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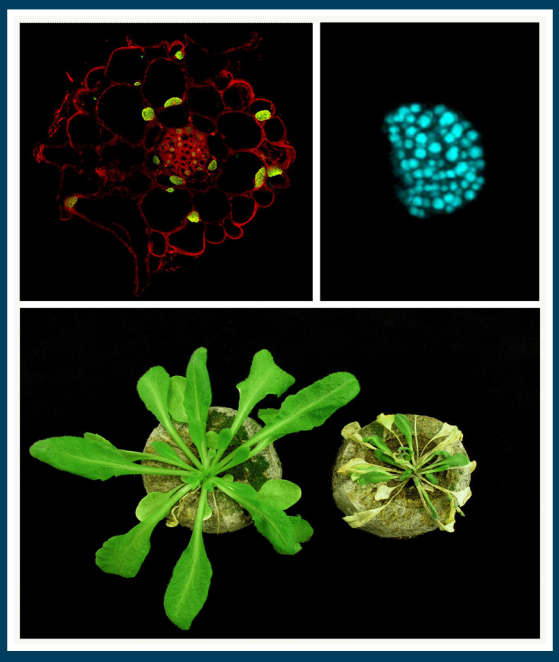

NUCLEAR COMPONENTS AND DYNAMICS DURING PLANT INNATE IMMUNITY

Topic Editors
Susana Rivas and Laurent Deslandes

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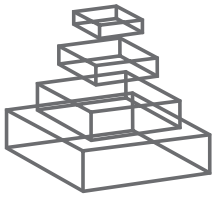

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ISSN 1664-8714
ISBN 978-2-88919-226-7
DOI 10.3389/978-2-88919-226-7

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NUCLEAR COMPONENTS AND DYNAMICS DURING PLANT INNATE IMMUNITY

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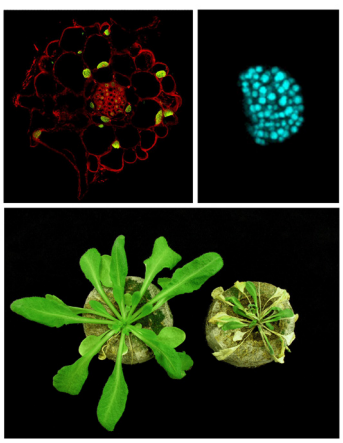

Upper left : Confocal image of an *Arabidopsis* root transversal section showing the nuclear accumulation of a bacterial effector fused to the green fluorescent protein.

Upper right : Confocal image of a *Nicotiana benthamiana* leaf epidermal cell nucleus showing *Agrobacterium*-mediated transient expression of a bacterial effector fused to the cyan fluorescent protein.

Bottom: Resistant (left) and susceptible (right) phenotypical responses of *Arabidopsis* plants challenged with the root bacterial pathogen *Ralstonia solanacearum.*

In plants, efficient immune responses against microbial infection depend on the ability to rapidly couple pathogen recognition to downstream signaling responses. In this context, plant immunity requires highly dynamic responses that involve multiple organelles during the recognition and signaling events associated to defense. Nuclear dynamics plays a critical role in plant immunity based to the growing number of reports revealing that nuclear localization of pathogen effectors, plant disease resistance proteins, and key plant components, including transcription factors and regulators, are essential for immunity.

Following their delivery into plant cells, a significant number of effector proteins from different pathogenic microorganisms, including viruses, oomycetes, fungi, nematodes, and bacteria, are targeted to the nucleus by co-opting the host nuclear import machinery. This suggests that effectors may manipulate host transcription or directly target essential host nuclear components for the benefit of the pathogen. Indeed, pathogen-induced transcriptional regulation in host cells plays a crucial role in the establishment of plant defense

and associated plant cell death responses. Along these lines, it has been estimated that about 25% of Arabidopsis genes are transcriptionally regulated in response to pathogen infection and a significant number of transcription factors are involved in the defense gene regulation. Moreover, spatial restriction of defense regulators by the nuclear envelope as well as their stimulus-induced nuclear translocation provide an important mechanism for defense regulation, as their level of nuclear accumulation determines the magnitude of the defense response. In addition, nuclear translocation of effectors may also affect subcellular localization of their cognate resistance proteins in a process that is essential for plant immunity. Finally, mutations in plant cellular factors involved in the transport of macromolecules through the nuclear envelope compromise plant resistance signaling, underlining the importance of nucleocytoplasmic trafficking during plant innate immunity. Together, these findings situate the nucleus at the forefront of the mutual recognition between plants and pathogens.

In this Research Topic, we aim to provide an open-access update on the current knowledge about the importance of nuclear components – both from the "microbial side" and from the "plant side"- and nuclear dynamics during the establishment of plant immune responses.

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frontiers in
PLANT SCIENCE

EDITORIAL
published: 22 November 2013
doi: 10.3389/fpls.2013.00481

Nuclear components and dynamics during plant innate immunity

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Edited by:
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Keywords: plant cell nucleus, immune receptor, plant immunity, transcription factors, defence regulator, effector, nucleo-cytoplamic trafficking, hormone signaling

In plants, efficient immune responses against microbial infection depend on the ability to rapidly couple pathogen recognition to downstream signaling responses. In this context, plant immunity requires highly dynamic responses that involve multiple organelles during the recognition and signaling events associated with defense. Nuclear dynamics play a critical role in plant immunity based on the growing number of reports revealing that nuclear localization of pathogen effectors, plant disease resistance proteins, and key plant components, including transcription factors and regulators, are essential for immunity. This Research Topic provides an overview of the current knowledge about the importance of nuclear components—both from the "microbial side" and from the "plant side"—and nuclear dynamics during the establishment of plant immune responses.

Mutations in plant cellular factors involved in the transport of macromolecules through the nuclear envelope compromise plant resistance signaling, underlining the importance of nucleocytoplasmic trafficking during plant innate immunity. The Mini-Review article by Gaouar and Germain (2013) describes the importance of nuclear mRNA export during plant immune responses whereas Wirthmueller et al. (2013) discuss importin-α-mediated nuclear translocation and how microbial effectors may compete with host cargo proteins for nuclear uptake.

Following their delivery into plant cells, a significant number of effector proteins from different pathogenic microorganisms, including viruses, oomycetes, fungi, nematodes, and bacteria, are targeted to the nucleus by co-opting the host nuclear import machinery. This suggests that effectors may manipulate host transcription or directly target essential host nuclear components for the benefit of the pathogen. Indeed, pathogen-induced transcriptional regulation in host cells plays a crucial role in the establishment of plant defense and associated plant cell death responses. Several articles in this Research Topic highlight these ideas. Quentin et al. (2013) describe effectors from plant parasitic nematodes that target host nuclei and possibly interact with nuclear proteins to establish feeding cells in infected plants. In their Original Research article, Ma et al. (2013) show that nuclear localization of the Avr2 effector from the xylem-colonizing fungus *Fusarium oxysporum* is required to trigger I2-mediated resistance in tomato plants, whereas Stam et al. (2013) show the diversity of nuclear functions of CRN effectors from the oomycete *Phytophthora infestans*. Finally, the Opinion article by Noël et al. (2013) discusses recent advances in predicting target sequences of nuclear-targeted TAL effectors from the plant pathogenic bacteria of the genus *Xanthomonas*.

It has been estimated that about 25% of *Arabidopsis* genes are transcriptionally regulated in response to pathogen infection and a significant number of transcription factors are involved in the defense gene regulation. Raffaele and Rivas (2013) review our current knowledge of the transcriptional control of plant defenses with a focus on the MYB family of transcription factors and, within this family, the *Arabidopsis* MYB protein AtMYB30, which is a positive regulator of disease resistance. The Review article by Gimenez-Ibanez and Solano (2013) discusses the nuclear crosstalk of jasmonate and salicylate signaling with other hormone pathways during the fine-tuning of a robust plant defence response.

Spatial restriction of immune receptors and defense regulators by the nuclear envelope as well as their stimulus-induced nuclear translocation provide an important mechanism for defense regulation, as their level of nuclear accumulation determines the magnitude of the defense response. In addition, nuclear translocation of effectors may also affect subcellular localization of their cognate resistance proteins in a process that is essential for plant immunity. Bhattacharjee et al. (2013) review nuclear functions of different immune receptors and associated proteins, including transcription factors and defence regulators. Chang et al. (2013) discuss how nucleo-cytoplasmic partitioning of the barley MLA immune receptor triggers downstream transcriptional responses, thereby providing an efficient connection between pathogen perception and the plant immune response. Finally, in their Original Research Article, Heidrich et al. (2013) provide evidence that the *Arabidopsis* WRKY domain-containing immune receptor RRS1 contributes to temperature-conditioned autoimmune responses conferred by a second nuclear immune receptor, RPS4. These data suggest that RPS4 engages RRS1 to direct defence signaling.

In summary, recent findings from our rapidly evolving field situate the nucleus at the forefront of the mutual recognition between plants and pathogens. Integrating the knowledge on immunity-associated nuclear events within the outlook of whole cellular dynamics represents an exciting perspective for future research.

REFERENCES

Bhattacharjee, S., Garner, C. M., and Gassmann, W. (2013). New clues in the nucleus: transcriptional reprogramming in effector-triggered immunity. *Front. Plant Sci.* 4:364. doi: 10.3389/fpls.2013.00364

Chang, C., Zhang, L., and Shen, Q-H. (2013) Partitioning, repressing and derepressing: dynamic regulations in MLA immune receptor triggered defense signaling. *Front. Plant Sci.* 4:396. doi: 10.3389/fpls.2013.00396

Gaouar, O., and Germain, H. (2013). mRNA export: threading the needle. *Front. Plant Sci*. 4:59. doi: 10.3389/fpls.2013.00059

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

Heidrich, K., Tsuda, K., Blanvillain-Baufumé, S., Wirthmueller, L., Bautor, J., and Parker, J. E. (2013) Arabidopsis TNL-WRKY domain receptor RRS1 contributes to temperature-conditioned RPS4 auto-immunity. *Front. Plant Sci.* 4:403. doi: 10.3389/fpls.2013.00403

Ma, L., Cornelissen, B. J. C., and Takken, F. L. W. (2013) A nuclear localization for Avr2 from*Fusarium oxysporum* is required to activate the tomato resistance protein I-2. *Front. Plant Sci*. 4:94. doi: 10.3389/fpls.2013.00094

Noël, L. D., Denancé, N., and Szurek, B. (2013) Predicting promoters targeted by TAL effectors in plant genomes: from dream to reality. *Front. Plant Sci.* 4:333. doi: 10.3389/fpls.2013.00333

Quentin, M., Abad, P., and Favery, B. (2013) Plant parasitic nematode effectors target host defense and nuclear functions to establish feeding cells. *Front. Plant Sci.* 4:53. doi: 10.3389/fpls.2013.00053

Raffaele, S., and Rivas, S. (2013). Regulate and be regulated: integration of defense and other signals by the AtMYB30 transcription factor. *Front. Plant Sci.* 4:98. doi: 10.3389/fpls.2013.00098

Stam, R., Howden, A. J. M., Delgado-Cerezo, M., M. M. Amaro, T. M., Motion, G. B., Pham, J., et al. (2013) Characterization of cell death inducing Phytophthora capsici CRN effectors suggests diverse activities in the host nucleus. *Front. Plant Sci.* 4:387. doi:10.3389/fpls.2013.00387

Wirthmueller, L., Roth, C., Banfield, M. J., and Wiermer, M. (2013) Hop-on hop-off: importin-α-guided tours to the nucleus in innate immune signaling. *Front. Plant Sci*. 4:149. doi: 10.3389/fpls.2013.00149

Received: 02 November 2013; accepted: 06 November 2013; published online: 22 November 2013.

*Citation: Rivas S and Deslandes L (2013) Nuclear components and dynamics during plant innate immunity. Front. Plant Sci. **4**:481. doi: 10.3389/fpls.2013.00481*

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

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 22 March 2013
doi: 10.3389/fpls.2013.00059

mRNA export: threading the needle

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After mRNA biogenesis, several proteins interact with the messenger to ensure its proper export to the cytoplasm. Some of these proteins will bind RNA early on, at the onset of transcription by RNA polymerase II holoenzyme, while others will join later for downstream processing steps, such as poly-adenylation or splicing, or may direct mRNA ribonucleoprotein particle migration to the nucleopore. We recently discovered that *Arabidopsis* plant knockout for the protein MOS11 (MODIFIER OF SNC1, 11) partially suppresses autoimmune responses observed in the TNL-type [TIR/NBS/LRR (Toll-interleukin-like receptor/nucleotide-binding site/C-terminal leucine-rich repeat)] R gene gain-of-function variant *snc1* (*suppressor of npr1-1, constitutive 1*). This suppression of resistance to pathogens appears to be caused by a decrease in nuclear mRNA export in *mos11-1 snc1* plants. In humans, the putative ortholog of MOS11, CIP29 (29-kDa cytokine-induced protein), interacts with three proteins that are also involved in mRNA export: DDX39 (DEAD-box RNA helicase), TAF15 of the FUS family (FUSED IN SARCOMA), and ALY (ALWAYS EARLY), a protein implicated in mRNA export in mammalian systems. These proteins have received very little attention in plants. Here, we will discuss their particularities and role in mRNA export and biotic stress.

Keywords: mRNA export, ALY, MOS11, CIP29, TAF15b, TAFs, RNA helicase, snc1

INTRODUCTION

Messenger RNA export is a tightly regulated process, unique to eukaryotes, that enhances control over the timing and level of translation. Albeit mRNA export is unidirectional, unlike nucleocytoplasmic protein trafficking, it represents a more complex process. Before mRNA can actually be led through the conduit formed by the nucleopore (NP) across the nuclear envelope (NE), it must be adequately processed and guided toward the NP basket. As mRNA elongates, it is loaded with proteins; some of them will mark intron–exon junctions, helicases will keep mRNA unwound, the spliceosome complex will bind to single-stranded nucleic acid, some will induce mRNA cleavage in its 3′UTR so that it can then be 3′-poly-adenylated and bound with more proteins that recognize the poly-A tail. Additional proteins will serve as guides to direct mRNA to the NP. This association of proteins and one mRNA molecule is known as the messenger ribonucleoprotein particle (mRNP). In fact, mRNP thickness has been evaluated to be approximately 5–7 nm (Batisse et al., 2009). In comparison, the free, double-stranded DNA molecule has a width of 2 nm, which increases to 10 nm when DNA is bound to nucleosomes. Hence, the traditional representation of a free and linear mRNA molecule generally illustrated in textbooks does not depict reality; rather mRNP is virtually the size of the double-stranded DNA molecule bound to nucleosomes.

Messenger ribonucleoprotein particle does not journey from the nucleoplasm to the cytoplasm by linear progression. As Siebrasse et al. (2012) beautifully demonstrated recently by light sheet microscopy, bottleneck of mRNP export occurs on the nuclear side of the NE, more precisely at the NP basket. Accumulation of mRNP at this precise location is indicative of intensive mRNP remodeling required to remove mRNP nuclear proteins that will not engage in NP conduit along with mRNA during its migration toward the cytoplasm. The bulk of mRNP is exported by proteins of THO/TREX (transcription-export) complex (Strasser et al., 2002). However, the unidirectionality of transport is conferred, on the cytoplasmic side, by a RNA helicase that unloads the mRNP of key transport-related proteins, preventing its return to the nucleus (Tran et al., 2007; Stewart, 2010). TREX complex is highly conserved across species and orchestrates several steps between mRNA synthesis and export (Katahira, 2012).

Knowledge of mRNA export has been garnered in mammalian species, *Drosophila* and yeast. However, this crucial aspect of cellular biology and translation regulation has received very little attention in plants. Genetic screens designed to modify the autoimmune phenotype in the TNL-type [TIR–NBS–LRR (Toll-interleukin-like receptor/nucleotide-binding site/C-terminal leucine-rich repeat)] R gene gain-of-function variant *snc1* (*suppressor of npr1-1, constitutive 1*) have identified a number of proteins involved in nucleocytoplasmic transport (reviewed in Monaghan et al., 2010). MOS2, discovered in a *snc1* modifier screen, is the first protein potentially involved in mRNA export (Zhang et al., 2005). It encodes a nuclear protein of unknown function that possesses a RNA-binding domain. The G-patch found in MOS2 is also observed in eukaryotic RNA-processing proteins. The single *mos2* mutant displays enhanced disease susceptibility to *Pseudomonas syringae maculicola* (*P.s.m.*) and Avr-containing *Pseudomonas* strains, indicating that MOS2 is involved in both basal and effector-triggered immunity (Zhang et al., 2005). Interestingly, MOS2 was recently found to be sumoylated (Miller et al., 2010). Sumoylation appears to be a functional regulatory key of

proteins involved in mRNA export and processing (Meier, 2012). A number of studies targeting SUMO-enriched fraction or yeast two-hybrid with SUMO E2 or SUMO protease EARLY IN SHORT DAYS4 (ESD4) as baits discerned a significant number of proteins involved in RNA splicing and processing, DEAD/DEAH-box RNA helicases, and proteins with RNA-recognition motifs (Budhiraja et al., 2009; Elrouby and Coupland, 2010; Miller et al., 2010). MOS3 (also called SAR3/AtNup96), another protein discovered in *snc1* modifier screen (Zhang and Li, 2005), is an integral NP component of the conserved Nup107–Nup160 complex shown to be required for mRNA export (Parry et al., 2006). Similarly to *mos2*, single *mos3* mutant plants display enhanced disease susceptibility to *P.s.m.* and Avr-containing *Pseudomonas* strains linking MOS3 to both basal and effector-triggered immunity (Zhang and Li, 2005).

We recently reported the discovery of a novel *snc1* modifier, naming it *modifier of snc1, 11* (*mos11*), a suppressor of mRNA export (Germain et al., 2010). Unlike most previously identified *mos* mutants, including *mos2* and *mos3*, single *mos11* mutant does not display enhanced disease susceptibility, indicating that its role in immunity, observed in suppression of the *snc1* autoimmune phenotype, may be limited to R gene-mediated resistance. Although cellular function of the putative homolog of MOS11 in mammalian systems (CIP29, 29-kDa cytokine-induced protein) was not established, the physical interactors encountered in human cells are consistent with a role in mRNA export. In this review, we focus on MOS11 and interactors of the homolog of MOS11, found in non-plant systems. Since none of them has been described in plants, we will hypothesize their putative involvement in mRNA export and innate plant immunity.

