F., Bhaumik, M., Artus, J., Cohen-Tannoudji, M., Babinet, C., Pandolfi, P. P., and Dejean, A. (2005). The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. *Dev. Cell* 9, 769–779.

Nie, M., Xie, Y., Loo, J. A., and Courey, A. J. (2009). Genetic and proteomic evidence for roles of Drosophila SUMO in cell cycle control, Ras signaling, and early pattern formation. *PLoS ONE* 4, e5905. doi:10.1371/journal.pone.0005905

Novatchkova, M., Tomanov, K., Hofmann, K., Stuible, H. P., and Bachmair, A. (2012). Update on sumoylation: defining core components of the plant SUMO conjugation system by phylogenetic comparison. *New Phytol.* 195, 23–31.

Panse, V. G., Hardeland, U., Werner, T., Kuster, B., and Hurt, E. (2004). A proteome-wide approach identifies sumoylated substrate proteins in yeast. *J. Biol. Chem.* 279, 41346–41351.

Park, H. C., Choi, W., Park, H. J., Cheong, M. S., Koo, Y. D., Shin, G., Chung, W. S., Kim, W. Y., Kim, M. G., Bressan, R. A., Bohnert, H. J., Lee, S. Y., and Yun, D. J. (2011a). Identification and molecular properties of SUMO-binding proteins in Arabidopsis. *Mol. Cells* 32, 143–151.

Park, H. J., Kim, W. Y., Park, H. C., Lee, S. Y., Bohnert, H. J., and Yun, D. J. (2011b). SUMO and SUMOylation in plants. *Mol. Cells* 32, 305–316.

Pedrioli, P. G., Raught, B., Zhang, X. D., Rogers, R., Aitchison, J., Matunis, M., and Aebersold, R. (2006). Automated identification of SUMOylation sites using mass spectrometry and SUMmOn pattern recognition software. *Nat. Methods* 3, 533–539.

Perry, J. J., Tainer, J. A., and Boddy, M. N. (2008). A SIM-ultaneous role for SUMO and ubiquitin. *Trends Biochem. Sci.* 33, 201–208.

Saitoh, H., and Hinchey, J. (2000). Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J. Biol. Chem.* 275, 6252–6258.

Samara, N. L., and Wolberger, C. (2011). A new chapter in the transcription SAGA. *Curr. Opin. Struct. Biol.* 21, 767–774.

Saracco, S. A., Miller, M. J., Kurepa, J., and Vierstra, R. D. (2007). Genetic analysis of SUMOylation in Arabidopsis: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. *Plant Physiol.* 145, 119–134.

Schimmel, J., Larsen, K. M., Matic, I., Van Hagen, M., Cox, J., Mann, M., Andersen, J. S., and Vertegaal, A. C. O. (2008). The ubiquitin-proteasome system is a key component of the SUMO-2/3 cycle. *Mol. Cell. Proteomics* 7, 2107–2122.

Servet, C., Conde E Silva, N., and Zhou, D. X. (2010). Histone acetyltransferase AtGCN5/HAG1 is a versatile regulator of developmental and inducible gene expression in Arabidopsis. *Mol. Plant* 3, 670–677.

Song, C. P., Agarwal, M., Ohta, M., Guo, Y., Halfter, U., Wang, P., and Zhu, J. K. (2005). Role of an Arabidopsis AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. *Plant Cell* 17, 2384–2396.

Song, C. P., and Galbraith, D. W. (2006). AtSAP18, an orthologue of human SAP18, is involved in the regulation of salt stress and mediates transcriptional repression in Arabidopsis. *Plant Mol. Biol.* 60, 241–257.

Sridhar, V. V., Surendrarao, A., Gonzalez, D., Conlan, R. S., and Liu, Z. (2004). Transcriptional repression of target genes by LEUNIG and SEUSS, two interacting regulatory proteins for Arabidopsis flower development. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11494–11499.

Srilunchang, K. O., Krohn, N. G., and Dresselhaus, T. (2010). DiSUMO-like DSUL is required for nuclei positioning, cell specification and viability during female

gametophyte maturation in maize. *Development* 137, 333–345.

Sterner, D. E., Nathan, D., Reindle, A., Johnson, E. S., and Berger, S. L. (2006). Sumoylation of the yeast Gcn5 protein. *Biochemistry* 45, 1035–1042.

Stielow, B., Kruger, I., Diezko, R., Finkernagel, F., Gillemans, N., Kong-a-San, J., Philipsen, S., and Suske, G. (2010). Epigenetic silencing of spermatocyte-specific and neuronal genes by SUMO modification of the transcription factor Sp3. *PLoS Genet.* 6, e1001203. doi:10.1371/journal.pgen.1001203

Stielow, B., Sapetschnig, A., Kruger, I., Kunert, N., Brehm, A., Boutros, M., and Suske, G. (2008a). Identification of SUMO-dependent chromatin-associated transcriptional repression components by a genome-wide RNAi screen. *Mol. Cell* 29, 742–754.

Stielow, B., Sapetschnig, A., Wink, C., Kruger, I., and Suske, G. (2008b). SUMO-modified Sp3 represses transcription by provoking local heterochromatic gene silencing. *EMBO Rep.* 9, 899–906.

Tatham, M. H., Matic, I., Mann, M., and Hay, R. T. (2011). Comparative proteomic analysis identifies a role for SUMO in protein quality control. *Sci. Signal.* 4, rs4.

Ullmann, R., Chien, C. D., Avantaggiati, M. L., and Muller, S. (2012). An acetylation switch regulates SUMO-dependent protein interaction networks. *Mol. Cell* 46, 759–770.

van den Burg, H. A., Kini, R. K., Schuurink, R. C., and Takken, F. L. W. (2010). Arabidopsis small ubiquitin-like modifier paralogs have distinct functions in development and defense. *Plant Cell* 22, 1998–2016.

Vassileva, M. T., and Matunis, M. J. (2004). SUMO modification of heterogeneous nuclear ribonucleoproteins. *Mol. Cell. Biol.* 24, 3623–3632.

Vertegaal, A. C. O. (2011). Uncovering ubiquitin and ubiquitin-like signaling networks. *Chem. Rev.* 111, 7923–7940.

Vertegaal, A. C. O., Andersen, J. S., Ogg, S. C., Hay, R. T., Mann, M., and Lamond, A. I. (2006). Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. *Mol. Cell. Proteomics* 5, 2298–2310.

Vertegaal, A. C. O., Ogg, S. C., Jaffray, E., Rodriguez, M. S., Hay, R. T., Andersen, J. S., Mann, M., and Lamond, A. I. (2004). A proteomic study of SUMO-2 target proteins. *J. Biol. Chem.* 279, 33791–33798.

Vethantham, V., and Manley, J. L. (2009). "Emerging roles for SUMO in mRNA processing and metabolsim," in *SUMO regulation of cellular processes*, ed. V. G. Wilson (Dordrecht: Springer), 41–57.

Wagner, S. A., Beli, P., Weinert, B. T., Nielsen, M. L., Cox, J., Mann, M., and Choudhary, C. (2011). A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol. Cell. Proteomics* 10, M111 013284.

Wang, Y., Zhang, H., Chen, Y., Sun, Y., Yang, F., Yu, W., Liang, J., Sun, L., Yang, X., Shi, L., Li, R., Li, Y., Zhang, Y., Li, Q., Yi, X., and Shang, Y. (2009). LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer. *Cell* 138, 660–672.

Westman, B. J., Verheggen, C., Hutten, S., Lam, Y. W., Bertrand, E., and Lamond, A. I. (2010). A proteomic screen for nucleolar SUMO targets shows SUMOylation modulates the function of Nop5/Nop58. *Mol. Cell* 39, 618–631.

Wilkinson, K. A., and Henley, J. M. (2010). Mechanisms, regulation and consequences of protein SUMOylation. *Biochem. J.* 428, 133–145.

Wohlschlegel, J. A., Johnson, E. S., Reed, S. I., and Yates, J. R. III. (2006). Improved identification of SUMO attachment sites using C-terminal SUMO mutants and tailored protease digestion strategies. *J. Proteome Res.* 5, 761–770.

Wohlschlegel, J. A., Johnson, E. S., Reed, S. I., and Yates, J. R.III. (2004). Global analysis of protein sumoylation in Saccharomyces cerevisiae. *J. Biol. Chem.* 279, 45662–45668.

Wykoff, D. D., and O'shea, E. K. (2005). Identification of sumoylated proteins by systematic immunoprecipitation of the budding yeast proteome. *Mol. Cell. Proteomics* 4, 73–83.

Xu, G., and Jaffrey, S. R. (2011). The new landscape of protein ubiquitination. *Nat. Biotechnol.* 29, 1098–1100.

Xu, G., Paige, J. S., and Jaffrey, S. R. (2010). Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat. Biotechnol.* 28, 868–873.

Xu, X. M., Rose, A., Muthuswamy, S., Jeong, S. Y., Venkatakrishnan, S., Zhao, Q., and Meier, I. (2007). Nuclear pore anchor, the Arabidopsis homolog of Tpr/Mlp1/Mlp2/megator, is involved in mRNA export and SUMO homeostasis and affects diverse aspects of plant development. *Plant Cell* 19, 1537–1548.

Yost, H. J., and Lindquist, S. (1986). RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. *Cell* 45, 185–193.

Yu, C. W., Liu, X., Luo, M., Chen, C., Lin, X., Tian, G., Lu, Q., Cui, Y., and Wu, K. (2011). Histone deacetylase6 interacts with flowering LOCUS D and regulates flowering in Arabidopsis. *Plant Physiol.* 156, 173–184.

Zhang, H., Bishop, B., Ringenberg, W., Muir, W. M., and Ogas, J. (2012). The CHD3 remodeler pickle associates with genes enriched for trimethylation of histone H3 lysine 27. *Plant Physiol.* 159, 418–432.

Zhu, S., Goeres, J., Sixt, K. M., Bekes, M., Zhang, X. D., Salvesen, G. S., and Matunis, M. J. (2009). Protection from isopeptidase-mediated deconjugation regulates paralog-selective sumoylation of RanGAP1. *Mol. Cell* 33, 570–580.

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: 02 July 2012; accepted: 23 August 2012; published online: 17 September 2012.