MOS11

mos11 mutant was initially identified by T-DNA tagging in the *snc1* background. Typically, *snc1* plants are dwarfs that have elevated salicylic acid levels, constitutive *pathogenesis-related* genes expression and enhanced resistance to *Hyaloperonospora arabidopsidis* Noco2 and virulent *Pseudomonas syringae* strains. *mos11 snc1* plants do not fully revert to wild type-looking plants; *mos11* only partially suppresses the molecular and morphological features of *snc1* mutants. With inverse polymerase chain reaction, we identified a T-DNA insertion in At5g02770 and confirmed, by complementation with the wild type At5g02770 sequence, that this T-DNA insertion was causing the *mos11 snc1* phenotype (Germain et al., 2010). At5g02770 encodes a small protein of unknown function, and its sequence is unique in *Arabidopsis*. The human protein CIP29 (**Table 1**) is the closest homolog with a putative function found with BlastP (Fukuda et al., 2002). Insight into its putative role came with the identification of two yeast two-hybrid interactors of CIP29: the DEAD-box RNA helicases BAT1 and DDX39 (DEAD-box RNA helicase; Leaw et al., 2004). Patches of positively charged residues in CIP29 (and MOS11) indicate affinity for DNA or RNA and *in vitro* assays have demonstrated that CIP29 binds RNA (Sugiura et al., 2007). The observation that the RNA helicase activity of DDX39 was greatly enhanced in the presence of CIP29 provided a molecular role for CIP29 (Sugiura et al., 2007). In addition, these authors demonstrated that DDX39 could immunoprecipitate both CIP29 and ALY (ALWAYS EARLY). Dufu et al. (2010) established that recruitment of CIP29 to mRNA is capping- and splicing-dependent, positioning the role of CIP29 as a post-splicing event. Finally, the yeast ortholog of CIP29, Tho1 (Transcriptional defect of Hpr1 by overexpression), can suppress the RNA export defect of *hpr1*Δ when overexpressed (Jimeno et al., 2006). Using whole mount *in situ* total mRNA localization, we clearly determined that *mos11* and *mos11 snc1* plants manifest decreased mRNA export and accumulate poly-A mRNA in their nuclei (Germain et al., 2010). Surprisingly, despite a rather dramatic decrease in mRNA export, plant morphology appears to be virtually identical to that of wild type plants, indicating that all mRNA probably eventually reach the cytosol. The important transcriptional reprograming responsible for *snc1* elevated defense is likely to be dimmed in this mutant that shows nuclear mRNAs accumulation.

TAF15B

RNA polymerase II pre-initiation complex assembly requires the presence of general transcription factors (GTFs). Transcription factors for polymerase IID (TFIID), one of these GTFs, comprises a complex of several subunits that include TATA-box binding protein (TBP) and TBP-associated factors (TAFs). The combination of these proteins makes up the GTF TFIID, the major core promoter recognition factor that provides scaffolding for the assembly of other GTFs. TAFs can bind activators and other transcriptional regulators; some of them exert catalytic

Table 1 | CIP29 interacting proteins and their putative orthologs in *Arabidopsis*.

Human gene (NCBI gene ID)	*Arabidopsis* ortholog (TAIR gene ID)	Putative function	Reference
CIP29 (84324)	*MOS11* (At5g02770)	Activation of DDX39	Sugiura et al. (2007), Germain et al. (2010)
TAF15 (8148)	*AtTAF15b* (At5g58470)	Unknown	Sugiura et al. (2007)
DDX39B/UAP56/BAT1 (7919)	*AtRH15* (At5g11170)	DEAD-box RNA helicase	Aubourg et al. (1999)
	AtRH15b (At5g11200)		
ALY/REF/BEF/THOC4 (10189)	*AtALY1* (At5g59950)	TREX complex adaptor protein	Dufu et al. (2010)
	AtALY2 (At5g02530)		Pendle et al. (2005)
	AtALY3 (At1g66260)		Uhrig et al. (2004)
	AtALY4 (At5g37720)		Yelina et al. (2010)

activity modifying the histone code and transcriptional regulators. For example, TAF1 alone displays kinase, acetyltransferase, and ubiquitin-activating/conjugating enzyme activity, all in one protein. TAF1 can de-condense chromatin by H3–H4 histone acetylation and ubiquitination of histone H1; with its two kinase domains, it also directly phosphorylates some transcription factors (Dikstein et al., 1996; Mizzen et al., 1996; Pham and Sauer, 2000). Mining the *Arabidopsis* genome identified 18 putative TAFs, including TAF15a and TAF15b (Lago et al., 2004). AtTAF15b (**Table 1**) is the *Arabidopsis* homolog closest to the TAF protein that interacts in human cells with CIP29. Although some TAFs, e.g., TAF1, have been characterized in *Arabidopsis* (Bertrand et al., 2005; Benhamed et al., 2006), TAF15b has not. Since TAF15b does not contain any conserved catalytic domain that would provide insight into its putative molecular function, we investigated whether it has an effect on mRNA export. Whole mount *in situ* mRNA localization on two T-DNA lines (Salk_061974 and Sail_35_B06), inserted 17 bp apart in the third intron of At5g58470, did not reveal any significant changes in the amount of nuclear mRNA compared to wild type plants (unpublished). Thus, it is likely that TAF15b is not absolutely required for mRNA export under our assay conditions or that it is needed only for export of certain mRNAs, which is not possible to detect with poly-A mRNA localization. We are currently investigating the putative role of TAF15b in innate plant immunity and other cellular functions.

DEAD-BOX RNA HELICASE

Employing BlastP with CIP29-interacting RNA helicase, we identified two *Arabidopsis* proteins (At5g11170 and At5g11200) with identical e-values. Both helicases are separated by only two genes on chromosome 5 and show 100% identity at the amino acid level; neither has been studied in plants. Previous mining of *Arabidopsis* sequences unveiled the presence of 32 DEAD-box RNA helicases in *Arabidopsis* (Aubourg et al., 1999). Based on the nomenclature proposed by them, At5g11170 is AtRH15 (**Table 1**). However, At5g11200 was not identified by Aubourg et al. (1999), perhaps due to its high degree of similarity to At5g11170. Therefore, we suggest re-naming At5g11200 as AtRH15b. The new TAIR (The Arabidopsis Information Resource) annotation identifies At5g11170 and At5g11200 as homologs of human UAP56. Alternate UAP56 names are DDX39 and BAT1, and we refer to UAP56 as DDX39 since it has been linked to CIP29 as the human DDX39. Enzymes that tap into energy released by the hydrolysis of a nucleotide triphosphate to unwind double-stranded RNAs are defined as RNA helicases (de la Cruz et al., 1999), linking them to every possible step in mRNA biogenesis and function. In the context of mRNA export, the unwinding activity of helicases can make mRNA more or less accessible to mRNA-processing proteins, thereby facilitating the addition and removal of proteins, and are involved in mRNP remodeling at the NP basket site. As mentioned previously, CIP29 is known to amplify the RNA-unwinding activity of DDX39 (Sugiura et al., 2007). The precise roles of AtRH15 and AtRH15b are unknown, and since their amino acid sequences are identical, it is likely that they complement each other functionally. It would be interesting to assess whether MOS11 can increase the RNA-unwinding activity of AtRH15 and/or AtRH15b as CIP29 does for DDX39. It should be mentioned that AtRH15 (like many helicases) can be sumoylated (Meier, 2012).

Very few functional analyses of DEAD-box RNA helicases have been conducted in *Arabidopsis*. Genetic screening of plants showing cold sensitivity identified *LOS4* (LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES4; Gong et al., 2002), which encodes a DEAD-box RNA helicase that localizes to the nucleus and cytoplasm. *los4-1* mutant plants are very sensitive to chilling temperatures, particularly in the dark. LOS4 has also been shown to be required for mRNA export in a temperature-dependent manner, and LOS4–GFP (green fluorescent protein) fusion accumulates at the NE (Gong et al., 2002, 2005) where it may be involved in mRNP remodeling.

In human cells, the helicase DEAD-box protein 5 (Dbp5) is known to be required for the directionality of mRNA export (Tran et al., 2007). Dbp5 accumulates on the cytoplasmic side of the NE where it removes Nab2 from mRNP (Tran et al., 2007). Export functions as a cargo:carrier complexe; once mRNA has reached the cytosol, the carrier is disassembled to prevent cargo re-entry from the destination compartment (Stewart, 2010). The helicase that controls directionality in plants is unknown. However, LOS4 is the *Arabidopsis* helicase with the strongest sequence similarity to Dbp5; it accumulates at the nucleus outer membrane and is observed in the cytoplasm and nucleus (Gong et al., 2005). Two other helicases – *STRS1* and *STRS2* (*STRESS RESPONSE SUPPRESSOR*) corresponding to AtRH5 (At1g31970) and AtRH25 (At5g08620), respectively – have been shown to induce tolerance to salt, osmotic and heat stresses, suggesting that helicases suppress responses to abiotic stress (Kant et al., 2007). However, it is not known whether these helicases are involved in mRNA export. More than 30 helicases have been identified in *Arabidopsis* and it is conceivable that one helicase or a specific group of helicases may have specificity for groups of transcripts such as transcripts induced after biotic stress.

ALY

Counterparts of the metazoan *ALY* (also known as *REF*, *BEF*, and *THOC4*) and its *Saccharomyces cerevisiae* homolog *YRA1* exist in plants (**Table 1**); as in animals, the number of ALY-coding genes in plants varies by species (Uhrig et al., 2004; Dufu et al., 2010). The exact functions of plant ALYs are not yet known. Four *ALY* genes have been reported so far in *Arabidopsis thaliana*: At5g59950, At5g02530, At1g66260, and At5g37720 (hereafter referred to as *AtALY1*, *AtALY2*, *AtALY3*, and *AtALY4*; Uhrig et al., 2004; Pendle et al., 2005; Yelina et al., 2010). Despite the relatively moderate sequence similarity between AtALYs (55–71%), AtALY1, AtALY2, AtALY3, and AtALY4 respectively share 41, 42, 38, and 48% sequence identity with human ALY. All four AtALYs localize to the nucleoplasm and, with the exception of AtALY2, also accumulate in the nucleolus, a poorly studied compartment in plants (Uhrig et al., 2004; Pendle et al., 2005; Shaw and Brown, 2012). Microarray data from Genevestigator (https://www.genevestigator.com/gv/) indicate medium to high expression levels for the four *AtALY* genes in seedlings, inflorescences, and shoots; lack of expression data for other tissues, however, makes it difficult to determine whether or not *AtALY* gene expression is tissue-specific.

Current models of mRNA export in animals and yeast depict ALY as a conserved component of TREX complex along with THO subcomplex, UAP56 (Sub2 in yeast), TEX1, and CIP29 (Tho1 in yeast), as a *bona fide* component (Carmody and Wente, 2009; Dufu et al., 2010; Chi et al., 2012; Katahira, 2012). However, recruitment of TREX complex components to mRNAs appears to proceed via distinct mechanisms in metazoans and yeast, possibly because of differences in gene structure, as the yeast genome contains far less introns than animal genomes (Cheng et al., 2006). Briefly, ALY and UAP56 are recruited to mRNA in an interdependent manner; UAP56 is then displaced by the Nxf1–Nxt1/TAP–p15 (Mex67–Mtr2 in yeast) heterodimer which is involved in mRNP targeting of nuclear pore complex (NPC; Carmody and Wente, 2009; Dufu et al., 2010; Walsh et al., 2010; Chi et al., 2012; Katahira, 2012). In human cells, TREX complex is recruited mostly in a splicing- and cap-dependent manner and binds near the 5′ end of spliced mRNA through interaction between ALY and the cap-binding complex protein CBP80. This mRNA 5′-end positioning of TREX complex is thought to account for the 5′–3′ orientation of the mRNA molecule during its export (Cheng et al., 2006; Kohler and Hurt, 2007).

Experimental results on *Arabidopsis* support the hypothesis that THO/TREX complex is conserved not only in yeast and metazoans, but also in plants, and strongly indicate a role for this complex (or at least some of its components) in plant immunity (Furumizu et al., 2010; Yelina et al., 2010; Pan et al., 2012b). For example, AtTEX1 and AtTHO1 have been shown to be required for the biogenesis of a subset of *trans*-acting small interfering RNAs (tasiRNAs; Yelina et al., 2010). Interestingly, it is now known that, in Fabaceae and Solanaceae, tasiRNAs mediate nucleotide-binding leucine-rich repeat gene silencing in the absence of pathogen threats (Zhai et al., 2011; Li et al., 2012). Furthermore, AtHPR1, a component of THO subcomplex, was recently identified as a mediator of disease resistance. *hpr1-4* mutant plants display compromised mRNA export and lack basal resistance to virulent bacterial and fungal strains (Pan et al., 2012a). To our knowledge, HPR1 is the only free nuclear protein (not bound to the NE) known to affect both mRNA export and basal defense responses.

Arabidopsis and *Nicotiana benthamiana* ALY proteins have been found to interact with P19 silencing suppressor protein of *Tomato bushy stunt virus* (Uhrig et al., 2004; Canto et al., 2006). ALY–P19 interaction results in the relocalization of AtALY2, AtALY4, and two *N. benthamiana* ALY proteins from the nucleus to the cytoplasm; however, it also leads to accumulation of P19 in the nucleolus by those ALY proteins that do not re-localize to the cytoplasm (Uhrig et al., 2004; Canto et al., 2006). Interestingly, nucleolar targeting of P19 interferes with its silencing suppression activity (Canto et al., 2006). Unfortunately, the biological role of ALY–P19 interaction is still unknown.

Yeast two-hybrid and *in vitro* findings have raised the assumption that AtALY3 and AtALY4 likely interact with the nuclear enzyme poly(ADP-ribose) polymerase 1 (PARP1; Storozhenko et al., 2001). Although this interaction has not been demonstrated *in planta*, it is noteworthy that plant PARPs have been reported to mediate some of the immune responses triggered upon recognition of microbe-associated molecular patterns (Adams-Phillips et al., 2010).

In mammals, ALY is emerging as a versatile protein involved in processes other than mRNA export. Thus, it is thought to stabilize some viral transcripts independently of their export (Stubbs et al., 2012). Furthermore, misregulation of ALY expression has been associated with tumorigenesis (Dominguez-Sanchez et al., 2011). In plants, such versatility would be consistent with the multiplicity of *ALY* genes and the heterogeneity of ALY protein subnuclear distribution.

CONCLUDING REMARKS

This review highlights several similarities between the mechanistic aspects of animal and plant mRNA export processes. However, key questions remain to be answered that could promote understanding of plant mRNA export specificities. Which helicase is driving the directionality of export in plants? Does MOS11 possess helicase activity-enhancing capacity? What is the true role of the different ALYs, why are there four ALYs in plants, what role do they play in the nucleolus, can ALY bind suppressors of silencing other than P19? Plant nuclear proteome dynamics is still largely unknown, even more so the nuclear proteome of biotic or abiotic stressed plants. Although confocal imagery and genetics will remain core tools in resolving these issues, thorough proteomics analysis could lead geneticists on the right track. The fact that several pathogen virulence factors appear to be targeting the nucleus, combined with the observed high level of conservation of the mRNA export machinery suggest that the export machinery would represent a good target for pathogen effectors.

REFERENCES

Adams-Phillips, L., Briggs, A. G., and Bent, A. F. (2010). Disruption of poly(ADP-ribosyl)ation mechanisms alters responses of *Arabidopsis* to biotic stress. *Plant Physiol.* 152, 267–280.

Aubourg, S., Kreis, M., and Lecharny, A. (1999). The DEAD box RNA helicase family in *Arabidopsis thaliana*. *Nucleic Acids Res.* 27, 628–636.

Batisse, J., Batisse, C., Budd, A., Bottcher, B., and Hurt, E. (2009). Purification of nuclear poly(A)-binding protein Nab2 reveals association with the yeast transcriptome and a messenger ribonucleoprotein core structure. *J. Biol. Chem.* 284, 34911–34917.

Benhamed, M., Bertrand, C., Servet, C., and Zhou, D. X. (2006). *Arabidopsis* GCN5, HD1, and TAF1/HAF2 interact to regulate histone acetylation required for light-responsive gene expression. *Plant Cell* 18, 2893–2903.

Bertrand, C., Benhamed, M., Li, Y. F., Ayadi, M., Lemonnier, G., Renou, J. P., et al. (2005). *Arabidopsis* HAF2 gene encoding TATA-binding protein (TBP)-associated factor TAF1, is required to integrate light signals to regulate gene expression and growth. *J. Biol. Chem.* 280, 1465–1473.

Budhiraja, R., Hermkes, R., Muller, S., Schmidt, J., Colby, T., Panigrahi, K., et al. (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.

Canto, T., Uhrig, J. F., Swanson, M., Wright, K. M., and Macfarlane, S. A. (2006). Translocation of tomato bushy stunt virus P19 protein into the nucleus by ALY proteins compromises its silencing suppressor activity. *J. Virol.* 80, 9064–9072.

Carmody, S. R., and Wente, S. R. (2009). mRNA nuclear export at a glance. *J. Cell Sci.* 122, 1933–1937.

Cheng, H., Dufu, K., Lee, C. S., Hsu, J. L., Dias, A., and Reed, R. (2006). Human mRNA export machinery recruited to the 5′ end of mRNA. *Cell* 127, 1389–1400.

Chi, B., Wang, Q., Wu, G., Tan, M., Wang, L., Shi, M., et al. (2012). Aly and THO are required for assembly of the human TREX complex and association of TREX components with the spliced mRNA. *Nucleic Acids Res.* 41, 1294–1306.

de la Cruz, J., Kressler, D., and Linder, P. (1999). Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families. *Trends Biochem. Sci.* 24, 192–198.

Dikstein, R., Ruppert, S., and Tjian, R. (1996). TAFII250 is a bipartite protein kinase that phosphorylates the base transcription factor RAP74. *Cell* 84, 781–790.

Dominguez-Sanchez, M. S., Saez, C., Japon, M. A., Aguilera, A., and Luna, R. (2011). Differential expression of THOC1 and ALY mRNP biogenesis/export factors in human cancers. *BMC Cancer* 11:77. doi: 10.1186/1471-2407-11-77

Dufu, K., Livingstone, M. J., Seebacher, J., Gygi, S. P., Wilson, S. A., and Reed, R. (2010). ATP is required for interactions between UAP56 and two conserved mRNA export proteins, Aly and CIP29, to assemble the TREX complex. *Genes Dev.* 24, 2043–2053.

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.

Fukuda, S., Wu, D. W., Stark, K., and Pelus, L. M. (2002). Cloning and characterization of a proliferation-associated cytokine-inducible protein, CIP29. *Biochem. Biophys. Res. Commun.* 292, 593–600.

Furumizu, C., Tsukaya, H., and Komeda, Y. (2010). Characterization of EMU, the *Arabidopsis* homolog of the yeast THO complex member HPR1. *RNA* 16, 1809–1817.

Germain, H., Qu, N., Cheng, Y. T., Lee, E., Huang, Y., Dong, O. X., et al. (2010). MOS11: a new component in the mRNA export pathway. *PLoS Genet.* 6:e1001250. doi: 10.1371/journal.pgen.1001250

Gong, Z., Dong, C. H., Lee, H., Zhu, J., Xiong, L., Gong, D., et al. (2005). A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in *Arabidopsis*. *Plant Cell* 17, 256–267.

Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B., and Zhu, J. K. (2002). RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11507–11512.

Jimeno, S., Luna, R., Garcia-Rubio, M., and Aguilera, A. (2006). Tho1, a novel hnRNP, and Sub2 provide alternative pathways for mRNP biogenesis in yeast THO mutants. *Mol. Cell. Biol.* 26, 4387–4398.

Kant, P., Kant, S., Gordon, M., Shaked, R., and Barak, S. (2007). STRESS RESPONSE SUPPRESSOR1 and STRESS RESPONSE SUPPRESSOR2, two DEAD-box RNA helicases that attenuate *Arabidopsis* responses to multiple abiotic stresses. *Plant Physiol.* 145, 814–830.

Katahira, J. (2012). mRNA export and the TREX complex. *Biochim. Biophys. Acta* 1819, 507–513.

Kohler, A., and Hurt, E. (2007). Exporting RNA from the nucleus to the cytoplasm. *Nat. Rev. Mol. Cell Biol.* 8, 761–773.

Lago, C., Clerici, E., Mizzi, L., Colombo, L., and Kater, M. M. (2004). TBP-associated factors in *Arabidopsis*. *Gene* 342, 231–241.

Leaw, C. L., Ren, E. C., and Choong, M. L. (2004). Hcc-1 is a novel component of the nuclear matrix with growth inhibitory function. *Cell. Mol. Life Sci.* 61, 2264–2273.

Li, F., Pignatta, D., Bendix, C., Brunkard, J. O., Cohn, M. M., Tung, J., et al. (2012). MicroRNA regulation of plant innate immune receptors. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1790–1795.

Meier, I. (2012). mRNA export and sumoylation – lessons from plants. *Biochim. Biophys. Acta* 1819, 531–537.

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.

Mizzen, C. A., Yang, X. J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., et al. (1996). The TAFII250 subunit of TFIID has histone acetyltransferase activity. *Cell* 87, 1261–1270.

Monaghan, J., Germain, H., Weihmann, T., and Li, X. (2010). Dissecting plant defence signal transduction: modifier of snc1 in *Arabidopsis*. *Can. J. Plant Pathol.* 32, 35–42.

Pan, H., Liu, S., and Tang, D. (2012a). The THO/TREX complex functions in disease resistance in *Arabidopsis*. *Plant Signal. Behav.* 7, 422–424.

Pan, H., Liu, S., and Tang, D. (2012b). HPR1, a component of the THO/TREX complex, plays an important role in disease resistance and senescence in *Arabidopsis*. *Plant J.* 69, 831–843.

Parry, G., Ward, S., Cernac, A., Dharmasiri, S., and Estelle, M. (2006). The *Arabidopsis* SUPPRESSOR OF AUXIN RESISTANCE proteins are nucleoporins with an important role in hormone signaling and development. *Plant Cell* 18, 1590–1603.

Pendle, A. F., Clark, G. P., Boon, R., Lewandowska, D., Lam, Y. W., Andersen, J., et al. (2005). Proteomic analysis of the *Arabidopsis* nucleolus suggests novel nucleolar functions. *Mol. Biol. Cell* 16, 260–269.

Pham, A. D., and Sauer, F. (2000). Ubiquitin-activating/conjugating activity of TAFII250, a mediator of activation of gene expression in *Drosophila*. *Science* 289, 2357–2360.

Shaw, P., and Brown, J. (2012). Nucleoli: composition, function, and dynamics. *Plant Physiol.* 158, 44–51.

Siebrasse, J. P., Kaminski, T., and Kubitscheck, U. (2012). Nuclear export of single native mRNA molecules observed by light sheet fluorescence microscopy. *Proc. Natl. Acad. Sci. U.S.A.* 109, 9426–9431.

Stewart, M. (2010). Nuclear export of mRNA. *Trends Biochem. Sci.* 35, 609–617.

Storozhenko, S., Inze, D., Van Montagu, M., and Kushnir, S. (2001). *Arabidopsis* coactivator ALY-like proteins, DIP1 and DIP2, interact physically with the DNA-binding domain of the Zn-finger poly(ADP-ribose) polymerase. *J. Exp. Bot.* 52, 1375–1380.

Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., et al. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. *Nature* 417, 304–308.

Stubbs, S. H., Hunter, O. V., Hoover, A., and Conrad, N. K. (2012). Viral factors reveal a role for REF/Aly in nuclear RNA stability. *Mol. Cell. Biol.* 32, 1260–1270.

Sugiura, T., Sakurai, K., and Nagano, Y. (2007). Intracellular characterization of DDX39, a novel growth-associated RNA helicase. *Exp. Cell Res.* 313, 782–790.

Tran, E. J., Zhou, Y., Corbett, A. H., and Wente, S. R. (2007). The DEAD-box protein Dbp5 controls mRNA export by triggering specific RNA:protein remodeling events. *Mol. Cell* 28, 850–859.

Uhrig, J. F., Canto, T., Marshall, D., and Macfarlane, S. A. (2004). Relocalization of nuclear ALY proteins to the cytoplasm by the tomato bushy stunt virus P19 pathogenicity protein. *Plant Physiol.* 135, 2411–2423.

Walsh, M. J., Hautbergue, G. M., and Wilson, S. A. (2010). Structure and function of mRNA export adaptors. *Biochem. Soc. Trans.* 38, 232–236.

Yelina, N. E., Smith, L. M., Jones, A. M., Patel, K., Kelly, K. A., and Baulcombe, D. C. (2010). Putative *Arabidopsis* THO/TREX mRNA export complex is involved in transgene and endogenous siRNA biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13948–13953.

Zhai, J., Jeong, D. H., De Paoli, E., Park, S., Rosen, B. D., Li, Y., et al. (2011). MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. *Genes Dev.* 25, 2540–2553.

Zhang, Y., Cheng, Y. T., Bi, D., Palma, K., and Li, X. (2005). MOS2, a protein containing G-patch and KOW motifs, is essential for innate immunity in *Arabidopsis thaliana*. *Curr. Biol.* 15, 1936–1942.

Zhang, Y., and Li, X. (2005). A putative nucleoporin 96 is required for both basal defense and constitutive resistance responses mediated by suppressor of npr1-1, constitutive 1. *Plant Cell* 17, 1306–1316.

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: 20 January 2013; paper pending published: 06 February 2013; accepted: 04 March 2013; published online: 22 March 2013.

*Citation: Gaouar O and Germain H (2013) mRNA export: threading the needle. Front. Plant Sci. **4**:59. doi: 10.3389/fpls.2013.00059*

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

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 21 May 2013
doi: 10.3389/fpls.2013.00149

Hop-on hop-off: importin-α-guided tours to the nucleus in innate immune signaling

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Nuclear translocation of immune regulatory proteins and signal transducers is an essential process in animal and plant defense signaling against pathogenic microbes. Import of proteins containing a nuclear localization signal (NLS) into the nucleus is mediated by nuclear transport receptors termed importins, typically dimers of a cargo-binding α-subunit and a β-subunit that mediates translocation through the nuclear pore complex. Here, we review recent reports of importin-α cargo specificity and mutant phenotypes in plant- and animal–microbe interactions. Using homology modeling of the NLS-binding cleft of nine predicted *Arabidopsis* α-importins and analyses of their gene expression patterns, we discuss functional redundancy and specialization within this transport receptor family. In addition, we consider how pathogen effector proteins that promote infection by manipulating host cell nuclear processes might compete with endogenous cargo proteins for nuclear uptake.

Keywords: importin-α, nuclear protein import, nucleocytoplasmic transport, *Arabidopsis*, innate immunity

HOP-ON HOP-OFF: IMPORTIN-MEDIATED NUCLEAR PROTEIN IMPORT

In eukaryotic cells, nuclear transport receptors (NTRs) of the importin-α family recognize and bind to canonical nuclear localization signal (NLS)-containing cargo proteins in the cytoplasm and link them to importin-β, the NTR that facilitates passage of the ternary complex through the nuclear pore complex (NPC) into the nucleus. Cargos may contain one (monopartite) or two (bipartite) NLS sequence motifs and directional binding to and release from the importin-α/β heterodimer is imposed by the nucleotide-binding state of Ran, a small guanosine-5′-triphosphatase (GTPase) that cycles between GTP-bound nuclear and guanosine-5′-diphosphate (GDP)-bound cytoplasmic states (Terry et al., 2007; Meier and Somers, 2011). The RanGDP-RanGTP gradient across the nuclear envelope (NE) is generated by the asymmetric distribution of two regulators, RanGAP in the cytoplasm and RanGEF in the nucleus that is associated with chromatin and drives nuclear cargo release upon binding of RanGTP to importin-β. After dissociation of the import complex and cargo delivery into the nucleus, importin-β bound to RanGTP is recycled to the cytoplasm, whereas importin-α interacts with the RanGTP-bound export receptor CAS for recycling of cargo-free importin-α back to the cytoplasm. In the cytoplasm, RanGAP stimulates GTP hydrolysis on Ran to release the importins for another round of import (Stewart, 2007).

α-importins typically consist of an N-terminal auto-inhibitory importin-β-binding (IBB) domain followed by a series of ten armadillo (ARM) repeats that form the NLS-binding cleft (Goldfarb et al., 2004; **Figures 1A,B**). The flexible IBB domain not only connects importin-α to importin-β but also contains a cluster of basic amino acids that competes with NLS-cargos for binding to the ARM-repeat domain of importin-α. Thus, the IBB domain is involved in regulating both formation of the trimeric import complex in the cytoplasm and release of cargo in the nucleus after the IBB domain is freed from importin-β by RanGTP (Görlich et al., 1996a; Kobe, 1999; Stewart, 2007). Following cargo release in the nucleus α-importin is exported to the cytoplasm by a complex of the export carrier CAS and RanGTP (Goldfarb et al., 2004; Matsuura and Stewart, 2004).

Stimulus-induced nuclear translocation and/or accumulation of signaling molecules and transcriptional regulators are essential for the coordinated relay of defense signals in both plant and animal innate immune responses to microbial pathogens. Inside the nucleus, these signals direct the expression of defense-related genes. In addition, it has become increasingly evident that not only do host resistance components show dynamic partitioning between the cytoplasm and the nucleus, but also that a significant number of animal and plant pathogen virulence factors exploit host cell nuclear import pathways to act directly within the nucleus and promote disease. In this review, we provide an overview of recent studies reporting importin-α cargo selectivity in animal and plant innate immunity and discuss potential promiscuity within the *Arabidopsis* import receptor family. We also consider how microbial virulence factors may hijack the nuclear import machinery to manipulate host cell nuclear processes.

IMPORTIN-α PARALOGS IN *Arabidopsis thaliana*

Although the *Saccharomyces cerevisiae* genome encodes only a single importin-α (Yano et al., 1992), several paralogs have been reported in most higher eukaryotes – seven in humans, six in mouse, three in *Drosophila*, five in rice, and nine in *Arabidopsis* (Ouyang et al., 2007; Ratan et al., 2008; Hu et al., 2010; Kelley et al., 2010; Merkle, 2011). Conceivably, expansion of the *importin-α* gene family in multicellular eukaryotes reflects adaptation toward a more complex regulation of nuclear import. Several mammalian *importin-α* paralogs show tissue-specific expression patterns (Köhler et al., 1997; Tsuji et al., 1997; Yasuhara et al., 2007), and nuclear import of some cargo proteins is preferentially mediated by specific importin-α adapters (Miyamoto et al., 1997; Nadler et al., 1997; Köhler et al., 1999; Melén et al., 2003; Quensel et al., 2004). In *Arabidopsis importin-α1-4*, α*6*, and α*9* are ubiquitously expressed (**Figure 1C**). However, there is controversy from different profiling techniques regarding the levels and tissue-specificity of *importin-α5*, α*7*, and α*8* expression (Meyers et al., 2004; Bhattacharjee et al., 2008; Hruz et al., 2008; Huang et al., 2010). For example, although Huang et al. (2010) report specific expression of *importin-α8* in rosette/cauline leaves and flowers, a search for genes regulated by the male germ line-specific transcription factor (TF) DUO1 suggests that *importin-α8* is a DUO1 target gene that is specifically expressed in the male germ line (Borg et al., 2011). These data indicate that importin-α8 may have a distinct function during pollen development. Notably, importin-α8 does not have an IBB domain (**Figure 1B**) suggesting that it lacks both the capacity to bind importin-β and the auto-inhibitory mechanisms that are conserved in the other α-importins. Therefore, it remains to be tested if importin-α8 can function as a NTR and whether the loss of the IBB domain is a consequence of specialization in pollen development.

The comparably high number of α-importins in *Arabidopsis* can only partially be rationalized by tissue-specific expression of single paralogs. Alternatively, multiple paralogs might have evolved to transport specific cargos. Indeed, the NLS from the rice COP1 protein binds *in vitro* the two rice importins α1a and α1b, but not importin-α2 (Jiang et al., 2001). This, and other examples outlined below, provides evidence for cargo specificity of α-importins and it appears likely that higher eukaryotes are equipped with an array of α-importins that accumulate to different levels and exhibit different affinities for distinct cargos. Transcriptional and post-translational regulation of importin-α protein levels in response to environmental stimuli would constitute a flexible system to alter nuclear import kinetics and specificities in changing environments.

SEQUENCE DIVERSITY IN *Arabidopsis* α-IMPORTINS

Resolved crystal structures of α-importins from yeast, human, mouse, and rice revealed strong structural conservation of the ARM repeat domains that form the NLS binding sites (Conti et al., 1998; Kobe, 1999; Fontes et al., 2003; Chang et al., 2012). ARM repeats from yeast, human, and mouse α-importins can be superimposed with a root mean square deviation of less than 1.8Å and amino acids that contribute to the NLS binding sites occupy very similar positions in these structures. We used homology modeling to characterize conservation of the NLS binding site among the nine *Arabidopsis* α-importins. As in α-importins from other species, a conserved array of Trp/Asn pairs protruding from the third helix of the ARM repeats (H3) forms the core of the major and minor NLS binding sites in *Arabidopsis* α-importins (**Figure 1A**). Previous comparative analysis revealed that major determinants of specificity are (i) the amino acid positioned three residues upstream of the conserved Trp, and (ii) residues that constitute the loops connecting the H3 and H1 helices (Marfori et al., 2012). Notably, the Trp/Asn array at the minor NLS binding site is not entirely conserved in plant α-importins (**Figure 1D** and **Table 1**). As some plant NLSs specifically bind to the minor NLS binding site (Chang et al., 2012) it will be interesting to test whether these divergent amino acids determine binding to specific NLSs.

IMPORTIN-α CARGO SPECIFICITY IN ANIMAL IMMUNE RESPONSES

Both animal and plant innate immune systems have evolved pattern recognition receptors (PRRs) to detect microbe-associated molecular patterns (MAMPs) and defend against pathogens (Nürnberger et al., 2004; Ausubel, 2005). In addition to MAMP detection, the plant innate immune system also imparts pathogen-specific recognition via nucleotide-binding/leucine-rich repeat immune sensors (NLRs) that detect the actions of isolate-specific pathogen virulence factors, termed effectors (Jones and Dangl, 2006). In contrast, animal NLRs detect MAMPs inside host cells (Kanneganti et al., 2007a; Ronald and Beutler, 2010; Maekawa et al., 2011). Activation of both NLRs and PRRs initiates signaling cascades that convey the biotic stress stimulus into the host cell nucleus to alter defense gene expression. Thus, stimulus-induced changes in the NPC permeability of signal transducers, immune and transcriptional regulators represent an important mechanism for controlling defense-associated gene expression.

Changes in nuclear translocation rates are often achieved via post-translational protein modifications leading to conformational changes that expose or conceal NLSs or nuclear export sequences (NESs). For example, gene expression changes in mammalian innate immunity are largely governed by the induced nuclear translocation of the NF-κB family of Rel-type TFs. Nuclear accumulation of NF-κB is controlled by its association with IκB proteins. Depending on the type of IκB, these proteins either sequester NF-κB in the cytoplasm by masking its NLS, or prevent its ability to bind to chromatin due to a strong NES in IκB that directs dominant nuclear export over nuclear import (Johnson et al., 1999; Huang et al., 2000; Malek et al., 2001). Signal-dependent phosphorylation by IκB-kinase targets IκB for proteolysis, thereby allowing NF-κB nuclear import to activate defense gene expression. In human cells, the closely related importins α3 and α4 are the two main isoforms responsible for nuclear import of NF-κB p50/p65 heterodimers following IκB degradation. Whereas the major NLS binding site of importin-α3 binds to p50, the minor NLS binding site mediates association with p65 (Fagerlund et al., 2005).