Citation: Mazur MJ and van den Burg HA (2012) Global SUMO proteome responses guide gene regulation, mRNA biogenesis, and plant stress responses. Front. Plant Sci. ***3****:215. doi: 10.3389/fpls.2012.00215*

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

APPENDIX

Table A1 | The overlap between the SUMO interactome and proteins that interact with topless (TPL) and TPL-related 1–4 from Arabidopsis.

AGI code	Gene name	Annotation
At1g07310	–	Calcium-dependent lipid-binding (CaLB domain) family protein
At1g10760	SEX1	STARCH EXCESS 1; Encodes an α-glucan, water dikinase required for starch degradation. Involved in cold-induced freezing tolerance.
At1g23190	PGM3	Cytosolic phosphoglucomutase (PGM
At1g43170	RP1	RIBOSOMAL PROTEIN 1 (RP1)
At1g62300	WRKY6	Regulates Phosphate1 (Pho1) expression in response to low phosphate (Pi) stress
At1g67090	RBCS1A	Ribulose bisphosphate carboxylase small chain 1A
At2g01350	QPT	QUINOLINATE PHOSHORIBOSYLTRANSFERASE, involved in NAD biosynthesis
At2g19520	FVE (MSI4)	Homolog of the mammalian retinoblastoma-associated protein (RbAp), one component of a histone deacetylase (HDAC) complex involved in transcriptional repressionControls flowering. protein_coding (FVE); (ACG1); (ATMSI4); (NFC4); (NFC04);MULTICOPY SUPPRESSOR OF IRA1 4 (MSI4)
At2g45640	SAP18	Interacts with SIN3, HDA19/HDA6 co-repressors complex
At3g01090	AKIN10	NF1-related protein kinase that physically interacts with SCF subunit SKP1/ASK1 and 20S proteosome subunit PAD1. It can also interact with PRL1 DWD-containing protein. Based on *in vitro* degradation assays and cul4cs and prl1 mutants, there is evidence that AKIN10 is degraded in a proteasome-dependent manner, and that this depends on a CUL4-PRL1 E3 ligase protein_coding SNF1 KINASE HOMOLOG 10 (KIN10) SNF1 KINASE HOMOLOG 10 (AKIN10);SNF1 KINASE HOMOLOG 10 (KIN10); (KIN10)
At3g02550	AS2	Asymmetric leaves 2, LOB domain-containing protein 41 (LBD41); CONTAINS InterPro DOMAIN
At3g10390	FLD	SWIRM domain-containing protein found in histone deacetylase complexes in mammals
At3g10480	NAC050	Transcription factor
At3g10490	NAC052	Transcription factor
At3g13920	EIF4A1	Eukaryotic translation initiation factor 4A-1
At3g16500	IAA26 (PAP1)	Phytochrome-associated protein 1
At3g17900	Unknown	Gene of unknown function
At3g20770	EIN3	Ethylene-insensitive3), a nuclear transcription factor that initiates downstream transcriptional cascades for ethylene responses
At3g52250	–	Protein with a putative role in mRNA splicing.
At4g11660	AT-HSFB2B	HEAT-SHOCK TRANSCRIPTION FACTOR B2B
At4g17330	unknown	Gene of unknown function
At4g29130	HXK1	GLUCOSE INSENSITIVE 2 (GIN2);HEXOKINASE 1 (HXK1); Functions as a glucose sensor to interrelate nutrient, light, and hormone signaling networks for controlling growth and development in response to the changing environment.
At5g28540	BIP	Luminal binding protein BiP, an ER-localized member of the HSP70 family
At5g42020	BIP2	Luminal binding protein (BiP2) involved in polar nuclei fusion during proliferation of endosperm nucle
At5g43700	IAA4	INDOLE-3-ACETIC ACID INDUCIBLE 4 (IAA4);AUXIN INDUCIBLE 2–11 (ATAUX2–11)
At5g44800	CHR4 (PKR1)	Chromatin remodeling 4 (CHR4), Pickle related 1
At5g55070	–	Dihydrolipoamide succinyltransferase;
At5g66140	PAD2	PROTEASOME ALPHA SUBUNIT D2 (PAD2); Encodes alpha5 subunit of 20S proteosome complex involved in protein degradation

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 27 June 2012
doi: 10.3389/fpls.2012.00142

mADP-RTs: versatile virulence factors from bacterial pathogens of plants and mammals

Lennart Wirthmueller* and Mark J. Banfield*

Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich, UK

Edited by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

Reviewed by:
Christian Lindermayr, Helmholtz Zentrum München – German Research Center for Environmental Health, Germany
Jesus V. Jorrin Novo, University of Cordoba, Spain

****Correspondence:***
Lennart Wirthmueller and Mark J. Banfield, Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK. e-mail: lennart.wirthmueller@jic.ac.uk; mark.banfield@jic.ac.uk

Mono ADP-ribosyltransferases (mADP-RTs) are a family of enzymes that cleave NAD^+ and covalently attach the ADP-ribosyl moiety to target proteins. mADP-RTs are well established as important virulence factors of bacteria that infect mammals. Cholera toxin, pertussis toxin, and diphtheria toxin are three of the best-known examples of mADP-RTs. They modify host target proteins in order to promote infection and/or killing of the host cell. Despite low sequence similarity at the primary amino acid level, mADP-RTs share a conserved core catalytic fold and structural biology has made important contributions to elucidating how mADP-RTs modify mammalian host targets. Recently, mADP-RTs were shown to be present in plant pathogenic bacteria, suggesting that mADP-RTs are also important virulence factors of plant pathogens. Crystal structures of plant pathogenic bacterial mADP-RTs are also now available. Here we review the structure/function of mADP-RTs from pathogens of mammals and plants, highlighting both commonalities and differences.

Keywords: pathogen effector, mono-ADP-ribosyltransferase, crystal structure, plant innate immunity

A COMMON FOLD MEDIATES MERCILESS KILLING OR SUBTLE MANIPULATION

Several important pathogenic bacteria use secreted proteins, frequently termed "effectors," to modify the physiology of their host cells to promote infection. One family of effectors encodes a mono-ADP-ribosyltransferase (mADP-RT) enzymatic activity. The manipulating activities of mADP-RTs often culminate in killing the infected host cell and therefore many pathogen secreted mADP-RTs are referred to as toxins (Holbourn et al., 2006). ADP-ribosylating toxins typically comprise several distinct domains that mediate attachment to host cell receptors, translocation into the host cell and mADP-ribosylation (**Figure 1**; Deng and Barbieri, 2008). Some mADP-RTs from Gram-negative bacteria rely on host cell delivery by the bacterial type three secretion system (T3SS) and consist only of the catalytic domain preceded by a type three secretion signal and host cell targeting domains (Deng and Barbieri, 2008). The catalytic domain mediates cleavage of an ADP-ribose moiety from NAD^+ and its subsequent transfer onto an amino acid of the target protein (often Cys, Arg, Asn, or Diphthamide – a modified form of His). In most cases mADP-ribosylation of target proteins leads to their inactivation. Transfer of an ADP-ribose moiety onto the $G_{S\alpha}$ subunit of heteromeric G proteins by cholera toxin (CT) results in constitutive production of cAMP and leads to the severe diarrhea associated with infection by *Vibrio cholerae* (Cassel and Pfeuffer, 1978; Vanden Broeck et al., 2007). Diphtheria toxin (DT) from *Corynebacterium diphtheriae* mADP-ribosylates eukaryotic elongation factor-2, leading to its inactivation and interference with mRNA translation that can have detrimental effects in the host organism (Collier, 1967; Honjo et al., 1968; Collier, 2001).

Despite a low level of sequence similarity, the catalytic domains of most mADP-RTs share a common core αβ fold (Holbourn et al., 2006). A series of anti-parallel β-strands provides structural support for the active site. This active site comprises a hydrophobic NAD^+ binding cleft and a conserved Glu residue that promotes cleavage of NAD^+ at the anomeric carbon of the nicotinamide ribose and subsequent transfer of the mADP-ribose moiety onto the target amino acid. The α helices and loops connecting the β-strands are structurally less conserved but make important contributions to the NAD^+ binding cleft and mediate substrate binding. The enzymatic activity of several mADP-RTs is regulated by an active site loop located adjacent to the catalytic cleft. This loop can undergo significant re-orientation to allow access to the active site upon substrate binding (Bell and Eisenberg, 1996; Sun et al., 2004; O'Neal et al., 2005).

Most mADP-RTs retain three structurally conserved features (Holbourn et al., 2006): (1) The arom-H/R motif is composed of an aromatic amino acid followed by His or Arg. This motif either contributes to NAD^+ binding or supports the structural integrity of the NAD^+ binding site; (2) The ARTT loop contains the conserved catalytic Glu residue required for NAD^+ cleavage and transferase activity. The catalytic Glu is often part of a Q/E-X-E motif in which the presence of Glu or Gln two residues upstream appears to determine substrate specificity for either Arg or Asn mADP-ribosylation; and (3) The STS motif that maintains the structure of the active site through hydrogen bonds with the catalytic Glu, and other conserved residues of the NAD^+ binding site. However, the STS motif is less conserved in DT and related structures.