Innate immune responses in *Drosophila* are also controlled at the level of nuclear transport. Upon activation of the Toll signaling cascade, NF-κB/Rel-type TFs translocate to the nucleus

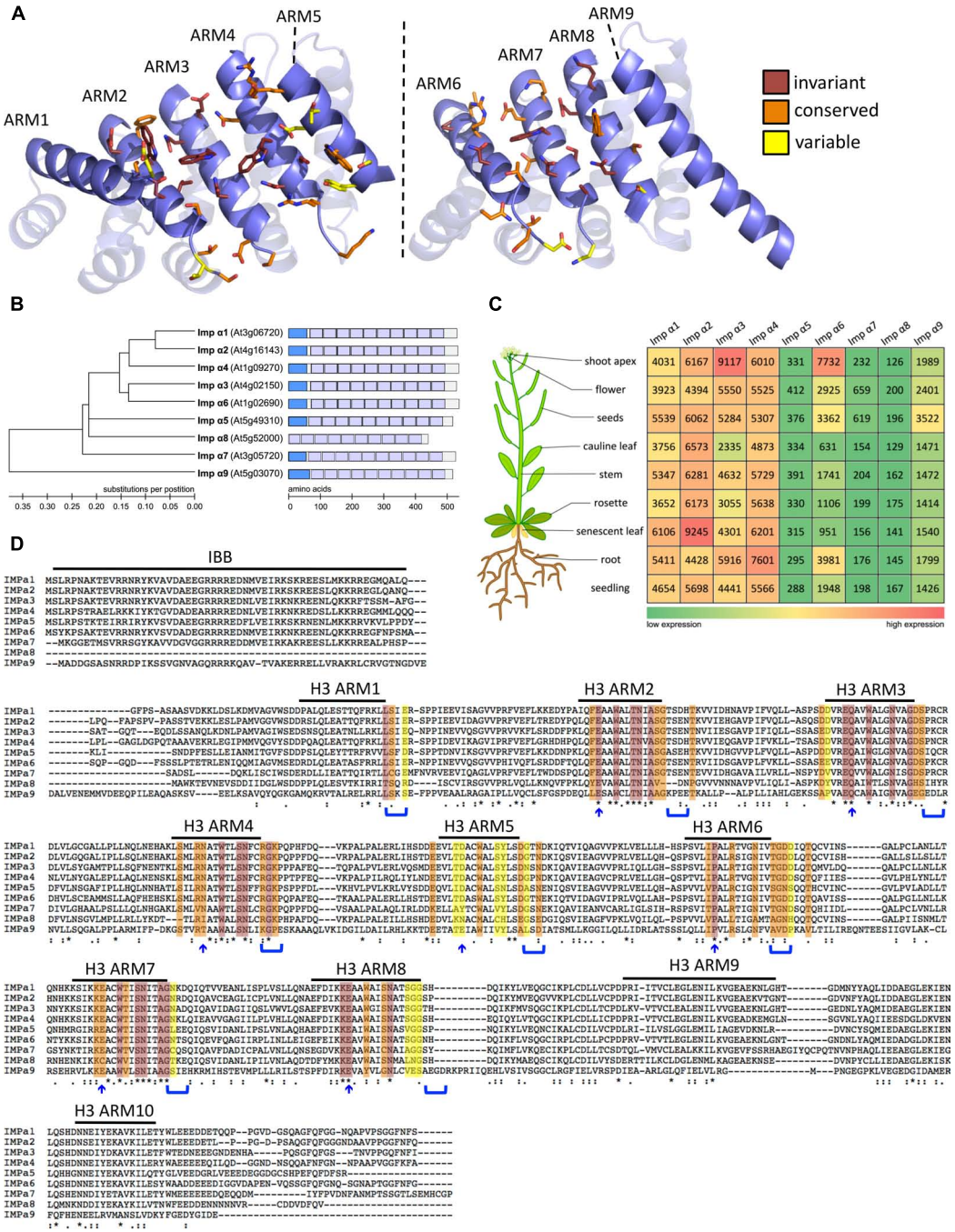

FIGURE 1 | Expression profile and sequence comparison of *Arabidopsis* importins α1-9. (A) Homology model of the ARM repeat domain of *Arabidopsis* importins α1-9 based on the structure of rice importin-α1a (RCSB identifier 4B8J, Chang et al., 2012). Left image: major NLS binding site. Right image: minor NLS binding site. Amino acids that are likely to contribute to the NLS binding sites are shown in stick representation. The color code indicates the level of conservation in *Arabidopsis* α-importins. **(B)** Phylogenetic tree constructed using neighbor joining in Molecular Evolutionary Genetics Analysis (MEGA) v4.0 (Tamura et al., 2007). Importin-α9 was used to root the tree. Scale bar represents amino acid substitutions per position. Schematic representation: The different protein domains are depicted as boxes within the full length protein sequence. Importin-β-binding domains are shown in dark blue and the ten Armadillo repeat domains are shown in light blue. Scale bar shows number of amino acids. **(C)** Gene expression data were gathered from the Genevestigator database (https://www.genevestigator.com/gv/; Hruz et al., 2008). Data referring to whole tissues were chosen for comparison of expression levels. Numbers represent linear signal intensity values of the given gene in the indicated tissues. Heat map indicates low signal intensity (green) to high signal intensity (red). **(D)** Multiple sequence alignment of full-length protein sequences performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Larkin et al., 2007). Color code for conservation as in A. Blue arrows and parenthesis indicate candidate amino acids that are predicted to contribute to the NLS binding sites based on analysis of yeast, mouse, and human α-importins (Marfori et al., 2012). Variations in these motifs are likely to determine specificity of α-importins for NLS binding.

Table 1 | Some plant α-importins diverge from the otherwise conserved pattern of amino acids protruding from ARM H3 helices that form the core of the NLS binding sites. The amino acid pairs denoted as consensus sequence (column two) are conserved in α-importins from yeast, human, mouse, and *Drosophila*, as well as the remaining α-importins from *Arabidopsis* and rice. Amino acids in blue bold font indicate divergence from the consensus sequence whereas "cons." indicates conservation of the consensus sequence.

ARM repeat	Consensus sequence	At importinα5	At importinα8	At importinα9	Os importin Os07g48880	Os importinα2
ARM2	Trp/Asn	cons.	cons.	cons.	cons.	cons.
ARM3	Trp/Asn	cons.	cons.	cons.	cons.	cons.
ARM4	Trp/Asn	cons.	cons.	cons.	cons.	cons.
ARM5	Trp/Tyr	Trp/**Asn**	**Met**/**His**	cons.	cons.	cons.
ARM6	Arg/Asn	cons.	**Leu**/**Ala**	cons.	**Thr**/**Arg**	cons.
ARM7	Trp/Asn	cons.	cons.	cons.	**Leu**/Asn	cons.
ARM8	Trp/Asn	cons.	cons	**Tyr**/Asn	cons.	**Tyr**/Asn

in a process that is dependent on nuclear transport factor-2 (NTF-2), an essential component of nuclear trafficking that acts as nuclear import receptor for RanGDP to replenish the nuclear Ran pool (Ribbeck et al., 1998; Smith et al., 1998; Bhattacharya and Steward, 2002). Whether NTF-2 directly binds Rel proteins or indirectly affects their nuclear import rates by regulating the function of *Drosophila* α-importins remains to be determined.

Like NF-κB, signal transducers and activators of transcription (STAT) proteins are a family of latent cytoplasmic TFs, consisting of seven members in mammals. Upon cytokine activation of the canonical STAT-signaling pathway, tyrosine phosphorylation induces STAT homo- or hetero-dimerization and subsequent importin-α-dependent nuclear import (Lim and Cao, 2006). Activated STAT1 homodimers and STAT1/STAT2 heterodimers interact with importin-α5 (Melén et al., 2001; Fagerlund et al., 2002) whereas RNAi-mediated silencing of *importin-α3* but not of other tested *importin-α* family members impairs nuclear translocation of STAT3, but not of STAT1 (Liu et al., 2005). This indicates that different α-importins can have distinct STAT protein binding preferences.

Further examples of vertebrate immune regulatory proteins that contain NLSs and can shuttle into the nucleus are the NLRs CIITA and NLRC5. Both these proteins function through association with DNA-binding proteins to regulate MHC class II and class I gene expression, respectively (Spilianakis et al., 2000; Cressman et al., 2001; Meissner et al., 2012). Correlating potential importin-α binding specificities for CIITA and NLRC5 remains to be determined.

IMPORTIN-α CARGO SPECIFICITY IN PLANT INNATE IMMUNITY

In rice, the intracellular kinase domain of the PRR XA21 carries a functional NLS and translocates to the nucleus after cleavage from the activated receptor, probably to modulate transcription (Park and Ronald, 2012). Also, several NLRs exhibit nucleocytoplasmic partitioning, including *Arabidopsis* RPS4, snc1 and RRS1-R, tobacco N, barley MLAs, and potato Rx (Deslandes et al., 2003; 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). Except for MLA and Rx, these proteins possess predicted NLSs and it appears that mono- or bipartite NLSs are widespread among *Arabidopsis* NLRs (Shen and Schulze-Lefert, 2007; Caplan et al., 2008; Liu and Coaker, 2008). However, experimental proof for the function of these motifs has only been provided for RPS4 (Wirthmueller et al., 2007) and it is not understood how nucleocytoplasmic partitioning of these immune sensors is regulated.

Besides NLRs, the dynamic translocation of several plant immune regulatory proteins is a key factor in defense pathway regulation. In healthy *Arabidopsis* cells, the transcriptional co-activator NPR1 is retained partially in the cytoplasm as a homo-oligomeric complex. Changes in the cell's redox potential, induced by the defense hormone salicylic acid, promotes release of NPR1 monomers and their nuclear accumulation, presumably via exposure of an obscured NLS (Kinkema et al., 2000; Mou et al., 2003; Tada et al., 2008). A negative regulator of cell death, the *Arabidopsis* zinc finger protein LSD1, antagonizes transcriptional activity of the nucleocytoplasmic shuttling leucine-zipper TF, bZIP10, by sequestering bZIP10 in the cytoplasm. Dissociation in response to pathogens is thought to unmask the NLS of bZIP10, permitting its nuclear translocation and expression of target genes (Kaminaka et al., 2006). Another report suggests that LSD1 itself localizes to nuclei, as *Pisum sativum* LSD1 is nuclear when transiently expressed in *Arabidopsis* protoplasts. *Ps*LSD1 nuclear localization is mediated by its zinc finger motifs that interact with several *Arabidopsis* α-importins and may constitute a novel NLS (He et al., 2011). The cell death pathway repressed by LSD1 depends on the activities of EDS1 and PAD4, two key regulators of basal resistance and immunity triggered by Toll interleukin-1 receptor (TIR)-type NLRs (Aarts et al., 1998; Feys et al., 2001; Wiermer et al., 2005). EDS1 harbors a predicted NLS and NES and forms dynamic nucleocytoplasmic complexes with PAD4 and SAG101, yet NTR binding-specificities responsible for nuclear targeting remain elusive (Feys et al., 2005; Garcia et al., 2010).

Evidence of importin-α cargo specificity in plants comes from a report by Kanneganti et al. (2007b). Silencing of *Nicotiana benthamiana importin-α1* and *α2* inhibits nuclear targeting of the

transiently expressed *Phytophthora infestans* effectors Nuk6 and Nuk7 while nuclear import of Nuk12 is unaffected.

Constitutive immune signaling induced by a point mutation in SNC1, an *Arabidopsis* TIR-type NLR, is partially suppressed by mutations in *importin-α3* (Palma et al., 2005). A pool of active snc1 protein is found in nuclei and auto-immunity is abolished by a snc1-NES fusion (Cheng et al., 2009). Overexpression of GFP-tagged SNC1-4 (a mutant version of snc1-1) in wild type *Arabidopsis* protoplasts results in an entirely nuclear accumulation of the fusion protein, while the same construct is nucleocytoplasmic in protoplasts lacking importin-α3 (Zhu et al., 2010). Although this makes importin-α3 a candidate NTR of SNC1-4 it remains to be tested whether SNC1-4 binds importin-α3 directly. Alternatively, importin-α3 may be required for nuclear import of signaling components activated by snc1. Partial suppression of the *snc1-1* phenotype by knock-out of *importin-α3* indicates that other α-importins might work redundantly with importin-α3 in *snc1*-triggered immunity.

A knock-out of *Arabidopsis importin-α4* results in a *rat* (resistant to *Agrobacterium* transformation) phenotype (Bhattacharjee et al., 2008). Transformation by *Agrobacterium* requires active nuclear import of the transfer DNA/protein complex (T-complex). Two *Agrobacterium* effectors, VirD2 and VirE2 are essential for plant transformation and both proteins carry NLSs, providing a molecular link between the T-complex and the host's nuclear import machinery (Gelvin, 2010; Pitzschke and Hirt, 2010). Although VirE2 and VirD2 can interact with several *Arabidopsis* α-importins, only a knock-out of *importin-α4* impairs host transformation (Bhattacharjee et al., 2008). Significantly, the *rat* phenotype is not only complemented by *importin-α4* overexpression but also by overexpression of six other *Arabidopsis α-importins*. This suggests that although importin-α4 is the most relevant NTR for the T-complex other α-importins can complement loss of importin-α4 function when their protein levels are increased. These results are in agreement with findings in yeast which show that nuclear import of different NLSs, with varying affinities for importin-α, is largely governed by the rate of NLS/importin-α complex formation (Riddick and Macara, 2005; Hodel et al., 2006; Timney et al., 2006). Thus, nuclear import rates can be elevated by either increasing protein levels of the cargo or importin-α, or by increasing the affinity of the NLS for the NTR.

HOLD ON TIGHT - NUCLEAR PATHOGEN EFFECTORS AND THE IMPORTIN-α/NLS AFFINITY CONTROVERSY

Notably, the "optimal" binding affinity of a NLS for importin-α is still controversial. Several *in vitro* studies reported dissociation constants in the low nanomolar range based on indirect affinity measurements (Hodel et al., 2001; Timney et al., 2006; Kosugi et al., 2008). Two other studies determined NLS/importin-α affinities *in vitro* by isothermal titration calorimetry and found K_d values of ∼3 and ∼48 μM, respectively (Ge et al., 2011; Lott et al., 2011). K_d values in the low nanomolar range are difficult to reconcile with the finding that *in vivo* importin-α-mediated nuclear import cannot be saturated even by ∼20-fold molar excess of NLS-cargo suggesting that the actual dissociation constants in the cytoplasm are significantly higher, possibly due to competitive binding of other cytoplasmic proteins to importin-α (Timney et al., 2006). Indeed, a non-invasive FRET/FLIM approach revealed K_d values in the low micromolar range in mammalian cells and substantiates the idea that formation of the NLS/importin-α complex in the cytoplasm is the rate-limiting event for nuclear import (Cardarelli et al., 2009). Artificial NLS peptides with extremely low K_d values interfere with dissociation of the NLS/importin-α complex in the nucleus and prevent recycling of importin-α to the cytoplasm (Kosugi et al., 2008). Consequently, these peptides inhibit nuclear import. Whether some cargo proteins with high-affinity NLS such as the cap-binding complex remain bound to importin-α in the nucleus is still matter of discussion (Görlich et al., 1996b; Dias et al., 2009, 2010).

A significant number of host-targeted pathogen effector proteins localize entirely to host cell nuclei, indicating active nuclear import or passive diffusion through the NPC and sequestration in the nucleus (Deslandes and Rivas, 2011; Caillaud et al., 2012a,b). Generally, nuclear localization correlates with NLS motifs in the primary sequence suggesting that these effectors exploit the host cell's nuclear import machinery for nuclear translocation. To what extent nuclear-targeted effectors need to compete with endogenous cargos is not understood. Effectors presumably act at relatively low protein levels to prevent activation of host defense. Given their low abundance and requirement for efficient nuclear targeting, effector NLSs might be an interesting source of high-affinity NLSs. Positioning effector NLSs within the above functional affinity limits will reveal whether pathogens evolved atypical NLS motifs that promote efficient nuclear import of effectors. Given the importance of nucleocytoplasmic transport for some immune pathways it has been hypothesized that microbial effectors might not only exploit but also manipulate or mimic components of the nuclear translocation machinery to subvert defense signaling. It is known that some animal viruses interfere with nucleocytoplasmic trafficking (Cohen et al., 2012), however, for microbial pathogens experimental proof for this hypothesis is lacking.

The reports discussed in this review substantiate the idea that tissue-specific expression, importin-α protein levels and sequence variation in the NLS binding cleft determine which importin-α functions as NTR for a cargo protein. However, more thorough analyses of plant NLS/importin-α complexes both *in vitro* and *in vivo* using emerging quantitative cell biology approaches are required to understand the complex regulation of nuclear import. Finally, many nuclear proteins do not have canonical NLS motifs. Although other import routes such as direct binding to importin-β (Marfori et al., 2011) or binding to other NTRs (Genoud et al., 2008) can account for some of these observations, the quest for novel NLSs continues.

ACKNOWLEDGMENTS

We apologize to all colleagues whose work could not be cited due to space limitations. We thank Jacqueline Monaghan for critical reading of the manuscript. Mark J. Banfield acknowledges the BBSRC (grant BB/J004553/1), the John Innes Foundation and the Gatsby Charitable Foundation for funding. Lennart Wirthmueller is supported by a FEBS long-term fellowship. Charlotte Roth and Marcel Wiermer acknowledge the Deutsche Forschungsgemeinschaft (DFG) for funding.

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J., and Parker, J. E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10306–10311.

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

Bhattacharjee, S., Lee, L., and Oltmanns, H. (2008). IMPa-4, an *Arabidopsis* importin-α isoform, is preferentially involved in *Agrobacterium*-mediated plant transformation. *Plant Cell* 20, 2661–2680.

Bhattacharya, A., and Steward, R. (2002). The *Drosophila* homolog of NTF-2, the nuclear transport factor-2, is essential for immune response. *EMBO Rep.* 3, 378–383.

Borg, M., Brownfield, L., Khatab, H., Sidorova, A., Lingaya, M., and Twell, D. (2011). The R2R3 MYB transcription factor DUO1 activates a male germline-specific regulon essential for sperm cell differentiation in *Arabidopsis*. *Plant Cell* 23, 534–549.

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

Caillaud, M.-C., Piquerez, S. J. M., Fabro, G., Steinbrenner, J., Ishaque, N., Beynon, J., et al. (2012a). Subcellular localization of the Hpa RxLR effector repertoire identifies a tonoplast-associated protein HaRxL17 that confers enhanced plant susceptibility. *Plant J.* 69, 252–265.