Dependent on the conservation of additional motifs that form the active site, mADP-RTs can be annotated as DT- or CT-like in their folds. Intriguingly, both groups are not limited to bacterial toxins. The DT group also includes the mammalian poly-ADP-ribosyltransferases (PARPs) that regulate diverse cellular

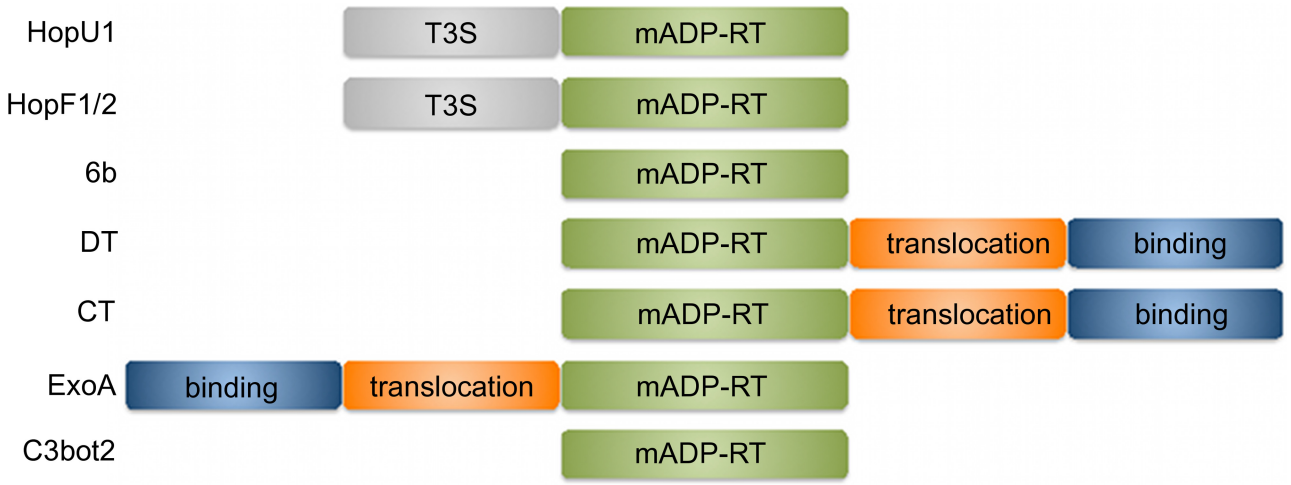


FIGURE 1 | Functional domains of mADP-RTs discussed in this review. T3S, type 3 secretion peptide; mADP-RT, mono-ADP-ribosylating catalytic domain; binding, host cell receptor binding; translocation, host cell translocation.

mechanisms including apoptosis, DNA repair, and intracellular transport (Schreiber et al., 2006). The CT group is also found in a class of eukaryotic extracellular enzymes, the Ecto-ARTs (Di Girolamo et al., 2005). Plants possess PARPs, but examples of mADP-RTs in plants have yet to be found (Wang et al., 2011a; Lamb et al., 2012). Recent structural studies on virulence effectors of plant pathogenic bacteria have revealed examples of both DT- and CT-like folds in several bacterial effector proteins, and most of these effectors have mADP-RT activity. In contrast to the fatal consequences associated with delivery of mADP-ribosylating toxins to host cells, but consistent with the (hemi-) biotrophic nature of plant pathogenic bacteria, these effectors appear to induce more subtle modifications to plant innate immunity pathways or the physiology of the infected cell. Molecular targets of selected mADP-RTs are shown in **Table 1**.

HopU1: TARGETING RNA-BINDING PROTEINS

The effector HopU1 from *Pseudomonas syringae* strain DC3000 is delivered through the T3SS into host cells where it interferes with plant immune responses (Fu et al., 2007). HopU1 is an active mADP-RT and several RNA binding proteins have been identified as *in vitro* targets of this effector. One of these RNA binding proteins, GRP7, regulates circadian mRNA oscillations and is also required for immunity towards *P. syringae* DC3000 (Heintzen et al., 1997; Fu et al., 2007).

Table 1 | Delivery mechanisms and molecular targets of mADP-RT effectors discussed in this review.

mADP-RT effector	Pathogen	Host cell delivery	Target protein(s) and amino acid	Role in pathogenesis	Reference
HopU1	*Pseudomonas syringae*	T3SS	*At* GRP7, Arg49	Suppression of PAMP-triggered immunity	Fu et al. (2007), Jeong et al. (2011)
HopF1	*P. syringae*	T3SS	Unknown	Unknown	Singer et al. (2004)
HopF2	*P. syringae*	T3SS	*At* MKK5, RIN4	Suppression of defense signaling	Wang et al. (2010), Wilton et al. (2010)
6b	*Agrobacterium spec.*	T-DNA	*At* SE, AGO1	Alteration of plant hormone levels	Wang et al. (2011b)
C3bot2	*Clostridium botulinum*	Unknown	Eukaryotic Rho GTPases (A/B/C), Asn41	Disintegration of actin cytoskeleton	Chardin et al. (1989)
Cholera toxin	*Vibrio cholerae*	Receptor-mediated endocytosis	$G_{S\alpha}$, Arg201	Activation of heteromeric G protein $G_{S\alpha}$	Cassel and Pfeuffer (1978)
Diphtheria toxin	*Corynebacterium diphtheriae*	Receptor-mediated endocytosis	Eukaryotic EF-2, conserved diphthamide residue	Inhibition of protein synthesis	Collier (1967), Honjo et al. (1968)
ExotoxinA	*P. aeruginosa*	Receptor-mediated endocytosis	Eukaryotic EF-2, conserved diphthamide residue	Inhibition of protein synthesis	Iglewski et al. (1977)

Recently, the crystal structure of HopU1 has been determined (Jeong et al., 2011). This showed that the HopU1 catalytic subunit shares structural homology to mADP-RTs of the CT-class. For example, 86 Cα atoms of HopU1 can be superimposed onto the structure of the *Clostridium botulinum* mADP-RT C3bot2 with a root mean square deviation of 2.12Å. The two opposing β sheets, that support the ARTT loop with the catalytic Glu and the STS motif (STT in HopU1), align well with the corresponding β sheets of C3bot2 (**Figure 2A**). In both structures the first Ser of the STS motif forms hydrogen bonds with the catalytic Glu.

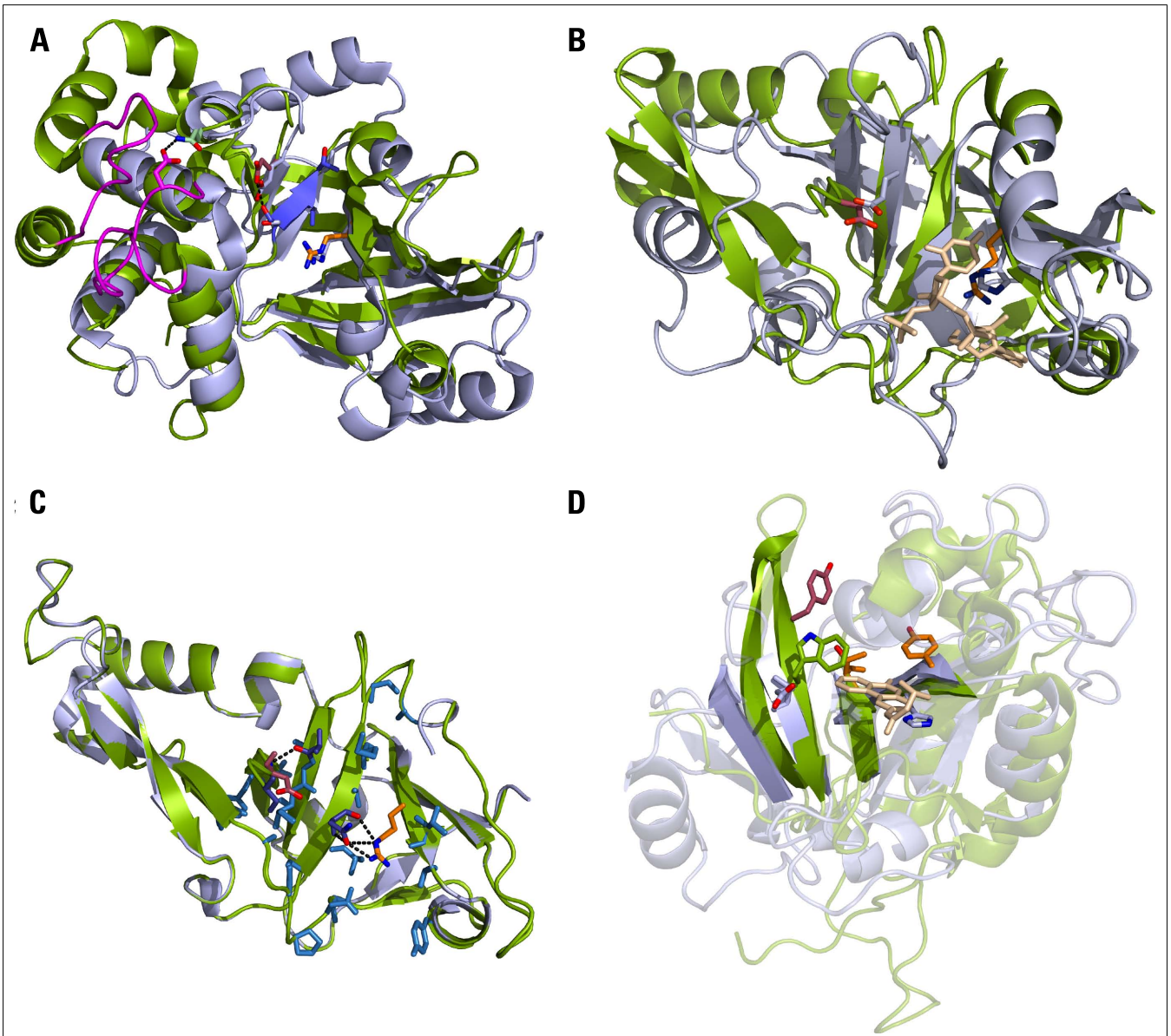


FIGURE 2 | Plant and animal pathogen mADP-RTs share the same protein fold. (A) HopU1 structure (green) superimposed on the catalytic subunit of C3bot2 (light blue). Highlighted in the HopU1 structure are the conserved Arg of the arom-H/S motif (orange), the STS motif (blue) and the catalytic Glu (dark red). The corresponding amino acids of C3bot2 are shown as sticks. The two loops that mediate binding to GRP7 are shown in magenta. PDB: 3U0J (HopU1), 1R45 (C3bot2). **(B)** HopF1 structure (green) superimposed on the catalytic subunit of DT (light blue) in complex with the NAD^+ analog adenylyl 3′,5′ uridine 3′ monophosphate (beige). Highlighted in the HopF1 structure are the conserved Arg of the arom-H/S motif (orange) and the catalytic Asp (dark red). The corresponding amino acids of DT are shown as sticks. PDB: 1S21 (HopF1), 1DDT (DT). **(C)** Homology model of HopF2 (green) based on the HopF1 structure (light blue). Amino acids colored in blue contribute to the active site and are conserved in both effectors. These also include the catalytic Asp174/175 (dark red) and Arg 72/71 (orange) as well as Asn135/134 and Ala187/188 (purple) that form hydrogen bonds with Arg and Asp, respectively. **(D)** *Agrobacterium vitis* 6b structure (green) superimposed on the catalytic subunit of Exotoxin A (light blue) in complex with the inhibitor PJ34 (beige). Highlighted is the conserved arrangement of b-strands with 6b residues Tyr66 and Thr93 (orange) as well as the proposed catalytic Tyr153 (red). The catalytic Glu553 and His440 of Exotoxin A are shown as sticks. The amino acid of 6b that is located closest to the position of the catalytic Glu of Exotoxin A is Trp155 (green). PDB: 3AQ2 (6b), 1XK9 (Exotoxin A).

Whereas the catalytic subunits of mADP-RTs are structurally conserved, the regions mediating substrate binding can adopt more divergent conformations. The structure of HopU1 revealed two protruding loops that are not conserved in other mADP-RTs (Jeong et al., 2011). Ala substitutions in both of these loops were shown to affect GRP7 binding *in vitro* and abolish mADP-ribosylation of GRP7, suggesting they are essential for substrate recognition. Two Arg residues located in the GRP7 RNA binding domain have been previously identified as potential HopU1 target amino acids (Fu et al., 2007). Using a proteomics approach Jeong et al. (2011) further demonstrate that at least one residue, Arg49, is mADP-ribosylated by HopU1 *in vitro*. This is consistent with the close proximity of the putative HopU1 active site and Arg47/Arg49 in a structural model of the HopU1/GRP7 RNA binding domain complex. Interestingly, GRP7 Arg49 makes direct contact to RNA (Schöning et al., 2007). Therefore, HopU1 specifically targets an amino acid of GRP7 that is essential for RNA binding.

HopF1: IN SEARCH OF A TARGET

Structural homology to mADP-RTs has also been observed for other T3SS effectors from *P. syringae*. The crystal structure of HopF1 (formerly AvrPphF) from the bean-infecting *P. syringae* pathovar *phaseolicola* reveals structural similarity to DT (**Figure 2B**; Singer et al., 2004). However, to date, no mADP-RT activity has been shown for HopF1 *in vitro*, or in a plant cell extract (Singer et al., 2004). One explanation for this lack of activity could be changes in the amino acid composition at key positions compared to DT. This includes the catalytic Glu (Glu148, DT-numbering), which is an Asp in HopF1 (Asp174) and His21, part of the arom-H/S motif in DT (Bennett and Eisenberg, 1994), which is replaced by an Arg in HopF1 (Arg72). Interestingly, the side chains of each of these residues occupy almost identical positions in the active sites (**Figure 2B**). However, in the case of the DT(Glu148)/HopF1(Asp174) substitution, the residues originate from different secondary structure elements (β7 in DT and β6 in HopF1).

Despite the lack of mADP-RT activity under the conditions tested, Arg72 and Asp174 are still required for the virulence and avirulence activities of HopF1. Whilst wild type HopF1 enhances growth of *P. syringae* on the susceptible bean cultivar Tendergreen ∼eightfold, Ala substitutions of either Arg72 or Asp174 completely abolish this virulence activity (Singer et al., 2004). In bean cultivar Red Mexican the *R1* disease resistance gene confers recognition of HopF1 (Tsiamis et al., 2000). This recognition event requires both Arg72 and Asp174 as *P. syringae* expressing the corresponding Ala substitutions evades recognition on Red Mexican (Singer et al., 2004). Therefore, even though HopF1 does not exhibit mADP-RT activity under the conditions tested, the conserved cleft forming the putative active site is essential for both HopF1's virulence activity and recognition on resistant host plants. The side chains of both Arg72 and Asp174 form hydrogen bonds to other residues located within the cleft and these intramolecular interactions would be impaired by Ala substitutions, suggesting that the structural integrity of the cavity is required for HopF1's virulence function and recognition by R1. It is conceivable that the interface supported by Arg72 and Asp174 forms a binding site for virulence target/s of HopF1. As the same binding site is required for HopF1 recognition on resistant bean cultivars this recognition event might be mediated by a "decoy" protein mimicking a virulence target (van der Hoorn and Kamoun, 2008). In this scenario modification of the decoy protein would trigger defense gene activation by the R1 resistance protein.

HopF2: TARGETING KINASE CASCADES

In contrast to HopF1, the sequence related *P. syringae* strain DC3000 effector HopF2 is known to be an active mADP-RT (Wang et al., 2010). HopF2 contributes to virulence of *P. syringae* as it enhances growth of the bacteria in *Arabidopsis* and tomato (Robert-Seilaniantz et al., 2006; Wilton et al., 2010). When delivered via the T3SS of a non-pathogenic *Pseudomonas* strain, HopF2 suppresses MAMP-triggered MAP-kinase activation and direct interaction of HopF2 with several MAP-kinase-kinases (MKKs) has been shown *in vitro* and *in planta* (Wang et al., 2010). Using biotin-labeled NAD^+ as co-factor, HopF2 is able to transfer biotin-ADP-ribose onto MKK5 and this impairs phosphorylation of MPK6 (Wang et al., 2010). Interestingly, HopF2 appears to modify the constitutively active $MKK5^{DD}$ mutant more efficiently than the wild type form, suggesting that the effector may preferentially target activated MKKs. ADP-ribosylation of MKK5 is dependent on a conserved Arg in the C-terminus of MKK5 and an Ala substitution of this residue largely impairs MKK5-mediated activation of the defense marker gene FRK1. Hence, one virulence activity of HopF2 is to interfere with MKK signaling to suppress MAMP-triggered immunity (Wang et al., 2010).

When over-expressed in *Arabidopsis*, HopF2 also interferes with activation of the resistance protein RPS2 by its cognate *P. syringae* effector AvrRpt2 (Wilton et al., 2010). AvrRpt2 is a Cys protease that cleaves *Arabidopsis* RIN4 and the disappearance of RIN4 triggers RPS2 activation (Axtell and Staskawicz, 2003; Mackey et al., 2003). HopF2 interferes with AvrRpt2-mediated cleavage of RIN4 and *in vitro* assays show that HopF2 binds to and mADP-ribosylates RIN4 (Wang et al., 2010; Wilton et al., 2010). Therefore, a possible mechanism of HopF2 interference with RIN4 cleavage is mADP-ribosylation of an amino acid in the RIN4 peptide that is cleaved by AvrRpt2 (Day et al., 2005; Kim et al., 2005).

HopF2 elicits a hypersensitive response in the non-host plant *Nicotiana tabacum* (Robert-Seilaniantz et al., 2006). In striking accordance to the requirement of Arg72 and Asp174 residues for HopF1 virulence function and recognition, the corresponding two amino acids of HopF2 (Arg71 and Asp175) are essential for both the virulence and avirulence activities of HopF2 (Wang et al., 2010). HopF2 has 48% amino acid identity and 92% amino acid similarity to HopF1. The level of sequence conservation between the two effectors allows building of a reliable homology model of HopF2 based on the HopF1 structure (**Figure 2C**). According to this homology model, amino acids forming the putative active site, including the Arg/Asp pair critical for effector virulence and recognition, are conserved in both effectors. The enzymatic activity of HopF2 suggests that replacement of the catalytic Glu residue, which is highly conserved in ADP-ribosylating toxins, by Asp174 in HopF1 does not explain why

HopF1 is enzymatically inactive. Based on the structural conservation of the active site in both effectors and the requirement of corresponding amino acids for virulence and avirulence functions it would be surprising if only HopF2 but not HopF1 functions as a mADP-RT. Hence, it would be informative to re-test whether HopF1 has mADP-RT function under conditions used for HopF2.

Based on the finding that Ala substitutions of HopF2 Arg71 or Asp175 abolish MKK5 binding, Wang and co-workers (Wang et al., 2010) suggest that Arg71/Asp175 are more likely to be involved in substrate binding than in catalysis. However, both functions are likely to depend on the structural integrity of the active site and loss of hydrogen bonding mediated by Arg71 and Asp175 might affect its overall conformation. In a similar manner, substitutions of the conserved Glu 233/235 in the HopU1 ARTT loop impair not only mADP-ribosylating activity, but also decrease binding to GRP7 (Jeong et al., 2011).

AGROBACTERIUM 6b: A DIVERGENT mADP-RT

Plant pathogen effectors adopting the mADP-RT core fold are not limited to *P. syringae*. The *Agrobacterium* 6b protein, encoded on the Ti plasmids of *A. tumefaciens* and *A. vitis*, shows structural homology to mADP-ribosylating toxins such as Exotoxin A from *P. aeruginosa* (Wang et al., 2011b). Although the 6b protein is dispensable for crown gall formation in a natural infection, ectopic expression of 6b in several host species is sufficient to induce tumors, probably by altering auxin and cytokinin physiology of the host cell (Hooykaas et al., 1988; Tinland et al., 1989). Further, ectopic expression of 6b in *Arabidopsis* leads to formation of serrated leaves, a phenotype also observed in mutants deficient in microRNA (miRNA) metabolism (Wang et al., 2011b). Indeed, the accumulation of several miRNAs is altered in plants expressing 6b and the reduced levels of one particular miRNA, miR319, could provide a direct link to activation of auxin signaling (Navarro et al., 2006). 6b interacts with two proteins involved in miRNA processing, SE and AGO1, *in vitro* and in plant cells (Kidner and Martienssen, 2004; Yang et al., 2006; Wang et al., 2011b). Thus, manipulation of the miRNA processing machinery, to alter hormone levels in host cells, appears to be one virulence mechanism of 6b proteins.

Despite the lack of sequence similarity, the structure of 6b can be superimposed on Exotoxin A and CT with a root mean square deviation of <4Å (**Figure 2D**; Wang et al., 2011b). Although the overall position of the central β-sheets in both proteins is conserved, dramatic changes can be observed at amino acids forming the putative active site of 6b (Wang et al., 2011b). There is a notable lack of residues in 6b that could functionally substitute for the Arg/His of the arom-H/S motif and the catalytic Glu in CT/Exotoxin A. Remarkably, the authors still provide evidence that 6b is an active mADP-RT: (1) the morphological phenotype induced by 6b expression in *Arabidopsis* can be rescued by application of the less-hydrolysable NAD^+ analog TAD; (2) in the presence of the putative target, SE, 6b is able to hydrolyze the NAD^+ analog ε-NAD^+ *in vitro* and this activity is dependent on three Tyr and one Thr residue that contribute to the active site; (3) the hydrolytic activity of 6b is enhanced >20-fold in presence of an *Arabidopsis* ARF protein. This observation is in striking accordance with activation of CT by human ARF6-GTP (O'Neal et al., 2005). ARF6-GTP binding to CT leads to a conformational change in the active site loop rendering the catalytic cleft accessible to NAD^+ and the substrate. A similar active site loop occluding the NAD^+ binding site in absence of ARF is found in the 6b structures. Thus, it appears that an ARF-GTP-dependent activation mechanism has been conserved in mADP-RTs of mammalian and plant pathogens.