Caillaud, M.-C., Wirthmueller, L., Fabro, G., Piquerez, S. J. M., Asai, S., Ishaque, N., et al. (2012b). Mechanisms of nuclear suppression of host immunity by effectors from the *Arabidopsis* downy mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa). *Cold Spring Harb. Symp. Quant. Biol.* [Epub ahead of print].

Caplan, J., Padmanabhan, M., and Dinesh-Kumar, S. P. (2008). Plant NB-LRR immune receptors: from recognition to transcriptional reprogramming. *Cell Host Microbe* 3, 126–135.

Cardarelli, F., Bizzarri, R., Serresi, M., Albertazzi, L., and Beltram, F. (2009). Probing nuclear localization signal-importin alpha binding equilibria in living cells. *J. Biol. Chem.* 284, 36638–36646.

Chang, C.-W., Counago, R. L. M., Williams, S. J., Boden, M., and Kobe, B. (2012). Crystal structure of rice importin-α and structural basis of its interaction with plant-specific nuclear localization signals. *Plant Cell* 24, 5074–5088.

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.

Cohen, S., Etingov, I., and Panté, N. (2012). Effect of viral infection on the nuclear envelope and nuclear pore complex. *Int. Rev. Cell Mol. Biol.* 299, 117–159.

Conti, E., Uy, M., and Leighton, L. (1998). Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin-α. *Cell* 94, 193–204.

Cressman, D. E., O'Connor, W. J., Greer, S. F., Zhu, X. S., and Ting, J. P. (2001). Mechanisms of nuclear import and export that control the subcellular localization of class II transactivator. *J. Immunol.* 167, 3626–3634.

Deslandes, L., and Rivas, S. (2011). The plant cell nucleus: a true arena for the fight between plants and pathogens. *Plant Signal. Behav.* 6, 42–48.

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.

Dias, S. M. G., Cerione, R. A., and Wilson, K. F. (2010). Unloading RNAs in the cytoplasm. *Nucleus* 1, 139–143.

Dias, S. M. G., Wilson, K. F., Rojas, K. S., Ambrosio, A. L. B., and Cerione, R. A. (2009). The molecular basis for the regulation of the cap-binding complex by the importins. *Nat. Struct. Mol. Biol.* 16, 930–937.

Fagerlund, R., Kinnunen, L., Köhler, M., Julkunen, I., and Melén, K. (2005). NF-kB is transported into the nucleus by importin a3 and importin a4. *J. Biol. Chem.* 280, 15942–15951.

Fagerlund, R., Melen, K., Kinnunen, L., and Julkunen, I. (2002). Arginine/lysine-rich nuclear localization signals mediate interactions between dimeric STATs and importin α5. *J. Biol. Chem.* 277, 30072–30078.

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

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

Fontes, M. R., Teh, T., Jans, D., Brinkworth, R. I., and Kobe, B. (2003). Structural basis for the specificity of bipartite nuclear localization sequence binding by importin-alpha. *J. Biol. Chem.* 278, 27981–27987.

Garcia, A. V., Blanvillain-Baufume, S., Huibers, R. P., Wiermer, M., Li, G., Gobbato, E., et al. (2010). Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS Pathog.* 6:e1000970. doi: 10.1371/journal.ppat.1000970

Ge, Q., Nakagawa, T., Wynn, R. M., Chook, Y. M., Miller, B. C., and Uyeda, K. (2011). Importin-alpha protein binding to a nuclear localization signal of carbohydrate response element-binding protein (ChREBP). *J. Biol. Chem.* 286, 28119–28127.

Gelvin, S. B. (2010). Finding a way to the nucleus. *Curr. Opin. Microbiol.* 13, 53–58.

Genoud, T., Schweizer, F., Tscheuschler, A., Debrieux, D., Casal, J. J., Schäfer, E., et al. (2008). FHY1 mediates nuclear import of the light-activated phytochrome A photoreceptor. *PLoS Genet.* 4:e1000143. doi: 10.1371/journal.pgen.1000143

Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004). Importin α: a multipurpose nuclear-transport receptor. *Trends Cell Biol.* 14, 505–514.

Görlich, D., Henklein, P., Laskey, R. A., and Hartmann, E. (1996a). A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. *EMBO J.* 15, 1810–1817.

Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., et al. (1996b). Importin provides a link between nuclear protein import and U snRNA export. *Cell* 87, 21–32.

He, S., Huang, K., Zhang, X., Yu, X., Huang, P., and An, C. (2011). The LSD1-type zinc finger motifs of *Pisum sativa* LSD1 are a novel nuclear localization signal and interact with importin alpha. *PLoS ONE* 6:e22131. doi: 10.1371/journal.pone.0022131

Hodel, A. E., Harreman, M. T., Pulliam, K. F., Harben, M. E., Holmes, J. S., Hodel, M. R., et al. (2006). Nuclear localization signal receptor affinity correlates with in vivo localization in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 281, 23545–23556.

Hodel, M. R., Corbett, A. H., and Hodel, A E. (2001). Dissection of a nuclear localization signal. *J. Biol. Chem.* 276, 1317–1325.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., et al. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics* 2008, 420747.

Hu, J., Wang, F., Yuan, Y., Zhu, X., Wang, Y., Zhang, Y., et al. (2010). Novel importin-alpha family member Kpnα7 is required for normal fertility and fecundity in the mouse. *J. Biol. Chem.* 285, 33113–33122.

Huang, J.-G., Yang, M., Liu, P., Yang, G.-D., Wu, C.-A., and Zheng, C.-C. (2010). Genome-wide profiling of developmental, hormonal or environmental responsiveness of the nucleocytoplasmic transport receptors in *Arabidopsis*. *Gene* 451, 38–44.

Huang, T. T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000). A nuclear export signal in the N-terminal regulatory domain of IκBα controls cytoplasmic localization of inactive NF-κB/IκBαcomplexes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1014–1019.

Jiang, C. J., Shoji, K., Matsuki, R., Baba, A., Inagaki, N., Ban, H., et al. (2001). Molecular cloning of a novel importin alpha homologue from rice, by which Constitutive Photomorphogenic 1 (COP1) nuclear localization signal (NLS)-protein is preferentially nuclear imported. *J. Biol. Chem.* 276, 9322–9329.

Johnson, C., Van Antwerp, D., and Hope, T. J. (1999). An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IκBα. *EMBO J.* 18, 6682–6693.

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

Kaminaka, H., Näke, C., Epple, P., Dittgen, J., Schütze, K., Chaban, C., et al. (2006). bZIP10-LSD1 antagonism modulates basal defense and cell death in *Arabidopsis* following infection.*EMBO J.* 25, 4400–4411.

Kanneganti, T.-D. D., Bai, X., Tsai, C.-W. W., Win, J., Meulia, T., Goodin, M., et al. (2007b). A functional genetic assay for nuclear trafficking in plants. *Plant J.* 50, 149–158.

Kanneganti, T. D., Lamkanfi, M., and Nunez, G. (2007a). Intracellular

NOD-like receptors in host defense and disease. *Immunity* 27, 549–559.

Kelley, J. B., Talley, A. M., Spencer, A., Gioeli, D., and Paschal, B. M. (2010). Karyopherin alpha7 (KPNA7), a divergent member of the importin alpha family of nuclear import receptors. *BMC Cell Biol.* 11:63. doi: 10.1186/1471-2121-11-63

Kinkema, M., Fan, W. H., and Dong, X. N. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350.

Kobe, B. (1999). Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin alpha. *Nat. Struct. Biol.* 6, 388–397.

Köhler, M., Ansieau, S., Prehn, S., Leutz, A., Haller, H., and Hartmann, E. (1997). Cloning of two novel human importin-α subunits and analysis of the expression pattern of the importin-α protein family. *FEBS Lett.* 417, 104–108.

Köhler, M., Speck, C., Christiansen, M., Bischoff, R., Prehn, S., Haller, H., et al. (1999). Evidence for distinct substrate specificities of importin α family members in nuclear protein import evidence for distinct substrate specificities of importin α family members in nuclear protein import. *Mol. Cell Biol.* 19, 7782–7791.

Kosugi, S., Hasebe, M., Entani, T., Takayama, S., Tomita, M., and Yanagawa, H. (2008). Design of peptide inhibitors for the importin αβ nuclear import pathway by activity-based profiling. *Chem. Biol.* 15, 940–949.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948.

Lim, C. P., and Cao, X. (2006). Structure, function, and regulation of STAT proteins. *Mol. Biosyst.* 2, 536–550.

Liu, J., and Coaker, G. (2008). Nuclear trafficking during plant innate immunity. *Mol. Plant* 1, 411–422.

Liu, L., McBride, K. M., and Reich, N. C. (2005). STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-α3. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8150–8155.

Lott, K., Bhardwaj, A., Sims, P. J., and Cingolani, G. (2011). A minimal nuclear localization signal (NLS) in human phospholipid scramblase 4 that binds only the minor NLS-binding site of importin alpha1. *J. Biol. Chem.* 286, 28160–28169.

Maekawa, T., Kufer, T. A., and Schulze-Lefert, P. (2011). NLR functions in plant and animal immune systems: so far and yet so close. *Nat. Immunol.* 12, 817–826.

Malek, S., Chen, Y., Huxford, T., and Ghosh, G. (2001). IkBβ, but not IkBα, functions as a classical cytoplasmic inhibitor of NF-κB dimers by masking both NF-κB nuclear localization sequences in resting cells. *J. Biol. Chem.* 276, 45225–45235.

Marfori, M., Lonhienne, T. G., Forwood, J. K., and Kobe, B. (2012). Structural basis of high-affinity nuclear localization signal interactions with importin-α. *Traffic* 13, 532–548.

Marfori, M., Mynott, A., Ellis, J. J., Mehdi, A. M., Saunders, N. F. W., Curmi, P. M., et al. (2011). Molecular basis for specificity of nuclear import and prediction of nuclear localization. *Biochim. Biophys. Acta* 1813, 1562–1577.

Matsuura, Y., and Stewart, M. (2004). Structural basis for the assembly of a nuclear export complex. *Nature* 432, 872–877.

Meier, I., and Somers, D. E. (2011). Regulation of nucleocytoplasmic trafficking in plants. *Curr. Opin. Plant Biol.* 14, 538–546.

Meissner, T. B., Li, A., Liu, Y. J., Gagnon, E., and Kobayashi, K. S. (2012). The nucleotide-binding domain of NLRC5 is critical for nuclear import and transactivation activity. *Biochem. Biophys. Res. Commun.* 418, 786–791.

Melén, K., Fagerlund, R., Franke, J., Kohler, M., Kinnunen, L., and Julkunen, I. (2003). Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein. *J. Biol. Chem.* 278, 28193–28200.

Melén, K., Kinnunen, L., and Julkunen, I. (2001). Arginine/lysine-rich structural element is involved in interferon-induced nuclear import of STATs. *J. Biol. Chem.* 276, 16447–16455.

Merkle, T. (2011). Nucleo-cytoplasmic transport of proteins and RNA in plants. *Plant Cell Rep.* 30, 153–176.

Meyers, B. C., Lee, D. K., Vu, T. H., Tej, S. S., Edberg, S. B., Matvienko, M., et al. (2004). *Arabidopsis* MPSS. An online resource for quantitative expression analysis 1 [w]. *Analysis* 135, 801–813.

Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., et al. (1997). Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors. *J. Biol. Chem.* 272, 26375–26381.

Mou, Z., Fan, W. H., Dong, X. N., and Carolina, N. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944.

Nadler, S. G., Tritschler, D., Haffar, O. K., Blake, J., Bruce, A. G., and Cleaveland, J. S. (1997). Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences. *J. Biol. Chem.* 272, 4310–4315.

Nürnberger, 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.

Ouyang, S., Zhu, W., Hamilton, J., Lin, H., Campbell, M., Childs, K., et al. (2007). The TIGR rice genome annotation resource: improvements and new features. *Nucleic Acids Res.* 35, D883–D887.

Palma, K., Zhang, Y., and Li, X. (2005). An importin alpha homolog, MOS6, plays an important role in plant innate immunity. *Curr. Biol.* 15, 1129–1135.

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

Pitzschke, A., and Hirt, H. (2010). New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. *EMBO J.* 29, 1021–1032.

Quensel, C., Friedrich, B., Sommer, T., Hartmann, E., and Kohler, M. (2004). In vivo analysis of importin α proteins reveals cellular proliferation inhibition and substrate specificity. *Mol. Cell. Biol.* 24, 10246–10255.

Ratan, R., Mason, D. A., Sinnot, B., Goldfarb, D. S., and Fleming, R. J. (2008). *Drosophila* importin alpha1 performs paralog-specific functions essential for gametogenesis. *Genetics* 178, 839–850.

Ribbeck, K., Lipowsky, G., Kent, H. M., Stewart, M., and Gorlich, D. (1998). NTF2 mediates nuclear import of Ran. *EMBO J.* 17, 6587–6598.

Riddick, G., and Macara, I. G. (2005). A systems analysis of importin-α-βmediated nuclear protein import. *J. Cell Biol.* 168, 1027–1038.

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

Shen, Q. H., and Schulze-Lefert, P. (2007). Rumble in the nuclear jungle: compartmentalization, trafficking, and nuclear action of plant immune receptors. *EMBO J.* 26, 4293–4301.

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, 4195–4115.

Smith, A., Brownawell, A., and Macara, I. G. (1998). Nuclear import of Ran is mediated by the transport factor NTF2. *Curr. Biol.* 8, 1403–1406.

Spilianakis, C., Papamatheakis, J., and Kretsovali, A. (2000). Acetylation by PCAF enhances CIITA nuclear accumulation and transactivation of major histocompatibility complex class II genes. *Mol. Cell. Biol.* 20, 8489–8498.

Stewart, M. (2007). Molecular mechanism of the nuclear protein import cycle. *Nat. Rev. Mol. Cell Biol.* 8, 195–208.

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

Tameling, W. I. L., 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.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.

Terry, L. J., Shows, E. B., and Wente, S. R. (2007). Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. *Science* 318, 1412–1416.

Timney, B. L., Tetenbaum-Novatt, J., Agate, D. S., Williams, R., Zhang, W., Chait, B. T., et al. (2006). Simple kinetic relationships and non-specific competition govern nuclear import rates in vivo. *J. Cell Biol.* 175, 579–593.

Tsuji, L., Takumi, T., Imamoto, N., and Yoneda, Y. (1997). Identification of novel homologues of mouse

importin α, the α subunit of the nuclear pore-targeting complex, and their tissue-specific expression. *FEBS Lett.* 416, 30–34.

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

Wirthmueller, L., Zhang, Y., Jones, J. D. G., 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.

Yano, R., Oakes, M., Yamaghishi, M., Dodd, J. A., and Nomura, M. (1992). Cloning and characterization of SRPI, a suppressor of temperature-sensitive RNA polymerase I mutations, in *Saccharomyces cerevisiae.Mol. Cell. Biol.* 12, 5640–5651.

Yasuhara, N., Shibazaki, N., Tanaka, S., Nagai, M., Kamikawa, Y., Oe, S., et al. (2007). Triggering neural differentiation of ES cells by subtype switching of importin-alpha. *Nat. Cell Biol.* 9, 72–79.

Zhu, Y., Qian, W., and Hua, J. (2010). Temperature modulates plant defense responses through NB-LRR proteins. *PLoS Pathog.* 6:e1000844. doi: 10.1371/journal.ppat.1000844

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

Received: 04 February 2013; accepted: 02 May 2013; published online: 21 May 2013.

Citation: Wirthmueller L, Roth C, Banfield MJ and Wiermer M (2013) Hop-on hop-off: importin-α-guided tours to the nucleus in innate immune signaling. Front. Plant Sci. ***4****:149. doi: 10.3389/fpls.2013.00149*

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

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 13 March 2013
doi: 10.3389/fpls.2013.00053

Plant parasitic nematode effectors target host defense and nuclear functions to establish feeding cells

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Plant parasitic nematodes are microscopic worms, the most damaging species of which have adopted a sedentary lifestyle within their hosts. These obligate endoparasites have a biotrophic relationship with plants, in which they induce the differentiation of root cells into hypertrophied, multinucleate feeding cells (FCs). Effectors synthesized in the esophageal glands of the nematode are injected into the plant cells via the syringe-like stylet and play a key role in manipulating the host machinery. The establishment of specialized FCs requires these effectors to modulate many aspects of plant cell morphogenesis and physiology, including defense responses. This cell reprogramming requires changes to host nuclear processes. Some proteins encoded by parasitism genes target host nuclei. Several of these proteins were immunolocalized within FC nuclei or shown to interact with host nuclear proteins. Comparative genomics and functional analyses are gradually revealing the roles of nematode effectors. We describe here these effectors and their hypothesized roles in the unique feeding behavior of these pests.

Keywords: root-knot nematodes, cyst nematodes, effectors, plant nuclei, feeding cells

INTRODUCTION

Plant parasitic nematodes (PPNs) are small roundworms comprising about 4,000 species infesting roots of thousands of plant species and causing tremendous crop yield losses worldwide (Blok et al., 2008). The sedentary endoparasites, root-knot nematodes, (RKNs, *Meloidogyne* spp.) and cyst nematodes (CNs, *Globodera* spp. and *Heterodera* spp.), are among the most economically damaging PPNs. These parasites are obligate biotrophs that can feed only on the cytoplasm of living cells. Thus, both RKNs and CNs establish an intimate relationship with their host plants, inducing the redifferentiation of root cells into specialized multinucleate feeding cells (FCs). RKNs cause the formation and maintenance of five to seven giant cells, whereas CNs induce a syncytium (**Figure 1**). The first sign of giant cell induction by RKNs is the formation of one or several binucleate cells. These cells then go on to become multinucleate, through repeated nuclear divisions (karyokinesis) without cell division (Caillaud et al., 2008b). The hyperplasia and hypertrophy of the surrounding cells lead to the formation of the typical gall. In syncytia, the initial FC expands into the vascular tissue by the progressive and local dissolution of cell walls, resulting in the fusion of hundreds of neighboring root cells (Sobczak and Golinowski, 2011).