How do 6b proteins catalyze NAD^+hydrolysis without the conserved catalytic Glu? Although the detailed reaction mechanisms of most mADP-RTs remain to be elucidated, the conserved Glu is generally assumed to play a critical role in stabilizing the bound NAD^+ molecule in a transition state that renders the anomeric carbon of the nicotinamide ribose more vulnerable to nucleophilic attack by the substrate (Holbourn et al., 2006). It has been proposed that Tyr153 might perform the same function in 6b (Wang et al., 2011b). Consistent with this, the 6B Tyr153Ala mutant is impaired in ε-NAD^+ hydrolysis. However, it is debatable whether amino acids less electronegative than Glu or Asp would be able to stabilize the NAD^+ transition state. It is equally plausible that Tyr153, together with Tyr66 and Tyr121, contributes to the structural integrity of the active site.

An alternative explanation for retention of enzymatic activity in absence of a catalytic Glu is suggested by research on mammalian PARPs. Human PARP10 and PARP14, which both lack the conserved Glu in the catalytic core motif, do not function as PARPs (Kleine et al., 2008). However, both enzymes show mADP-ribosylating activity. Based on their findings, Kleine et al. (2008) proposed an alternative substrate-assisted catalytic mechanism where the catalytic Glu is provided not by the enzyme but by the substrate. It is conceivable that the enzymatic activity of *Agrobacterium* 6b effectors relies on a similar mechanism.

The work reviewed here suggests that several plant pathogenic bacteria evolved host-targeted enzymes with mADP-RT activity to manipulate the physiology and immune system in infected host cells. Notably, mADP-RTs from plant pathogens appear to show greater structural diversity than secreted mADP-RTs from mammalian pathogens, or the eukaryotic Ecto-ARTs. How some of these effectors retain enzymatic activity despite considerable changes in the active site is an intriguing question. The approaches summarized here need to be extended to include mADP-RT structures in the presence of non-hydrolysable NAD^+ analogs and complemented with more sophisticated enzymatic analysis. Elucidating how this structurally diverged group of host-targeted effectors catalyzes transfer of mADP-ribose onto target proteins, and defining the their target specificity, will not only provide new insights into manipulation of plant immunity but also extend our functional understanding of mADP-RTs.

ACKNOWLEDGMENTS

We apologize to colleagues whose work could not be included in this review due to space constraints. Lennart Wirthmueller is supported by a FEBS long-term fellowship. Mark J. Banfield acknowledges the BBSRC (grant BB/J004553/1), the John Innes Foundation and the Gatsby Charitable Foundation for funding.

REFERENCES

Axtell, M. J., and Staskawicz, B. J. (2003). Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* 112, 369–377.

Bell, C. E., and Eisenberg, D. (1996). Crystal structure of diphtheria toxin bound to nicotinamide adenine dinucleotide. *Biochemistry* 35, 1137–1149.

Bennett, M. J., and Eisenberg, D. (1994). Refined structure of monomeric diphtheria toxin at 2.3Å resolution. *Protein Sci.* 3, 1464–1475.

Cassel, D., and Pfeuffer, T. (1978). Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. U.S.A.* 75, 2669–2673.

Chardin, P., Boquet, P., Madaule, P., Popoff, M. R., Rubin, E. J., and Gill, D. M. (1989). The mammalian G protein rhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J.* 8, 1087–1092.

Collier, R. J. (1967). Effect of diphtheria toxin on protein synthesis: inactivation of one of the transfer factors. *J. Mol. Biol.* 25, 83–98.

Collier, R. J. (2001). Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. *Toxicon* 39, 1793–1803.

Day, B., Dahlbeck, D., Huang, J., Chisholm, S. T., Li, D., and Staskawicz, B. J. (2005). Molecular basis for the RIN4 negative regulation of RPS2 disease resistance. *Plant Cell* 17, 1295–1305.

Deng, Q., and Barbieri, J. T. (2008). Molecular mechanisms of the cytotoxicity of ADP-ribosylating toxins. *Annu. Rev. Microbiol.* 62, 271–288.

Di Girolamo, M., Dani, N., Stilla, A., and Corda, D. (2005). Physiological relevance of the endogenous mono-(ADP-ribosyl)ation of cellular proteins. *FEBS J.* 272, 4565–4575.

Fu, Z. Q., Guo, M., Jeong, B. R., Tian, F., Elthon, T. E., Cerny, R. L., Staiger, D., and Alfano, J. R. (2007). A type III effector ADP-ribosylates RNA-binding proteins and quells plant immunity. *Nature* 447, 284–288.

Heintzen, C., Nater, M., Apel, K., and Staiger, D. (1997). AtGRP7, a nuclear RNA-binding protein as a component of a circadian-regulated negative feedback loop in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 94, 8515–8520.

Holbourn, K. P., Shone, C. C., and Acharya, K. R. (2006). A family of killer toxins. Exploring the mechanism of ADP-ribosylating toxins. *FEBS J.* 273, 4579–4593.

Honjo, T., Nishizuka, Y., and Hayaishi, O. (1968). Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J. Biol. Chem.* 243, 3553–3555.

Hooykaas, P. J., Dulk-Ras, H., and Schilperoort, R. A. (1988). The *Agrobacterium tumefaciens* T-DNA gene *6b* is an *onc* gene. *Plant Mol. Biol.* 11, 791–794.

Iglewski, B. H., Liu, P. V., and Kabat, D. (1977). Mechanism of action of *Pseudomonas aeruginosa* exotoxin: adenosine diphosphate-ribosylation of mammalian elongation factor 2 *in vitro* and *in vivo*. *Infect. Immun.* 15, 138–144.

Jeong, B. R., Lin, Y., Joe, A., Guo, M., Korneli, C., Yang, H., Wang, P., Yu, M., Cerny, R. L., Staiger, D., Alfano, J. R., and Xu, Y. (2011). Structure function analysis of an ADP-ribosyltransferase type III effector and its RNA-binding target in plant immunity. *J. Biol. Chem.* 286, 43272–43281.

Kidner, C. A., and Martienssen, R. A. (2004). Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. *Nature* 428, 81–84.

Kim, H. S., Desveaux, D., Singer, A. U., Patel, P., Sondek, J., and Dangl, J. L. (2005). The *Pseudomonas syringae* effector AvrRpt2 cleaves its C-terminally acylated target, RIN4, from *Arabidopsis* membranes to block RPM1 activation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6496–6501.

Kleine, H., Poreba, E., Lesniewicz, K., Hassa, P. O., Hottiger, M. O., Litchfield, D. W., Shilton, B. H., and Lüscher, B. (2008). Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation. *Mol. Cell* 32, 57–69.

Lamb, R. S., Citarelli, M., and Teotia, S. (2012). Functions of the poly-ADP-ribose- polymerase superfamily in plants. *Cell. Mol. Life Sci.* 69, 175–189.

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.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J. D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312, 436–439.

O'Neal, C. J., Jobling, M. G., Holmes, R. K., and Hol, W. G. (2005). Structural basis for the activation of cholera toxin by human ARF6-GTP. *Science* 309, 1093–1096.

Robert-Seilaniantz, A., Shan, L., Zhou, J. M., and Tang, X. (2006). The *Pseudomonas syringae* pv. *tomato* DC3000 type III effector HopF2 has a putative myristoylation site required for its avirulence and virulence functions. *Mol. Plant Microbe Interact.* 19, 130–138.

Schöning, J. C., Streitner, C., Page, D. R., Hennig, S., Uchida, K., Wolf, E., Furuya, M., and Staiger, D. (2007). Auto-regulation of the circadian slave oscillator component AtGRP7 and regulation of its targets is impaired by a single RNA recognition motif point mutation. *Plant J.* 52, 1119–1130.

Schreiber, V., Dantzer, F., Ame, J. C., and de Murcia, G. (2006). Poly-(ADP-ribose): novel functions for an old molecule. *Nat. Rev. Mol. Cell Biol.* 7, 517–28.

Sun, J., Maresso, A. W., Kim, J. J. P., and Barbieri, J. T. (2004). How bacterial ADP-ribosylating toxins recognize substrates. *Nat. Struct. Mol. Biol.* 11, 868–876.

Singer, A. U., Desveaux, D., Betts, L., Chang, J. H., Nimchuk, Z., Grant, S. R., Dangl, J. L., and Sondek, J. (2004). Crystal structures of the type III effector protein AvrPphF and its chaperone reveal residues required for plant pathogenesis. *Structure* 12, 1669–1681.

Tinland, B., Huss, B., Paulus, F., Bonnard, G., and Otten, L. (1989). *Agrobacterium tumefaciens 6b* genes are strain-specific and affect the activity of auxin as well as cytokinin genes. *Mol. Gen. Genet.* 219, 217–224.

Tsiamis, G., Mansfield, J. W., Hockenhull, R., Jackson, R. W., Sesma, A., Athanassopoulos, E., Bennett, M. A., Stevens, C., Vivian, A., Taylor, J. D., and Murillo, J. (2000). Cultivar-specific avirulence and virulence functions assigned to avrPphF in *Pseudomonas syringae* pv. *phaseolicola*, the cause of bean halo-blight disease. *EMBO J.* 19, 3204–3214.

Vanden Broeck, D., Horvath, C., and De Wolf, M. J. (2007). *Vibrio cholerae*: cholera toxin. *Int. J. Biochem. Cell Biol.* 39, 1771–1775.

van der Hoorn, R. A., and Kamoun, S. (2008). From guard to decoy: a new model for perception of plant pathogen effectors. *Plant Cell* 20, 2009–2017.

Wang, H., Liang, Q., Cao, K., and Ge, X. (2011a). Endogenous protein mono-ADP-ribosylation in *Arabidopsis thaliana*. *Planta* 233, 1287–1292.

Wang, M., Soyano, T., Machida, S., Yang, J. Y., Jung, C., Chua, N. H., and Yuan, Y. A. (2011b). Molecular insights into plant cell proliferation disturbance by Agrobacterium protein 6b. *Genes Dev.* 64–76.

Wang, Y., Li, J., Hou, S., Wang, X., Li, Y., Ren, D., Chen, S., Tang, X., and Zhou, J. M. (2010). A *Pseudomonas syringae* ADP-ribosyltransferase inhibits *Arabidopsis* mitogen-activated protein kinase kinases. *Plant Cell* 22, 2033–2044.