Fully differentiated FC is several hundred times the size of a normal root vascular cell. The cell walls thicken and ingrowths develop, facilitating solute exchange across the FC plasma membrane and sustaining nematode feeding until adult stages. Within the dense cytoplasm of the developing FC, subcellular organelles proliferate, their nuclei and nucleoli enlarge, and small secondary vacuoles are formed (**Figure 1**). FCs constitute the sole source of nutrients for the nematodes and are essential for their growth and reproduction. The complex changes in cellular morphology and physiology leading to FC establishment result from extensive changes to gene expression in the infected root cells. Patterns of host gene transcription have been compared by various techniques (Gheysen and Fenoll, 2002; Caillaud et al., 2008a), including, in particular, the recent genome-wide expression profiling of isolated giant cells or syncytia (Szakasits et al., 2009; Barcala et al., 2010; Damiani et al., 2012). These studies have led to the identification of many genes involved in diverse processes, such as cell cycle activation, cell wall modification, hormone and defense responses that are differentially expressed in FC formation. It remains unclear how this developmental switch allowing the nematodes to settle and resulting in changes to root cell morphology and the induction of FC occurs. However, it is now widely accepted that secreted nematode effectors play key roles in parasitism.

One of the characteristic features of PPNs is the presence of specialized esophageal gland cells allowing the production of proteins that are then secreted into the host through a hollow protrusible syringe-like stylet. The activity of these esophageal glands is developmentally regulated. Two subventral glands are particularly active during the preparasitic stages, secreting proteins involved in root invasion and larva migration, whereas a dorsal gland becomes hypertrophied and actively secretes effectors responsible for FC initiation and maintenance during the sedentary stages (Davis et al., 2008). In addition, proteins thought to be involved in parasitism are secreted into the apoplasm through the cuticle or via the chemosensory organs, the amphids. Various strategies have been used to identify nematode effectors. Proteomic approaches have been applied to purified *M. incognita* secretions (Bellafiore et al., 2008). Transcriptomic approaches, benefiting from recent advances in next-generation sequencing technologies, have made it possible to generate CN and RKN expressed sequence tags (ESTs) from various juvenile developmental stages

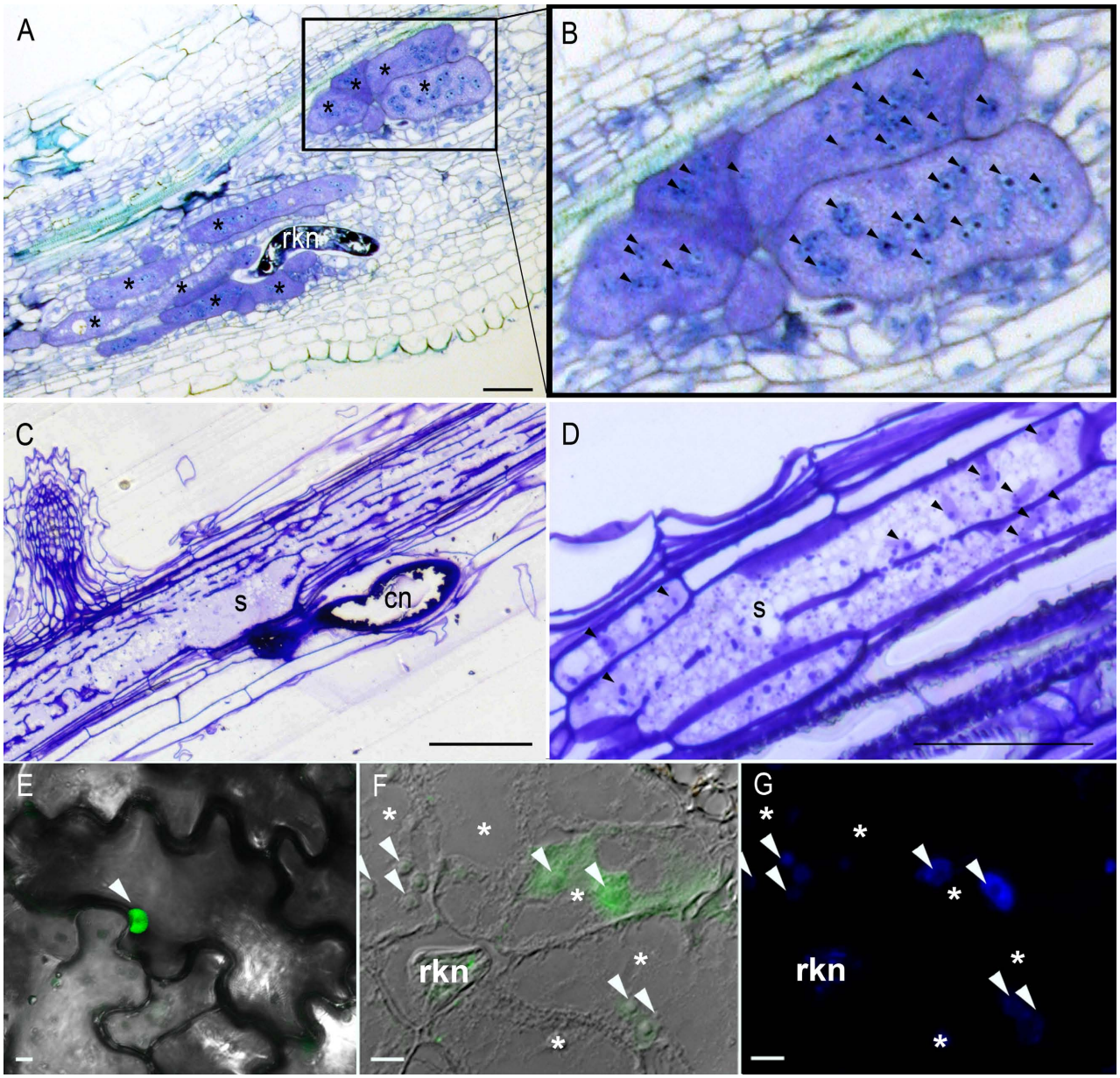

FIGURE 1 | Multinucleate and hypertrophied feeding cells induced by endoparasitic plant nematodes and nuclear localization of a RKN effector *in planta*. (A) Giant cells induced by the root-knot nematode *Meloidogyne chitwoodi* in pepper. **(B)** Multiple nuclei (arrowheads) are visible in the giant cells. **(C,D)** Syncytium induced by the cyst nematode *H. schachtii* in *Arabidopsis*. Cell wall dissolution results in a multinucleate cell. Toluidine blue-stained **(A,B)** or Crystal Violet-stained **(C,D)** longitudinal sections of infected roots 7 **(A,B)** or 10 **(C,D)** days after inoculation. **(E)** RKN effector MiEFF1::GFP accumulates in the nucleus (arrowhead) of tobacco epidermal leaf cells after agroinfiltration. **(F)** Immunolocalization of the secreted MiEFF1 in tomato galls 14 days after inoculation. FITC signal is observed at the tip of the stylet of a sedentary parasitic juvenile and in the nuclei (arrowheads) of giant cells. **(G)** DAPI-staining of nuclei of the section presented in **(F)**. rkn, root-knot nematode; *, giant cells; s, syncytium; cn, cyst nematode. Bars = 50 μm **(A–D)** or 10 μm **(E–G)**.

(Roze et al., 2008; Jones et al., 2009; Jaouannet et al., 2012; Haegeman et al., 2013), infected plant tissues (Haegeman et al., 2013), or microaspiration of the cytoplasmic content of the esophageal glands (Wang et al., 2001; Gao et al., 2003; Huang et al., 2004) or isolated whole glands (Maier et al., 2013). Finally, comparative genomics approaches have facilitated a major breakthrough in effector identification. Two RKN genomes are now available, for *M. incognita* and *M. hapla* (Abad et al., 2008; Opperman et al., 2008) and increasing amounts of genomic information are being released for the soybean CN *H. glycines* and the potato CN *G. pallida*. The increasing availability of such data has led to the prediction of large effector repertoires. *In situ* hybridization studies have confirmed the specific expression of several of these putative effectors in the esophageal glands, suggesting their probable

secretion into the host via the stylet and, thus, a role in infection. The identification of these effectors has made it possible to initiate functional analyses, which should make it possible to decipher the roles of these proteins in the targeting and manipulation of host functions (Haegeman et al., 2012; Hewezi and Baum, 2013). In this review, we describe nematode effectors that interact with host proteins or mimic host proteins, manipulating various aspects of plant physiology, including plant defense responses, and others that are targeted to the nucleus, where they may manipulate the nuclear machinery or bind to nucleotides (**Table 1**).

NEMATODE EFFECTORS HIJACK KEY CELLULAR FUNCTIONS

It remains unclear whether the nematode stylet perforates both the cell wall and the plasma membrane, to deliver effectors directly to the cytoplasm of the host cells. The apoplasm appears to be a major target of nematode effectors (Rosso et al., 2011; Vieira et al., 2011). However, nematode effectors may also be located within the host cells, where they may target different subcellular domains and assume highly diverse cellular functions (Haegeman et al., 2012; Hewezi and Baum, 2013). The first secreted proteins from PPNs to be characterized were cell wall-degrading and cell wall-modifying enzymes, such as β-1,4-endoglucanases, pectate lyases, polygalacturonases, and expansins, which are involved, in particular, in the invasion of root tissues by preparasitic juveniles and the migration of nematodes (Davis et al., 2011). These enzymes may also play an important role in FC formation, supporting the tremendous expansion of RKN-induced giant cells and facilitating syncytium formation. Effectors also target host enzymes to potentiate their function. Indeed, a *H. schachtii* (Hs) cellulose-binding protein, HsCBP, interacts with an *Arabidopsis* pectin methylesterase, potentially promoting the activity of this pectin-modifying enzyme or rendering cell wall polymers more accessible to other wall-degrading enzymes (Hewezi et al., 2008).

The *de novo* organogenesis underlying the construction of a nematode feeding site has a major impact on cell morphology and function as described above. Effectors mimicking plant compounds or binding to host proteins have been characterized. These molecules can affect plant signaling, hormone balance, and cell morphogenesis. The CNs secrete active CLAVATA3/ESR (CLE)-like proteins (Wang et al., 2005, 2011). In plants, CLE-like peptides play an essential role in meristem differentiation. These effectors seem to be secreted into the cytoplasm of host cells, from which they are transported to the plant apoplasm, where they mimic plant CLE signaling peptides and interact at the plasma

Table 1 | Nematode effectors mentioned in this review, that target host functions to establish feeding cells.

Effector	Predicted function	Host function	Identified plant target	Reference
***Globodera* spp.**				
GpRBP-1	SPRYSEC	Defense	NB-LRR-resistant protein potato GPA-2	Sacco et al. (2009)
GpCM	Chorismate mutase	Hormone and/or defense	–	Jones et al. (2003)
GrVAP1	Venom allergen protein	Defense	Papain-like cysteine protease Rcr3^{pim}	Lozano-Torres et al. (2012)
GrSPRYSEC-19	SPRYSEC	Defense	NB-LRR protein tomato SW5F	Rehman et al. (2009)
GrCLE1	CLE-like peptide	Hormone	Receptors AtCLV2 and AtBAM1 and 2	Guo et al. (2011)
***Heterodera* spp.**				
HgSYV46	CLE-like peptide	Hormone	–	Wang et al. (2005)
Hg30C02	Unknown	Defense	β-1,3-endoglucanase	Hamamouch et al. (2012)
HgCM	Chorismate mutase	Hormone and/or defense	–	Bekal et al. (2003)
HsCM	Chorismate mutase	Hormone and/or defense	–	Vanholme et al. (2009)
HsCBP	Cellulose-binding protein	Cell wall	Pectin methylesterase AtPME3	Hewezi et al. (2008)
HsCLE1 and 2	CLE-like peptide	Hormone	–	Wang et al. (2011)
Hs19C07	Unknown	Hormone	Plasma membrane auxin influx transporter AtLAX3	Lee et al. (2011)
Hs10A06	Unknown	Defense	Spermidine synthase AtSPDS2	Hewezi et al. (2010)
Hs4F01	Annexin-like	Defense	Oxidoreductase of the 2OG-Fe(II) oxygenase family	Patel et al. (2010)
HsUbil	Ubiquitin extension protein	Synthesis	–	Tytgat et al. (2004)
***Meloidogyne* spp.**				
MiCM	Chorismate mutase	Hormone and/or defense	–	Huang et al. (2005)
Mi8D05	Unknown	Transport	Tonoplast intrinsic protein AtTIP2	Xue et al. (2013)
MiCRT	Calreticulin	Defense	–	Jaouannet et al. (2013)
Mi16D10	CLE-like peptide	Transcription	Scarecrow-like transcription factor AtSCL6 and 11	Huang et al. (2006)
MiEFF1	Unknown	Unknown	–	Jaouannet et al. (2012)
MjNULG1	Unknown	Unknown	–	Lin et al. (2013)

membrane with leucine-rich repeat (LRR) receptor kinase family proteins, resulting in the formation and maintenance of syncytia (Guo et al., 2011). Another example of an effector having an impact on FC formation through the manipulation of host physiology is provided by Hs19C07, an effector that may modify hormone balance (Lee et al., 2011). Indeed, Hs19C07 interacts with the *Arabidopsis* plasma membrane auxin influx transporter LAX3, which modulates auxin influx in syncytia, thereby facilitating their development. Furthermore, both CNs and RKNs secrete proteins homologous to plant chorismate mutases (Bekal et al., 2003; Jones et al., 2003; Huang et al., 2005; Vanholme et al., 2009). The overexpression of nematode chorismate mutases *in planta* alters root growth (Doyle and Lambert, 2003), and it has been suggested that these effectors affect the auxin pool within the host cells. The recently characterized *M. incognita* effector Mi8D05 affects a different function of plant cells (Xue et al., 2013). Mi8D05 is essential for parasitism, as revealed by RNAi and overexpression approaches, and the overproduction of this effector strongly stimulates the growth of plant shoots. This effector has been shown to interact with a plant aquaporin tonoplast intrinsic protein (TIP2), suggesting a role in the regulation of solute and water transport within giant cells, promoting giant cell enlargement and nematode feeding.

Plants protect themselves against pathogen attacks through a combination of constitutive and induced strategies. The induction of plant defenses involves the recognition of compounds derived from the pathogen, called pathogen-associated molecular patterns (PAMPs). Pattern-triggered immunity (PTI) results from PAMP perception, leading to the activation of signaling pathways that restrict pathogen growth and promote host disease resistance (Jones and Dangl, 2006). No PAMPs have been described in nematodes, but secreted proteins and products of cell wall degradation may be recognized as such. Transcriptomic analysis has shown that a massive down-regulation of genes involved in plant defense is associated with the early stages of plant–nematode interaction (Jammes et al., 2005; Barcala et al., 2010; Damiani et al., 2012). This suggests that PPNs can suppress PTI. Various effectors that affect plant stress and defense responses have, indeed, been characterized (Smant and Jones, 2011). The nematode chorismate mutases mentioned above affect the plant shikimate pathway, thereby decreasing the synthesis of salicylic acid and phytoalexin through competition with chorismate, and preventing the triggering of host defense (Doyle and Lambert, 2003). Hs10A06 effector targets *Arabidopsis* spermidine synthase 2. Plants overproducing Hs10A06 are more susceptible to CNs and to bacterial and viral pathogens and produce smaller amounts of pathogenesis-related (PR) proteins. Hs10A06 acts on salicylic acid signaling and the antioxidant machinery, thereby protecting nematodes against plant defense responses (Hewezi et al., 2010). Similarly, the Hs4F01 annexin-like effector is secreted into the cytosol (Patel et al., 2010), where it interacts with an oxidoreductase of the 2OG-Fe(II) oxygenase family to prevent the triggering of host defense. Another CN effector, Hg30C02, interacts physically with a plant β-1,3-endoglucanase, a potential PR protein, and may thus be involved in defense suppression (Hamamouch et al., 2012). However, only *M. incognita* calreticulin (Mi-CRT), which suppresses defences induced by the PAMP elf18 when expressed *in planta,* has been shown to have a direct effect on PTI suppression (Jaouannet et al., 2013).

Plants have evolved resistance proteins that can recognize, either directly or indirectly, pathogen effectors, and induce effector-triggered immunity (ETI; Jones and Dangl, 2006). Several plant proteins conferring resistance to nematodes have been identified, mostly nucleotide-binding LRR (NB-LRR) proteins. However, very few nematode avirulence effectors have been identified (Smant and Jones, 2011). The CN effectors repertoire include a large family of secreted effectors containing a SPRY domain, named SPRYSECs (Jones et al., 2009; Rehman et al., 2009). One cytoplasmic *G. pallida* SPRYSEC (GpRBP-1) has been shown to be the avirulence target of the GPA-2 NB-LRR-resistant protein (Sacco et al., 2009). Remarkably, the GrSPRYSEC-19 protein has been shown to suppress the ETI mediated by several NB-LRR-resistant proteins, including GPA-2 (Postma et al., 2012). However, SPRYSEC-19 does not seem to mediate nematode resistance, despite interacting physically with SW5F, an SW5 NB-LRR-resistant protein from tomato (Rehman et al., 2009).

NEMATODE EFFECTORS TARGET HOST CELL NUCLEI

The manipulation of host cell processes, such as the cell cycle, gene expression, and immunity, almost certainly involves the targeting of the host nucleus by secreted effectors. Bioinformatic analyses of predicted effectors have revealed the presence of nuclear localisation signals (NLSs) in several secreted proteins from both CNs (Gao et al., 2003; Elling et al., 2007) and RKN (Huang et al., 2003; Roze et al., 2008), potentially allowing nuclear import. Proteomic studies have identified 486 proteins secreted by *M. incognita*, 66 of which were found to have a putative NLS, or DNA-binding or chromatin-binding domains (Bellafiore et al., 2008).

The use of green fluorescent protein (GFP)-fusions in transient expression assays has confirmed the nuclear localization of some of these effectors within plant cells (Elling et al., 2007; Jones et al., 2009). For instance, MiEFF1 is a small secreted protein of 122 amino acids (aa), with no predicted homologs in databases and no known functional domain. It has a NLS and localizes to the nucleus when transiently expressed in tobacco cells (**Figure 1E**; Jaouannet et al., 2012). Interestingly, some effectors are found in the cytoplasm when their full-length forms are produced *in planta,* but their truncated forms have a nuclear or nucleolar distribution, suggesting that they may be relocalized after modification of the protein within the host cell (Tytgat et al., 2004; Elling et al., 2007). In this way, the putative ubiquitin extension protein HsUbiI is delivered to the host cell cytoplasm, and the cleavable C-terminal domain of the protein is directed to the nucleolus, where it may be involved in ribosome synthesis and parasitism (Tytgat et al., 2004).