Wilton, M., Rajagopal, S., Elmore, J., Felsensteiner, C., Coaker, G., and Desvaux, D. (2010). The type III effector HopF2 Pto targets *Arabidopsis* RIN4 protein to promote *Pseudomonas syringae* virulence, *Proc. Natl. Acad. Sci. U.S.A.* 107, 2349–2354

Yang, L., Liu, Z., Lu, F., Dong, A., and Huang, H. (2006). SERRATE is a novel nuclear regulator in primary microRNA processing in *Arabidopsis*. *Plant J.* 47, 841–850.

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: 30 April 2012; paper pending published: 20 May 2012; accepted: 12 June 2012; published online: 27 June 2012.

Citation: Wirthmueller L and Banfield MJ (2012) mADP-RTs: versatile virulence factors from bacterial pathogens of plants and mammals. Front. Plant Sci. 3:142. doi: 10.3389/fpls.2012.00142

This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 16 July 2012
doi: 10.3389/fpls.2012.00160

Effector-triggered post-translational modifications and their role in suppression of plant immunity

Andrew J. M. Howden and Edgar Huitema*

Division of Plant Science, College of Life Sciences, University of Dundee at The James Hutton Institute, Dundee, UK

Edited by:
Jacqueline Monaghan, The Sainsbury Laboratory, UK

Reviewed by:
Benjamin Schwessinger, University of California at Davis, USA
Milena Roux, University of Copenhagen, Denmark

***Correspondence:**
Edgar Huitema, Division of Plant Science, College of Life Sciences, University of Dundee at The James Hutton Institute, Errol Road, Invergowrie, Dundee DD2 5DA, UK.
e-mail: e.huitema@dundee.ac.uk

Plant–pathogen interactions feature complex signaling exchanges between host and microbes that ultimately determine association outcomes. Plants deploy pattern recognition receptors to perceive pathogen-associated molecular patterns, mount pattern-triggered immunity (PTI), and fend off potential pathogens. In recent years an increasing number of defense-signaling components have been identified along with a mechanistic understanding of their regulation during immune responses. Post-translational modifications (PTMs) are now thought to play a crucial role in regulating defense signaling. In a bid to suppress PTI and infect their host, pathogens have evolved large repertoires of effectors that trigger susceptibility and allow colonization of host tissues. While great progress has been made in elucidating defense-signaling networks in plants and the activities of effectors in immune suppression, a critical gap exists in our understanding of effector mechanism-of-action. Given the importance of PTMs in the regulation of defense signaling, we will explore the question: how do effectors modify the post-translational status of host proteins and thus interfere with host processes required for immunity? We will consider how emerging proteomics-based experimental strategies may help us answer this important question and ultimately open the pathogens' effector black box.

Keywords: effector, PAMP-triggered immunity, effector-triggered susceptibility, post-translational modifications, direct effector-triggered modification, indirect effector-triggered modification

INTRODUCTION

Within their natural environment, plants are continuously challenged by a diverse array of pathogens such as viruses, bacteria, fungi, and oomycetes as well as nematodes and insects. In most cases, infection or disease is limited upon the perception of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by pattern recognition receptors (PRRs; Boller and Felix, 2009). Recognition results in PAMP or pattern-triggered immunity (PTI) and features a marked shift in transcriptional activity toward defense, as well as the production and secretion of defense-associated proteins and metabolites leading to increased levels of resistance (Jones and Dangl, 2006; Zipfel, 2009). With some of the principal players and processes identified in plant immune signaling networks, only now we are starting to appreciate the critical roles of regulatory mechanisms that ensure an appropriate response to a given biotic stress.

Protein post-translational modifications (PTMs) are ubiquitous in cell signaling networks and enable rapid alterations to the protein complement of the cell without need for new protein synthesis. In addition, PTMs provide enormous diversity to the proteome, allowing cells to respond with flexibility to a stimulus. PTMs regulate a wide array of processes within plants including growth, development, flowering, and defense (Kwon et al., 2006). Phosphorylation, ubiquitination, and SUMOylation have emerged as pivotal PTMs that plants employ to target and control the activity of immune regulators (Stulemeijer and Joosten, 2008). These findings (reviewed elsewhere in this issue) have highlighted the intricacies of an immune system that has adapted to an environment in which plants are bombarded by commensal, symbiotic as well as pathogenic organisms.

In a select few cases, plants are successfully invaded and colonized by microbes, which in turn can lead to the manifestation of disease. Disease development ultimately results from the perturbation of immune signaling networks, suppression of defense responses and modulation of metabolism in the host (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Pathogens have evolved strategies to actively evade or suppress immunity. Given the importance of PTMs in regulating immune signaling networks, it is likely that pathogens specifically target and perturb host PTM pathways implicated in defense.

The last decade has seen the identification of pathogen encoded secreted proteins (effectors) that upon secretion, manipulate host processes, perturb signaling, and induce effector-triggered susceptibility (ETS; Kamoun, 2007; Hann and Rathjen, 2010). During infection, pathogens secrete effectors that accumulate in the host intercellular space (apoplastic effectors) and target apoplastic or surface exposed host components. Pathogens can also secrete effectors that translocate across the host cell membrane (intracellular effectors) and upon delivery, travel to discrete subcellular compartments or organelles to target resident cellular processes (Kamoun, 2006; Block et al., 2008). Both effector classes are thought to modulate host (defense) signaling and perturb cellular processes required for PTI, ultimately leading to ETS. One example of effector driven host defense modulation is the manipulation of mitogen-activated protein kinase (MAPK) phosphorylation cascades, which ultimately leads

to altered defense gene expression and enhanced susceptibility (**Figures 1A,B**).

In recent years great progress has been made in elucidating defense-signaling networks in plants and identifying those pathogen effectors that can suppress immunity. However, an understanding of the mechanism-of-action of most effectors remains elusive. Given the lack of models describing effector mode-of-action and the importance of PTMs in defense signaling, we will explore the question: how do effectors modify the post-translational status of host proteins and thus interfere with host processes required for immunity? In this context, we will aim to consider how emerging proteomics-based experimental strategies may help us answer this important question, and ultimately open the pathogens' effector black box.

TWO MODELS DESCRIBE EFFECTOR-TRIGGERED PTMs

Effector-triggered susceptibility is achieved through the interaction between a pathogen effector and its host target that eventually impinges on immune signaling. Mechanistically, this interaction results in a modification of the target and its cellular fate (Kamoun, 2007). The host target protein may be modified by the addition or removal of a chemical group (PTM). The resulting PTM may trigger target protein degradation, altered protein conformation and activity or re-localization (Kwon et al., 2006). Effector-triggered target modification requires an enzymatic activity that is either provided by the effector or by a given host cellular pathway. These observations infer the presence of at least two mechanistic models (**Figure 1C**) that help explain effector-triggered target modification. Both simplified models will be discussed and explored here and examples supporting both models are also provided (**Table 1**).

MODEL 1: DIRECT EFFECTOR-TRIGGERED MODIFICATION

The first model (which we have named direct effector-triggered modification or DETM) assumes that a direct interaction between effector and target, combined with an enzymatic activity carried by the effector, ensures modification of the target. In recent years a number of plant pathogen effectors have been shown to modify host targets by direct interactions combined with a catalytic activity carried by the effector. The *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) effector AvrPtoB features an N-terminal kinase binding motif that aids binding to host receptor like kinases FLS2, BAK1, FEN, and CERK1 (Rosebrock et al., 2007; Goehre et al., 2008; Shan et al., 2008; Gimenez-Ibanez et al., 2009). The AvrPtoB C-terminus has been shown to exhibit E3 ubiquitin ligase activity, and is responsible for the degradation of FEN during *Pst* infection of tomato (Rosebrock et al., 2007). AvrPtoB binding to the PRRs FLS2 and CERK1, and their co-receptor BAK1 also leads to their degradation and enhanced virulence of *Pst* on *Arabidopsis* (Goehre et al., 2008; Shan et al., 2008; Gimenez-Ibanez et al., 2009). AvrPtoB induced degradation of factors important for immunity leads to increased susceptibility to *Pst* on both tomato and *Arabidopsis* (Rosebrock et al., 2007; Goehre et al., 2008), providing a compelling illustration of effector catalyzed modification events that lead to ETS. Besides targeting proteins to the host proteasome for degradation, effectors can also directly eliminate

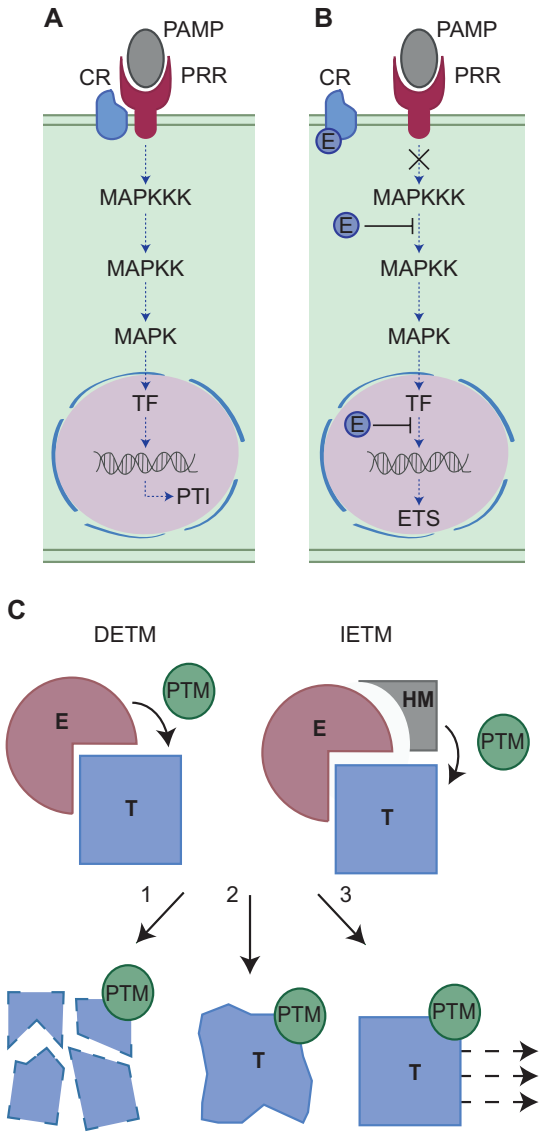


FIGURE 1 | (A,B) Modulation of host defense-signaling networks by pathogen effectors. One example of effector mediated host defense modulation is the manipulation of mitogen-activated protein kinase (MAPK) phosphorylation cascades. **(A)** Host defenses may be activated by the perception and binding of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) with the aid of a co-receptor (CR). Successful PAMP perception triggers a MAPK phosphorylation cascade resulting in transcription factor (TF) activation, defense gene expression and PAMP-triggered immunity (PTI). However, pathogen effectors (E) can manipulate this signaling pathway at a number of key steps **(B)** resulting in effector-triggered susceptibility (ETS). For example, the MAPK cascade can be blocked either by the effector perturbing CR-PRR activities or by the effector modifying MAPKs directly. Alternatively, nuclear targeting effectors may block defense gene induction, ultimately leading to enhanced susceptibility and the onset of disease. **(C)** Two models for effector-triggered post-translational modifications. In Model 1 (direct effector-triggered modification – DETM) the effector (E) binds the host target protein (T) and directly catalyzes its post-translational modification (PTM). In Model 2 (indirect effector-triggered modification – IETM) the effector binds the target protein and recruits a host machinery (HM) which catalyzes target PTM. The modified host protein may then be subject to proteasome mediated degradation (1), altered structural confirmation and activity (2), or re-localization (3).

Table 1 | Examples of direct effector-triggered modification (DETM) and indirect effector-triggered modification (IETM).

Effector	Pathogen/host	Target	Effector activity	Reference
DETM				
AvrPtoB	*Pst*/*S.l.* and *A.t.*	FLS2, BAK1, FEN, CERK1	E3 ubiquitin ligase	Rosebrock et al. (2007), Goehre et al. (2008), Shan et al. (2008), Gimenez-Ibanez et al. (2009)
AvrPphB	*Pst*/*S.l.* and *A.t.*	PBS1	Cysteine protease	Zhang et al. (2010)
AvrRpt2	*Pst*/*S.l.* and *A.t.*	RIN4	Cysteine protease	Mackey et al. (2002, 2003), Coaker et al. (2005), Kim et al. (2005)
AvrAC	*Xcc*/brassicas	BIK1, RIPK	Uridylyl transferase	Feng et al. (2012)
HopAI1	*Pst*/*S.l.* and *A.t.*	MPK3, MPK6	Phosphate lyase	Zhang et al. (2007)
HopU1	*Pst*/*S.l.* and *A.t.*	GRP7	Mono-ADP-ribosyltransferase	Jeong et al. (2011)
IETM				
AvrB	*Pst*/*S.l.* and *A.t.*	RIN4	Recruits host protein kinase	Liu et al. (2011)
AvrRpm1	*Pst*/*S.l.* and *A.t.*	RIN4	Recruits host protein kinase	Liu et al. (2011)
HopM1	*Pst*/*S.l.* and *A.t.*	AtMIN7	Recruits host proteasome	Nomura et al. (2006)
Unknown				
Avr3a	*P.i.*/*S.t.*	CMPG1	Stabilizes target	Bos et al. (2010)

Pathogen and host names: Pst, Pseudomonas syringae pv. tomato DC3000; Xcc, Xanthomonas campestris pv. Campestris; P.i., Phytophthora infestans; S.l., Solanum lycopersicum, A.t., Arabidopsis thaliana; S.t., Solanum tuberosum.

targets from the host cell. AvrRpt2 encodes a cysteine protease that is delivered into host cells during *Pst* infection where it is activated by the eukaryotic host factor cyclophilin (Coaker et al., 2005). Activated AvrRpt2 associates with the host plasma membrane and releases RIN4 from the host cell membrane by proteolysis. RIN4 dissociation from the membrane results in enhanced susceptibility, provided that RPS2, one of two resistance proteins guarding RIN4 is absent (Mackey et al., 2002, 2003; Kim et al., 2005).

Besides proteolytic degradation, DETM can also alter host protein phosphorylation status. MAPKs link PAMP perception to downstream defense gene expression (Pitzschke et al., 2009). The *Pst* effector HopAI1 interacts with the *Arabidopsis* MAP kinases MPK3 and MPK6. During PTI, both MPK3 and MPK6 are activated by the phosphorylation of a threonine residue by upstream MAPKKs. HopAI1 phosphate lyase activity however, removes the phosphate group from these residues, preventing MPK3 and MPK6 activation by PAMP induced MAPKKs. Since phosphate group removal cannot be reversed, both MPK3 and MPK6 are effectively inhibited, leading to dampening of PTI activated MAPK signaling cascades (Zhang et al., 2007).

MODEL 2: INDIRECT EFFECTOR-TRIGGERED MODIFICATION

Many C-terminal effector domains do not exhibit any sequence similarity to known enzymes. While some enzymatic functions have been elucidated after solving and comparing effector structures to known catalytic enzymes, there are an increasing number of effectors for which enzymatic function remains elusive. Although the presence of an unknown enzymatic function can never be excluded, this observation raises the possibility that in such cases, effectors modify their targets with the help of host-encoded enzymes, in a process that we have termed indirect effector-triggered modification (IETM). Examples of such mechanisms are sparse but one early report emanated from studies on the human papilloma virus oncoprotein E6. E6 was identified in oncogenic HPV strains and detailed studies of this protein led to the observation that in host cells, E6 recruits an E3 ubiquitin ligase that in turn, ubiquitinates the tumor suppressor protein p53. E6 induced p53 ubiquitination was found to mark the tumorigenesis suppressor for proteasomal degradation, providing a molecular explanation for the onset of cancer in infected cells (Scheffner et al., 1990).

One of the best characterized examples of IETM in plant–microbe interactions features the *P. syringae* effectors AvrB and AvrRpm1. Delivery of either AvrB or AvrRpm1 results in phosphorylation of its molecular target RIN4. Attempts aimed at demonstrating kinase activity of either effector have failed. However, the host kinase RIPK (RPM1-induced protein kinase) has been found to form a complex with RIN4 and AvrB, and this observation has helped to explain the effector dependent phosphorylation of RIN4. Delivery of AvrB inside host cells leads to recruitment of RIPK to an AvrB–RIN4–RIPK complex. RIPK phosphorylates RIN4 threonine residue 166 presumably suppressing PTI in the absence of RPM1 (Chung et al., 2011; Liu et al., 2011).

Another interesting example of IETM is the activity of the *Pst* effector HopM1. Within the host HopM1 interacts with the defense-associated protein AtMIN7, targeting it for degradation by the host proteasome and resulting in impaired cell wall-associated defenses. HopM1 is thought to act as an adapter protein which shows no similarity to proteins

involved in ubiquitination or proteolysis but instead recruits the host machinery to selectively remove a key defense protein (Nomura et al., 2006). These findings combined with an immense but yet elusive repertoire of effector domains with unknown function, hint that IETM is a common mechanism that drives effector induced target modifications during host–microbe associations.

There is now increasing evidence supporting the DETM and IETM models for effector activity. However, in some cases despite the effector target having been identified, the modification events remain elusive. The *Phytophthora infestans* RXLR effector AVR3a interacts with the host E3 ubiquitin ligase CMPG1, an interaction that protects this target from degradation and leads to its stabilization in host cells. The presence of AVR3a induces an increase in CMPG1 molecular mass, suggesting that specific PTM events underpin stabilization. The presence of AVR3a suppresses INF1-induced cell death and PTI, suggesting that CMPG1 modification perturbs key PTI signaling steps (Bos et al., 2010). Despite these observations and the recent elucidation of structures for two members from the AVR3a protein family in *Phytophthora capsici* (Boutemy et al., 2011; Yaeno et al., 2011), the exact function of AVR3a remains to be determined. The observation that AVR3a cell death suppression activity can be uncoupled from R3a (a NBS-LRR) mediated recognition of AVR3a, may suggest the presence of additional host factors that are recruited by AVR3a and are guarded by R3a.

EMERGING PROTEOMICS-BASED EXPERIMENTAL STRATEGIES WILL HELP US EXAMINE THE ROLE OF PTMs IN EFFECTOR ACTIVITY

With the availability of an ever increasing array of pathogen and host genomes, great advances have been made in the identification of putative pathogen effectors and the disease signaling networks these proteins may impact upon. Considering the immense diversity in functional effector domains, we are only now beginning to appreciate the vast but yet unexplored repertoire of novel activities encoded by microbial effectors and their possible roles in ETS. Given the recent advances in proteomics approaches, we will discuss experimental approaches that can be employed to further understand effector function in the context of the models described here.

IN SITU DETECTION OF EFFECTORS AND THEIR PUTATIVE TARGETS

Effectors have been shown to target specific subcellular compartments where they modulate distinct host processes (Kamoun, 2006; Block et al., 2008). The identification of host compartments targeted by pathogen effectors thus forms a critical requirement to understand function. Confocal microscopy-based localization studies are a powerful means to deduce effector targeting in plant cells. It should be noted however that during localization experiments, conditions that reflect a given host–microbe interaction cannot be easily reconstituted. Monitoring the proteome of host organelles during the course of infection represents a powerful tool for studying effector-triggered host modifications *in situ*. Organelle enrichment strategies, combined with LC–MS/MS would enable the identification of effectors which localize to particular organelles while simultaneously monitoring the relative abundance and post-translational status of host proteins. Recently, Drakakaki et al. (2012), used vesicle affinity purification combined with mass spectrometry to identify proteins of the trans-Golgi network in *Arabidopsis*. This method provided the sensitivity to reveal novel protein complexes and trafficking components. Similar strategies may be employed for studying organelle proteomes during infection.

Subcellular fractionation has the added benefit of enriching the protein sample for fractions of interest, making the detection of low abundance signaling proteins and their PTMs more achievable. Such strategies will have to be combined with quantitative proteomics techniques in order to monitor the relative abundance of proteins at a given time or location. Quantitative proteomics is technically challenging in plants due to their autotrophic nature and the resulting difficulties associated with whole proteome labeling. However, strategies are now available for labeling plant cell cultures using either isotopically labeled nitrogen compounds (^{15}N; Keinath et al., 2010) or stable isotope labeling by amino acids (SILAC; Schuetz et al., 2011), while whole plants may be isotopically labeled using ^{15}N (Schaff et al., 2008). In addition, label free proteomics is also likely to become more routine with improvements in the accuracy and reproducibility of mass spectrometers (Oeljeklaus et al., 2009).