Recently, immunolocalization approaches have shown two RKN effectors to be effectively delivered to giant cell nuclei (Jaouannet et al., 2012; Lin et al., 2013). Immunostainings demonstrate MiEFF1 is produced in the dorsal esophageal gland of the nematode and is secreted through the stylet into the giant cells, in which it is transported into the nuclei (**Figures 1F,G**; Jaouannet et al., 2012). Similarly, *M. javanica* MjNULG1a is a 274 aa protein of unknown function with two predicted NLS localizing in FC nuclei. Transgenic plants overproducing MjNULG1a are more

sensitive to RKN, and RNAi studies *in planta* have provided evidence of a role for this protein in nematode parasitism (Lin et al., 2013). Both MiEFF1 and MjNULG1a seem to be specific to early steps in parasitism, but it remains unclear whether these effectors are involved in giant cell formation. Identification of the host cell targets of these proteins is underway and should shed light on their functions.

Very few host targets of nematode effectors that could form part of the host nuclear machinery corrupted to promote parasitism have been identified to date with yeast two-hybrid approaches. The *M. incognita* effector Mi16D10, which encodes a novel 13-amino acid secretory peptide, appears to be important for nematode development, as shown by RNAi approaches, and it favors root growth when produced *in planta*. Two plant SCARECROW-like transcription factors that interact with the Mi16D10 protein have been identified (Huang et al., 2003, 2006). In plants, these transcription factors play a key role in regulating root meristem identity and root development, and Mi16D10 may thus function in the extensive transcriptional reprogramming responsible for FC ontogenesis. The CN effector Hs10A07 contains a NLS, but is generally located in the cytoplasm when produced in plant cells (Elling et al., 2007). However, Hs10A07 is translocated from the cytoplasm to the nucleus following its interaction with a specific *Arabidopsis* protein kinase. Once inside the nucleus, this Hs10A07 effector interacts with transcriptional regulators and plays a role in parasitism (Hewezi and Baum, 2013). The Hs32E03 effector is located in the nucleus following the transient expression of its gene in plant cells (Elling et al., 2007). During parasitism, this effector interacts with nuclear proteins, leading to its localization in nuclear bodies (Hewezi and Baum, 2013). However, it remains unclear how this particular pattern of nuclear localization promotes parasitism. Finally, some SPRYSEC proteins localize to the nucleus of plant cells when transiently produced *in planta* (Jones et al., 2009), and bimolecular fluorescence complementation assay have confirmed that SPRYSEC-19 interacts strongly with the tomato SW5F-resistant proteins in infiltrated tobacco cell nucleoli (Postma et al., 2012). The putative function of this interaction remains unknown, but it does not appear to be involved in the ETI suppression mediated by SPRYSEC-19.

CONCLUSION AND PERSPECTIVES

The identification of effectors is a major challenge in our understanding of the molecular aspects of plant–nematode interactions. Tremendous progress has been made toward the building of nematode effector repertoires since the completion of several genome sequencing. We still know little about the functions of these effectors and the host processes manipulated during the interaction to mediate the transformation of root cells into hypertrophied and multinucleate FCs. Functional characterization will be required to improve our understanding of the way in which these effectors promote host plant parasitism. Transformation procedures are currently lacking for PPNs, but such functional analysis should benefit from the recent development of RNAi approaches (Rosso et al., 2009), effector immunocytochemistry and the cellular imaging of feeding sites (Vieira et al., 2012a,b). A major breakthrough will result from identification of the plant targets of these effectors. Efforts to develop high-throughput approaches for such screening are already underway.

Host plant proteins targeted by effectors from many plant pathogenic microorganisms are being identified. It will be of particular interest to determine whether there are conserved parasitism strategies and whether nematodes and other plant pathogens target similar proteins. As obligate biotrophic parasites, nematodes must protect themselves against plant defenses and protect the host cells they need for feeding. PPNs may therefore target key components of the plant immune system corrupted during other plant–pathogen interactions. The GrVAP1 of CNs, Avr2 from the fungus *Cladosporium fulvum* and the EPIC1 and EPIC2B effectors from the oomycete *Phytophthora infestans* all target the same host papain-like cysteine protease, $Rcr3^{pim}$ (Lozano-Torres et al., 2012), the tomato Cf-2 protein mediating resistance to *G. rostochiensis* and *C. fulvum* in a $Rcr3^{pim}$-dependent manner.

Plant pathogens seem to target the plant nuclear machinery during infection (Bierne and Cossart, 2012; Caillaud et al., 2012; Deslandes and Rivas, 2012). Recent studies have shown that nematode effectors may indeed be localized to host nuclei or interact with host nuclear proteins. In addition, Hewezi and Baum (2013) have suggested that CN effectors recruit proteins involved in nucleocytoplasmic movement and nuclear dynamic during the parasitization of their hosts. These processes play an important role in several plant–pathogen interactions (Wiermer et al., 2007; Rivas, 2012). The identification of nematode effectors likely to bind DNA directly and affect host gene expression remains a major challenge. The molecular characterization of effectors and their plant targets is a key step toward understanding the factors determining nematode virulence, plant susceptibility or immunity and host range, and will open up new perspectives for controlling nematodes and other agronomically important pathogens.

ACKNOWLEDGMENTS

We wish to thank Miroslaw Sobczak (Warsaw University of Life Sciences) for the syncytium pictures, Caroline Djian-Caporalino for the giant cells images, and Maëlle Jaouannet and Marc Magliano for MiEFF1 immunolocalization pictures. Our work is funded by the French ANR-08-GENM-014 (SCRIPS) and ANR PCS-08-GENO-166 (NEMATARGET), and carried out in the SIGNALIFE Laboratoire d'Excellence (LabEx).

REFERENCES

Abad, P., Gouzy, J., Aury, J.-M., Castagnone-Sereno, P., Danchin, E. G. J., Deleury, E., et al. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nat. Biotech.* 26, 909–915.

Barcala, M., Garcia, A., Cabrera, J., Casson, S., Lindsey, K., Favery, B., et al. (2010). Early transcriptomic events in microdissected *Arabidopsis* nematode-induced giant cells. *Plant J.* 61, 698–712.

Bekal, S., Niblack, T. L., and Lambert, K. N. (2003). A chorismate mutase from the soybean cyst nematode *Heterodera glycines* shows polymorphisms that correlate with virulence. *Mol. Plant Microbe Interact.* 16, 439–446.

Bellafiore, S., Shen, Z., Rosso, M.-N., Abad, P., Shih, P., and Briggs, S. P. (2008). Direct Identification of the *Meloidogyne incognita* secretome reveals proteins with host cell reprogramming potential. *PLoS Pathog.* 4:e1000192. doi: 10.1371/journal.ppat.1000192

Bierne, H., and Cossart, P. (2012). When bacteria target the nucleus: the emerging family of

nucleomodulins. *Cell. Microbiol.* 14, 622–633.

Blok, V. C., Jones, J. T., Phillips, M. S., and Trudgill, D. L. (2008). Parasitism genes and host range disparities in biotrophic nematodes: the conundrum of polyphagy versus specialisation. *Bioessays* 30, 249–259.

Caillaud, M.-C., Dubreuil, G., Quentin, M., Perfus-Barbeoch, L., Lecomte, P., de Almeida Engler, J., et al. (2008a). Root-knot nematodes manipulate plant cell functions during a compatible interaction. *J. Plant Physiol.* 165, 104–113.

Caillaud, M.-C., Lecomte, P., Jammes, F., Quentin, M., Pagnotta, S., Andrio, E., et al. (2008b). MAP65-3 microtubule-associated protein is essential for nematode-induced giant cell ontogenesis in *Arabidopsis*. *Plant Cell* 20, 423–437.

Caillaud, M.-C., Wirthmueller, L., Fabro, G., Piquerez, S. J. M., Asai, S., Ishaque, N., et al. (2012). Mechanisms of nuclear suppression of host immunity by effectors from the *Arabidopsis* downy mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa). *Cold Spring Harb. Symp. Quant. Biol.* doi: 10.1101/sqb.2012.1177.015115 [Epub ahead of print].

Damiani, I., Baldacci-Cresp, F., Hopkins, J., Andrio, E., Balzergue, S., Lecomte, P., et al. (2012). Plant genes involved in harbouring symbiotic rhizobia or pathogenic nematodes. *New Phytol.* 194, 511–522.

Davis, E., Haegeman, A., and Kikuchi, T. (2011). "Degradation of the plant cell wall by nematodes," in *Genomics and Molecular Genetics of Plant-Nematode Interactions*, eds J. Jones, G. Gheysen, and C. Fenoll (Berlin: Springer), 225–272.

Davis, E. L., Hussey, R. S., Mitchum, M. G., and Baum, T. J. (2008). Parasitism proteins in nematode–plant interactions. *Curr. Opin. Plant Biol.* 11, 360–366.

Deslandes, L., and Rivas, S. (2012). Catch me if you can: bacterial effectors and plant targets. *Trends Plant Sci.* 17, 644–655.

Doyle, E. A., and Lambert, K. N. (2003). *Meloidogyne javanica* chorismate mutase 1 alters plant cell development. *Mol. Plant Microbe Interact.* 16, 123–131.

Elling, A. A., Davis, E. L., Hussey, R. S., and Baum, T. J. (2007). Active uptake of cyst nematode parasitism proteins into the plant cell nucleus. *Int. J. Parasitol.* 37, 1269–1279.

Gao, B., Allen, R., Maier, T., Davis, E. L., Baum, T. J., and Hussey, R. S. (2003). The parasitome of the phytonematode *Heterodera glycines*. *Mol. Plant Microbe Interact.* 16, 720–726.

Gheysen, G., and Fenoll, C. (2002). Gene expression in nematode feeding sites. *Annu. Rev. Phytopathol.* 40, 191–219.

Guo, Y., Ni, J., Denver, R., Wang, X., and Clark, S. E. (2011). Mechanisms of molecular mimicry of plant CLE peptide ligands by the parasitic nematode *Globodera rostochiensis*. *Plant Physiol.* 157, 476–484.

Haegeman, A., Bauters, L., Kyndt, T., Rahman, M. M., and Gheysen, G. (2013). Identification of candidate effector genes in the transcriptome of the rice root knot nematode *Meloidogyne graminicola*. *Mol. Plant Pathol.* doi: 10.1111/mpp.12014 [Epub ahead of print].

Haegeman, A., Mantelin, S., Jones, J. T., and Gheysen, G. (2012). Functional roles of effectors of plant-parasitic nematodes. *Gene* 492, 19–31.

Hamamouch, N., Li, C., Hewezi, T., Baum, T. J., Mitchum, M. G., Hussey, R. S., et al. (2012). The interaction of the novel 30C02 cyst nematode effector protein with a plant Beta-1,3-endoglucanase may suppress host defence to promote parasitism. *J. Exp. Bot.* 63, 3683–3695.

Hewezi, T., and Baum, T. J. (2013). Manipulation of plant cells by cyst and root-knot nematode effectors. *Mol. Plant Microbe Interact.* 26, 9–16.

Hewezi, T., Howe, P., Maier, T. R., Hussey, R. S., Mitchum, M. G., Davis, E. L., et al. (2008). Cellulose binding protein from the parasitic nematode *Heterodera schachtii* interacts with *Arabidopsis* pectin methylesterase: cooperative cell wall modification during parasitism. *Plant Cell* 20, 3080–3093.

Hewezi, T., Howe, P. J., Maier, T. R., Hussey, R. S., Mitchum, M. G., Davis, E. L., et al. (2010). *Arabidopsis* spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. *Plant Physiol.* 152, 968–984.

Huang, G., Dong, R., Allen, R., Davis, E. L., Baum, T. J., and Hussey, R. S. (2005). Two chorismate mutase genes from the root-knot nematode *Meloidogyne incognita*. *Mol. Plant Pathol.* 6, 23–30.

Huang, G., Dong, R., Allen, R., Davis, E. L., Baum, T. J., and Hussey, R. S. (2006). A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. *Mol. Plant Microbe Interact.* 19, 463–470.

Huang, G., Dong, R., Maier, T., Allen, R., Davis, E. L., Baum, T. J., et al. (2004). Use of solid-phase subtractive hybridization for the identification of parasitism gene candidates from the root-knot nematode *Meloidogyne incognita*. *Mol. Plant Pathol.* 5, 217–222.

Huang, G., Gao, B., Maier, T., Allen, R., Davis, E. L., Baum, T. J., et al. (2003). A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode *Meloidogyne incognita*. *Mol. Plant Microbe Interact.* 16, 376–381.

Jammes, F., Lecomte, P., de Almeida-Engler, J., Bitton, F., Martin-Magniette, M.-L., Renou, J. P., et al. (2005). Genome-wide expression profiling of the host response to root-knot nematode infection in *Arabidopsis*. *Plant J.* 44, 447–458.

Jaouannet, M., Magliano, M., Arguel, M. J., Gourgues, M., Evangelisti, E., Abad, P., et al. (2013). The root-knot nematode calreticulin Mi-CRT is a key effector in plant defense suppression. *Mol. Plant Microbe Interact.* 26, 97–105.

Jaouannet, M., Perfus-Barbeoch, L., Deleury, E., Magliano, M., Engler, G., Vieira, P., et al. (2012). A root-knot nematode-secreted protein is injected into giant cells and targeted to the nuclei. *New Phytol.* 194, 924–931.

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

Jones, J. T., Furlanetto, C., Bakker, E., Banks, B., Blok, V., Chen, Q., et al. (2003). Characterization of a chorismate mutase from the potato cyst nematode *Globodera pallida*. *Mol. Plant Pathol.* 4, 43–50.

Jones, J. T., Kumar, A., Pylypenko, L. A., Thirugnanasambandam, A., Castelli, L., Chapman, S., et al. (2009). Identification and functional characterization of effectors in expressed sequence tags from various life cycle stages of the potato cyst nematode *Globodera pallida*. *Mol. Plant Pathol.* 10, 815–828.

Lee, C., Chronis, D., Kenning, C., Peret, B., Hewezi, T., Davis, E. L., et al. (2011). The novel cyst nematode effector protein 19C07 interacts with the *Arabidopsis* auxin influx transporter LAX3 to control feeding site development. *Plant Physiol.* 155, 866–880.

Lin, B., Zhuo, K., Wu, P., Cui, R., Zhang, L.-H., and Liao, J. (2013). A novel effector protein, MJ-NULG1a, targeted to giant cell nuclei plays a role in *Meloidogyne javanica* parasitism. *Mol. Plant Microbe Interact.* 26, 55–66.

Lozano-Torres, J. L., Wilbers, R. H. P., Gawronski, P., Boshoven, J. C., Finkers-Tomczak, A., Cordewener, J. H. G., et al. (2012). Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10119–10124.

Maier, T. R., Hewezi, T., Peng, J., and Baum, T. J. (2013). Isolation of whole esophageal gland cells from plant-parasitic nematodes for transcriptome analyses and effector identification. *Mol. Plant Microbe Interact.* 26, 31–35.

Opperman, C. H., Bird, D. M., Williamson, V. M., Rokhsar, D. S., Burke, M., Cohn, J., et al. (2008). Sequence and genetic map of *Meloidogyne hapla*: a compact nematode genome for plant parasitism. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14802–14807.

Patel, N., Hamamouch, N., Li, C., Hewezi, T., Hussey, R. S., Baum, T. J., et al. (2010). A nematode effector protein similar to annexins in host plants. *J. Exp. Bot.* 61, 235–248.

Postma, W. J., Slootweg, E. J., Rehman, S., Finkers-Tomczak, A., Tytgat, T. O. G., van Gelderen, K., et al. (2012). The effector SPRYSEC-19 of *Globodera rostochiensis* suppresses CC-NB-LRR-mediated disease resistance in plants. *Plant Physiol.* 160, 944–954.

Rehman, S., Postma, W., Tytgat, T., Prins, P., Qin, L., Overmars, H., et al. (2009). A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic nematode *Globodera rostochiensis* interacts with a CC-NB-LRR protein from a susceptible tomato. *Mol. Plant Microbe Interact.* 22, 330–340.

Rivas, S. (2012). Nuclear dynamics during plant innate immunity. *Plant Physiol.* 158, 87–94.

Rosso, M. N., Jones, J. T., and Abad, P. (2009). RNAi and functional genomics in plant parasitic nematodes. *Annu. Rev. Phytopathol.* 47, 207–232.

Rosso, M. N., Vieira, P., de almeida Engler, J., and Castagnone-Sereno, P. (2011). Proteins secreted by root-knot nematodes accumulate in the extracellular compartment during root infection. *Plant Signal. Behav.* 6, 1232–1234.

Roze, E., Hanse, B., MIitreva, M., Van Holme, B., Bakker, J., and Smant, G. (2008). Mining the secretome of the root-knot nematode *Meloidogyne chitwoodi* for candidate parasitism genes. *Mol. Plant Pathol.* 9, 1–10.

Sacco, M. A., Koropacka, K., Grenier, E., Jaubert, M. J., Blanchard, A., Goverse, A., et al. (2009). The cyst nematode

SPRYSEC protein RBP-1 elicits Gpa2- and RanGAP2-dependent plant cell death. *PLoS Pathog.* 5:e1000564. doi: 10.1371/journal.ppat.1000564

Smant, G., and Jones, J. (2011). "Suppression of plant defences by nematodes," in *Genomics and Molecular Genetics of Plant-Nematode Interactions*, eds J. Jones, G. Gheysen, and C. Fenoll (Berlin: Springer), 273–286.

Sobczak, M., and Golinowski, W. (2011). "Cyst nematodes and syncytia," in *Genomics and Molecular Genetics of Plant-Nematode Interactions*, eds J. D. Jones, G. Gheysen, and C. Fenoll (Berlin: Springer), 61–82.

Szakasits, D., Heinen, P., Wieczorek, K., Hofmann, J., Wagner, F., Kreil, D. P., et al. (2009). The transcriptome of syncytia induced by the cyst nematode *Heterodera schachtii* in *Arabidopsis* roots. *Plant J.* 57, 771–784.