ENRICHING FOR PTMs

Our indirect model (IETM) of effector-induced PTMs raises a range of issues which should be considered when examining effector–target interactions. The indirect modification model assumes that the effector binds a host target protein and recruits host machinery which then catalyzes target PTM. Detecting the interaction between three partners (effector, target, and host machinery) is a challenge since the stoichiometry of each partner may not be equal. Conventional protein–protein interaction experiments using yeast-2-hybrid or tandem affinity purification may only detect the most abundant partners within this interaction. The target protein may be expressed at relatively low levels and the stoichiometry of the PTM often means that the modified protein represents a small proportion of the total pool for that protein. Enrichment strategies are therefore desirable if one wishes to survey the proteome for targets with a specific PTM. A range of strategies are now available for enriching proteins or peptides for specific PTMs, including antibody-based affinity enrichment and ionic interaction-based enrichment (Larsen et al., 2005; Zhao and Jensen, 2009). Nühse et al. (2007) used ionic interaction-based enrichment combined with a quantitative proteomics strategy to examine dynamic changes in the phosphorylation of *Arabidopsis* plasma membrane proteins treated with the bacterial elicitor flagellin. In a more recent study Mithoe et al. (2012) used ^{15}N metabolic labeling in combination with immunity-affinity purification to enrich and quantify tyrosine phosphorylated peptides upon flagellin perception in *Arabidopsis*. Combining tandem affinity purification with PTM enrichment strategies may provide the sensitivity to detect subtle effector induced PTM events.

OUTLOOK

The ability of pathogens to modify the post-translational state of host proteins represents a powerful means for the pathogen to tip the balance from immunity to susceptibility. Since most effectors have yet to be assigned a precise function, and given the enormous diversity that exists among those effectors identified to date, the expectation is that the future will see the identification of alternative effector-triggered PTMs and their substrates. However, a bottleneck is now forming in the characterization of effector activities, and is likely to build further as whole genome sequencing projects identify ever increasing numbers of putative effectors. The use of proteomics to monitor effector localization and host proteome dynamics is likely to emerge as a crucial tool that will enable effector activities to be linked with host PTM signaling pathways. An improved understanding of the mechanisms by which pathogens use their effector repertoire to manipulate host defense signaling, will prove invaluable for developing plant lines with improved pathogen resistance.

ACKNOWLEDGMENTS

The authors thank Remco Stam, Julietta Jupe, Tieme Heldeman, Jorunn Bos, and two referees for comments and suggestions. The Huitema lab is supported by the Royal Society of Edinburgh (co-funded by Marie-Curie) and the Biotechnology and Biological Sciences Research Council (BBSRC).

REFERENCES

Block, A., Li, G., Fu, Z. Q., and Alfano, J. R. (2008). Phytopathogen type III effector weaponry and their plant targets. *Curr. Opin. Plant Biol.* 11, 396–403.

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.

Bos, J. I. B., Armstrong, M. R., Gilroy, E. M., Boevink, P. C., Hein, I., Taylor, R. M., Tian, Z., Engelhardt, S., Vetukuri, R. R., Harrower, B., Dixelius, C., Bryan, G., Sadanandom, A., Whisson, S. C., Kamoun, S., and Birch, P. R. J. (2010). *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9909–9914.

Boutemy, L. S., King, S. R. F., Win, J., Hughes, R. K., Clarke, T. A., Blumenschein, T. M. A., Kamoun, S., and Banfield, M. J. (2011). Structures of *Phytophthora* RXLR effector proteins: a conserved but adaptable fold underpins funtional diversity. *J. Biol. Chem.* 286, 35834–35842.

Chung, E. H., da Cunha, L., Wu, A. J., Gao, Z., Cherkis, K., Afzal, A. J., Mackey, D., and Dangl, J. L. (2011). Specific threonine phosphorylation of a host target by two unrelated type III effectors activates a host innate immune receptor in plants. *Cell Host Microbe* 9, 125–136.

Coaker, G., Falick, A., and Staskawicz, B. (2005). Activation of a phytopathogenic bacterial effector protein by a eukaryotic cyclophilin. *Science* 308, 548–550.

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* 11, 539–548.

Drakakaki, G., Van De Ven, W., Pan, S., Miao, Y., Wang, J., Keinath, N. F., Weatherly, B., Jiang, L., Schumacher, K., Hicks, G., and Raikhel, N. (2012). Isolation and proteomic analysis of the SYP61 compartment reveal its role in exocytic trafficking in *Arabidopsis*. *Cell Res.* 22, 413–424.

Feng, F., Yang, F., Rong, W., Wu, X., Zhang, J., Chen, S., He, C., and Zhou, J.-M. (2012). A *Xanthomonas* uridine 5′-monophosphate transferase inhibits plant immune kinases. *Nature* 485, 114–118.

Gimenez-Ibanez, S., Hann, D. R., Ntoukakls, V., Petutschnig, E., Lipka, V., and Rathjen, J. P. (2009). AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. *Curr. Biol.* 19, 423–429.

Goehre, V., Spallek, T., Haeweker, H., Mersmann, S., Mentzel, T., Boller, T., De Torres, M., Mansfield, J. W., and Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr. Biol.* 18, 1824–1832.

Hann, D. R., and Rathjen, J. P. (2010). The long and winding road: virulence effector proteins of plant pathogenic bacteria. *Cell. Mol. Life Sci.* 67, 3425–3434.

Jeong, B.-R., Lin, Y., Joe, A., Guo, M., Korneli, C., Yang, H., Wang, P., Yu, M., Cerny, R. L., Staiger, D., Alfano, J. R., and Xu, Y. (2011). Structure function analysis of an ADP-ribosyltransferase type III effector and its RNA-binding target in plant immunity. *J. Biol. Chem.* 286, 43272–43281.

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

Kamoun, S. (2006). A catalogue of the effector secretome of plant pathogenic oomycetes. *Annu. Rev. Phytopathol.* 44, 41–60.

Kamoun, S. (2007). Groovy times: filamentous pathogen effectors revealed. *Curr. Opin. Plant Biol.* 10, 358–365.

Keinath, N. F., Kierszniowska, S., Lorek, J., Bourdais, G., Kessler, S. A., Shimosato-Asano, H., Grossniklaus, U., Schulze, W. X., Robatzek, S., and Panstruga, R. (2010). PAMP (pathogen-associated molecular pattern)-induced changes in plasma membrane compartmentalization reveal novel components of plant immunity. *J. Biol. Chem.* 285, 39140–39149.

Kim, M. G., Da Cunha, L., Mcfall, A. J., Belkhadir, Y., Debroy, S., Dangl, J. L., and Mackey, D. (2005). Two *Pseudomonas syringae* type III effectors inhibit RIM-regulated basal defense in *Arabidopsis*. *Cell* 121, 749–759.

Kwon, S. J., Choi, E. Y., Choi, Y. J., Ahn, J. H., and Park, O. K. (2006). Proteomics studies of post-translational modifications in plants. *J. Exp. Bot.* 57, 1547–1551.

Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., and Jorgensen, T. J. D. (2005). Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* 4, 873–886.

Liu, J., Elmore, J. M., Lin, Z.-J. D., and Coaker, G. (2011). A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. *Cell Host Microbe* 9, 137–146.

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.

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.

Mithoe, S. C., Boersema, P. J., Berke, L., Snel, B., Heck, A. J. R., and Menke, F. L. H. (2012). Targeted quantitative phosphoproteomics approach for the detection of phospho-tyrosine signaling in plants. *J. Proteome Res.* 11, 438–448.

Nomura, K., Debroy, S., Lee, Y. H., Pumplin, N., Jones, J., and He, S. Y. (2006). A bacterial virulence protein suppresses host innate immunity to cause plant disease. *Science* 313, 220–223.

Nühse, T. S., Bottrill, A. R., Jones, A. M. E., and Peck, S. C. (2007). Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. *Plant J.* 51, 931–940.

Oeljeklaus, S., Meyer, H. E., and Warscheid, B. (2009). Advancements in plant proteomics using quantitative mass spectrometry. *J. Proteomics* 72, 545–554.

Pitzschke, A., Schikora, A., and Hirt, H. (2009). MAPK cascade signalling networks in plant defence. *Curr. Opin. Plant Biol.* 12, 421–426.

Rosebrock, T. R., Zeng, L. R., Brady, J. J., Abramovitch, R. B., Xiao, F. M., and Martin, G. B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. *Nature* 448, 370–374.

Schaff, J. E., Mbeunkui, F., Blackburn, K., Bird, D. M., and Goshe, M. B. (2008). SILIP: a novel stable isotope labeling method for in planta quantitative proteomic analysis. *Plant J.* 56, 840–854.

Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus type-16 and type-18 promotes the degradation of P53. *Cell* 63, 1129–1136.

Schuetz, W., Hausmann, N., Krug, K., Hampp, R., and Macek, B. (2011). Extending SILAC to proteomics of

plant cell lines. *Plant Cell* 23, 1701–1705.

Shan, L., He, P., Li, J., Heese, A., Peck, S. C., Nuernberger, T., Martin, G. B., and Sheen, J. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host Microbe* 4, 17–27.

Stulemeijer, I. J. E., and Joosten, M. H. J. (2008). Post-translational modification of host proteins in pathogen-triggered defence signalling in plants. *Mol. Plant Pathol.* 9, 545–560.

Yaeno, T., Li, H., Chaparro-Garcia, A., Schornack, S., Koshiba, S., Watanabe, S., Kigawa, T., Kamoun, S., and Shirasu, K. (2011). Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 14682–14687.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y., and Zhou, J.-M. (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.

Zhang, J., Shao, F., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., and Zhou, J.-M. (2007). A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe* 1, 175–185.

Zhao, Y., and Jensen, O. N. (2009). Modification-specific proteomics: strategies for characterization of post-translational modifications using enrichment techniques. *Proteomics* 9, 4632–4641.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Curr. Opin. Plant Biol.* 12, 414–420.

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: 18 May 2012; paper pending published: 07 June 2012; accepted: 29 June 2012; published online: 16 July 2012.
*Citation: Howden AJM and Huitema E (2012) Effector-triggered post-translational modifications and their role in suppression of plant immunity. Front. Plant Sci. **3**:160. doi: 10.3389/fpls.2012.00160*
This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.