Tytgat, T., Vanholme, B., De Meutter, J., Claeys, M., Couvreur, M., Vanhoutte, I., et al. (2004). A new class of ubiquitin extension proteins secreted by the dorsal pharyngeal gland in plant parasitic cyst nematodes. *Mol. Plant Microbe Interact.* 17, 846–852.

Vanholme, B., Kast, P., Haegeman, A., Jacob, J., Grunewald, W., and Gheysen, G. (2009). Structural and functional investigation of a secreted chorismate mutase from the plant-parasitic nematode *Heterodera schachtii* in the context of related enzymes from diverse origins. *Mol. Plant Pathol.* 10, 189–200.

Vieira, P., Danchin, E. G. J., Neveu, C., Crozat, C., Jaubert, S., Hussey, R. S., et al. (2011). The plant apoplasm is an important recipient compartment for nematode secreted proteins. *J. Exp. Bot.* 62, 1241–1253.

Vieira, P., Engler, G., and de Almeida Engler, J. (2012a). Whole-mount confocal imaging of nuclei in giant feeding cells induced by root-knot nematodes in *Arabidopsis*. *New Phytol.* 195, 488–496.

Vieira, P., Banora, M. Y., Castagnone-Sereno, P., Rosso, M. -N., Engler, G., and de Almeida Engler, J. (2012b). An immunocytochemical procedure for protein localization in various nematode life stages combined with plant tissues using methylacrylate-embedded specimens. *Phytopathology* 102, 990–996.

Wang, J., Reploge, A., Hussey, R., Baum, T., Wang, X., Davis, E. L., et al. (2011). Identification of potential host plant mimics of CLAVATA3/ESR (CLE)-like peptides from the plant-parasitic nematode *Heterodera schachtii*. *Mol. Plant Pathol.* 12, 177–186.

Wang, X., Allen, R., Ding, X., Goellner, M., Maier, T., de Boer, J. M., et al. (2001). Signal peptide-selection of cDNA cloned directly from the esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. *Mol. Plant Microbe Interact.* 14, 536–544.

Wang, X., Mitchum, M. G., Gao, B., Li, C., Diab, H., Baum, T. J., et al. (2005). A parasitism gene from a plant-parasitic nematode with function similar to CLAVATA3/ESR (CLE) of *Arabidopsis thaliana*. *Mol. Plant Pathol.* 6, 187–191.

Wiermer, M., Palma, K., Zhang, Y., and Li, X. (2007). Should I stay or should I go? Nucleocytoplasmic trafficking in plant innate immunity. *Cell. Microbiol.* 9, 1880–1890.

Xue, B., Hamamouch, N., Li, C., Huang, G., Hussey, R. S., Baum, T. J., et al. (2013). The 8D05 parasitism gene of *Meloidogyne incognita* is required for successful infection of host roots. *Phytopathology* 103, 175–181.

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: 31 January 2013; paper pending published: 13 February 2013; accepted: 27 February 2013; published online: 13 March 2013.

*Citation: Quentin M, Abad P and Favery B (2013) Plant parasitic nematode effectors target host defense and nuclear functions to establish feeding cells. Front. Plant Sci. **4**:53. doi: 10.3389/fpls.2013.00053*

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

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 11 April 2013
doi: 10.3389/fpls.2013.00094

A nuclear localization for Avr2 from *Fusarium oxysporum* is required to activate the tomato resistance protein I-2

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Plant pathogens secrete effector proteins to promote host colonization. During infection of tomato xylem vessels, *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) secretes the Avr2 effector protein. Besides being a virulence factor, Avr2 is recognized intracellularly by the tomato I-2 resistance protein, resulting in the induction of host defenses. Here, we show that *AVR2* is highly expressed in root- and xylem-colonizing hyphae three days post inoculation of roots. Co-expression of *I-2* with *AVR2* deletion constructs using agroinfiltration in *Nicotiana benthamiana* leaves revealed that, except for the N-terminal 17 amino acids, the entire *AVR2* protein is required to trigger *I-2*-mediated cell death. The truncated Avr2 variants are still able to form homo-dimers, showing that the central region of Avr2 is required for dimerization. Simultaneous production of I-2 and Avr2 chimeras carrying various subcellular localization signals in *N. benthamiana* leaves revealed that a nuclear localization of Avr2 is required to trigger I-2-dependent cell death. Nuclear exclusion of Avr2 prevented its activation of I-2, suggesting that Avr2 is recognized by I-2 in the nucleus.

Keywords: disease resistance, effector, Avr2, *Fusarium oxysporum*, tomato, I-2

INTRODUCTION

Many plant pathogens employ small, secreted proteins called effectors, to facilitate infection and to establish disease (Ellis et al., 2009; Tyler, 2009). Effectors interfere with biological processes of the host to the benefit of the pathogen (Kamoun, 2006; Alfano, 2009). To counteract pathogens, plants evolved resistance (R) proteins to perceive the presence or actions of these effectors (Chisholm et al., 2006; Maekawa et al., 2011). Although some R proteins are cell-surface receptors, most of them are cytosolic proteins of the nucleotide-binding leucine-rich repeat (NLR) type. Effector perception leads to activation of "effector-triggered immunity" (ETI), a response that is typically associated with programmed cell death of the infected cells. The induced resistance responses restrict outgrowth of the pathogen from the infection site (Spoel and Dong, 2012).

Many bacterial pathogens, such as *Pseudomonas syringae*, employ a type III secretion system to directly deliver effectors into the cytosol of plant cells (Shames and Finlay, 2012). Concomitantly, most *R* genes controlling bacterial pathogens encode NLR immune receptors with a predicted cytosolic location. Plant pathogenic fungi and oomycetes lack a type III secretion system and they secrete their effectors directly into intercellular spaces such as the apoplast or the xylem sap. Although some resistance genes controlling pathogenic fungi encode extracellular immune receptors, such as the Cf and Ve proteins controlling, respectively, *Cladosporium fulvum* and *Verticillium dahliae* (Thomma et al., 2011), most encode intracellular receptors (Dodds and Rathjen, 2010). Resistance to haustorium-forming pathogens is typically conferred by cytosolic receptors (Maekawa et al., 2011). The effectors are secreted into the periplasmic space surrounding the haustorium, from which a subset is taken up by the host cell, allowing intracellular perception (Whisson et al., 2007; Dou et al., 2008; Rafiqi et al., 2010; Schornack et al., 2010; De Jonge et al., 2011). The conserved RxLR motif found in many oomycete effectors is likely involved in the uptake process, as mutations in this motif abolish uptake (Grouffaud et al., 2008). Resistance to xylem-colonizing fungal pathogens that do not form haustoria can also be mediated by cytosolic NLR resistance genes, suggesting the uptake of the corresponding effector from the xylem sap by the host cells. The mechanism by which these fungal effectors enter the host cell, and the subcellular localization where they are perceived by the host immune receptor, are as yet unknown.

The interaction between tomato and the xylem-colonizing fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) has emerged as a model system to study NLR-mediated recognition of xylem secreted effectors (Takken and Rep, 2010). *Fol* is a soil born pathogen that causes vascular wilt disease by colonizing the xylem vessels of roots and stems (Michielse and Rep, 2009). Resistance to *Fol* in tomato is conferred by so-called "immunity" or "*I*" genes, and three of these genes have been introgressed from wild *Solanum* relatives into commercial varieties: *I* (or *I-1*), *I-2,* and *I-3*. *I-2* has been cloned and encodes a classical NLR protein that mediates resistance upon recognition of the Avr2 effector protein from *Fol* (Simons et al., 1998; Houterman et al., 2009). *I-2* promoter-reporter studies revealed that the gene is specifically expressed in the parenchyma cells adjacent to the xylem vessels, but the subcellular localization of I-2 is unknown (Mes et al., 2000). Typically, ETI induces a

programmed cell death response. However, *I-2*-mediated resistance seems to be distinct, as *Fol* recognition triggers specific responses in the parenchymal cells, which include accumulation of phenolics, callose deposition, and formation tyloses (outgrowth of xylem contact cells) and gels in the infected vessels, but not cell death (Beckman, 2000; Takken and Rep, 2010).

Xylem sap proteomics of *Fol* infected tomato resulted in identification of the *Fol* Avr2 protein. The *AVR2* gene encodes a 15.7 kDa mature protein (after cleavage of the N-terminal signal peptide), without discernable sequence similarity to other proteins (Houterman et al., 2009). Avr2 is not only an avirulence determinant of *I-2,* it is also a virulence factor required for full virulence of the fungus on susceptible plants. Race 3 *Fol* strains that can overcome *I-2* carry amino acid substitutions in Avr2 that prevent its recognition by I-2 while retaining its virulence function (Houterman et al., 2009). Whereas I-2-mediated resistance typically does not involve a cell death response, such as response is induced upon co-expression of *AVR2* and *I-2* in *Nicotiana benthamiana* using agroinfiltration or upon Potato Virus X-mediated expression of *AVR2* in *I-2* tomato. The strongest cell death response was found upon expression of a truncated *AVR2* variant that is not secreted by the transformed host cells (Houterman et al., 2009). This potentiated response implies intracellular recognition of Avr2 by I-2 and suggests that during natural infection the effector is taken up from the xylem sap by the adjacent plant cells (Houterman et al., 2009).

To determine where in the plant Avr2 is being produced by the fungus, allowing its perception by I-2, we studied the *in planta* expression of *AVR2* during infection. Since Avr2 is perceived intracellularly by I-2, we also examined its subcellular localization and determined the subcellular localization where Avr2 activates I-2. Finally, Avr2 deletion studies were performed to identify the minimal region that is required for dimerization and I-2 activation.

MATERIALS AND METHODS

GENERATION OF TRANSGENIC *Fol* STRAINS

Homologous recombination was used to replace the *AVR2* gene with a cassette containing the gene of interest and a hygromycin resistance gene. To generate the *AVR2*-promoter-*RFP* construct, the terminator of *AVR2* gene was PCR amplified with primer combination FP2708/FP2663 listed in **Table A1** using *Fol*007 genomic DNA as template. The resulting amplicon was cloned into the *Kpn*I site of pRW2h:Δ*AVR2*. In this vector the hygromycin resistance gene cassette is flanked by 1266 bp and 717 bp of sequences upstream and downstream of the *AVR2* ORF (Houterman et al., 2009). The correct orientation of the *AVR2* terminator was confirmed by PCR using primer set FP1074/FP2663. The pRW2h: Δ*AVR2*-T vector was generated. RFP was amplified from the pGWB454 plasmid DNA using primer set FP2706/FP2707 listed in **Table A1** (Nakagawa et al., 2007). The obtained fragments were digested with *Spe*I, gel purified, and ligated into a *Spe*I digested pRW2h: Δ*AVR2*-T vector containing the *AVR2* terminator. The orientation of *RFP* constructs was confirmed by PCR with primer set FP1074/FP2707. The obtained plasmid pRW2h:*pAVR2:RFP* was transformed into *Agrobacterium tumefaciens* EHA105 and used for subsequent *A. tumefaciens*-mediated *Fol* transformation according to Rep et al. (2004). *Fol* transformants capable of growing on 100 $\mu g\,mL^{-1}$ hygromycin (Duchefa) were checked by PCR for the absence of the *AVR2* gene using primer pair FP1074/FP965. Presence of the right and left borders of these constructs was confirmed with primers annealing just outside the flanking sequences, these were FP745/FP1075 (right border) and FP659/FP1166 (left border), respectively. Out of the 150 hygromycin resistant transformants one genuine *AVR2* replacement mutant was identified based on the absence of *AVR2* and the presence of Monomeric red fluorescent protein (mRFP) and the hygromycin cassette in the *AVR2* locus (data not shown).

VECTOR CONSTRUCTION

For localization studies, the pENTR207:Δ*spAVR2* or pENTR207:*AVR2* plasmid, described previously (Houterman et al., 2009), was used to recombine *AVR2* or Δ*spAVR2* into binary vector pGWB454 and pGWB451 (Nakagawa et al., 2007) according to the Gateway protocol for LR recombination reaction (Invitrogen). In the pGWB454 constructs Avr2 is fused to an RFP tag present in the vector. In the construct pGWB451:Δ*AVR2* Avr2 is fused to a GFP tag present in the vector. To construct *NLS-*Δ*spAVR2:GFP,* a nuclear localization signal (NLS) was introduced into forward primer FP2959 and the fragment was amplified together with reverse primer FP2222 from the pGWB451:Δ*AVR2* plasmid. To construct Δ*spAVR2-NES: GFP*, part of the nuclear export signal (NES) was introduced into the reverse primer FP3483 and the fragment was amplified together with forward primer FP2525. The fragment obtained with this primer set was used as template for a second round of PCR using primer set FP2525/FP3482. To create *CBL-*Δ*spAVR2-NES:GFP*, first part of the myristoylation signal (CBL) (Batistic et al., 2008) was introduced into forward primer FP3479 and part of the NES was introduced into the reverse primer FP3483. The fragment obtained with this primer set was used as template for a second round of PCR using primer set FP3478/FP3482. The resulted fragment contained the complete CBL coding sequences in the N-terminus and a NES coding sequence in the C-terminus of *AVR2*. The fragment harboring the mutated CBL and NES coding sequences was generated using the same strategy but by using primer sets FP3481/FP3485 and FP3480/FP3484, respectively. Finally the four amplicons were digested with *Xba*I and *Sac*I, and ligated into pGWB451 digested with the same enzymes.

Three primer combinations: FP2684/FP1751, FP2699/FP1751, and FP1749/FP2685 were used to amplify truncated *AVR2* fragments from CTAPi:Δ*spAVR2*. Subsequently, gateway *attB* linkers were added via PCR using primers FP872 and FP873. The obtained PCR products were introduced into entry clone pDONR207 (Invitrogen, http://www.invitrogen.com/) using the Gateway protocol described by the manufacturer (Invitrogen). The hence obtained pENTR207::Δ*spAVR2-*Δ37 (N-terminal deletion-1), pENTR207::Δ*spAVR2-*Δ40, and pENTR207::Δ*spAVR2-*CTΔ11 (C-terminal deletion) plasmids were recombined into the binary vector CTAPi (Rohila et al.,

2004) using the Gateway protocol (Invitrogen). The resulting plasmids, CTAPi::Δ*spAVR2*-Δ37, CTAPi::Δ*spAVR2*-Δ40, and CTAPi::Δ*spAVR2*-CTΔ11, were used for agroinfiltration as described below.

To generate the constructs used for yeast-two hybrid experiments, the *AVR2* ORF, lacking the sequence encoding the signal peptide, was amplified using primer FP1873 and FP1874. As template the *AVR2* gene in CTAPi was used (Houterman et al., 2009). The obtained product, carrying *Nco*I and *Eco*RI restriction sites, was cloned into the pAS2-1 and pACT-2 (Clontech) vectors digested with the same restriction enzymes.

For co-immunoprecipication experiments binary vectors containing Avr2 were created. *Xba*I and *Bam*HI restriction sites flanking the Δsp*AVR2* coding sequence were introduced by PCR with primers FP2525 and FP2274 using CTAPi::Δsp*AVR2* as template (Houterman et al., 2009). The obtained product was sub-cloned into the vector SLDB3104 (Tameling et al., 2010) between the *Xba*I and *Bam*HI restriction sites to generate SLDB3104::Δsp*AVR2.* In the resulting plasmid Avr2 is fused to a C-terminal hemagglutinin (HA) and streptavidin-binding peptide (SBP) tag. All PCR primers were purchased from MWG (http://www.mwg-biotech.com), and sequences of all plasmids were confirmed by sequence analysis.

PROTEIN EXTRACTION AND IMMUNOBLOTTING

Infiltrated *N. benthamiana* leaves were harvested and pooled 24 h after agroinfiltration, and snap-frozen in liquid nitrogen. After grinding the tissue with a mortar and pestle, it was allowed to thaw in 2 ml protein extraction buffer per gram of tissue [25 mm Tris pH 8, 1 mm EDTA, 150 mm NaCl, 5 mm DTT, 0.1% NP-40, 1× Roche complete protease inhibitor cocktail (http://www.roche.com) and 2% PVPP]. Extracts were centrifuged at 12 000 *g,* 4°C for 10 min, and the supernatant was passed over four layers of Miracloth (http://www.calbiochem.com/miracloth) to obtain a total protein lysate. 40 μL samples were mixed with Laemmli sample buffer, and equal amounts of total protein were run on 13% SDS–PAGE gels and blotted on PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as a blocking agent. A 1:3000 dilution of anti-GFP antibody (VXA6455, Invitrogen), or 1:8000 dilution of anti-tandem affinity purification (TAP) tag antibody (PAP, P1291, Sigma P1291) linked to horseradish peroxidase were used. The secondary antibody goat-anti-rabbit (P31430, Pierce) was used as a 1:5000 dilution. The luminescent signal was visualized by ECL using BioMax MR film (Kodak, http://www.kodak.com).

For mass spectrometry analysis, protein extracts were spun for 10 min at 12,000g, and 1 ml supernatant was added to 100 μl bed volume of Streptavidin Sepharose High Performance beads (GE Healthcare). Protein extracts were incubated in a rotator for 3 h at 4°C, and washed four times with immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, and 5 mM DTT, and 0.15% Nonidet P-40). Elution was performed twice with two bed volumes of washing buffer containing 4 mM D-biotin (Sigma-Aldrich). 400 μl eluted fractions were pooled and precipitated with trichloroacetic acid. Pellets were washed with 100% acetone at −20°C. 40 μl samples were mixed with Laemmli sample buffer for MS and loaded on 12% SDS-PAGE gels cased in Hoefer Might Small SE250 mini gel equipment (Amersham Biosciences, AB, Uppsala). After gel electrophoreses Coomassie PageBlue™ (Fermentas) staining was used to visualize the proteins.

MASS SPECTROMETRY

The protein bands corresponding to the mass of the expected Avr2 monomer and dimer were sliced from the Coomassie stained gel. In-gel digestion was performed as described by Rep et al. (2002). The peptides obtained after the digestion were analyzed by MALDI-TOF/TOF MS as described by Krasikov et al. (2011). Acquired spectra were then searched with Mascot (Matrix Science, UK) against a *Fol* database. The *Fol* protein database used for the analysis was obtained from Fusarium Comparative Genome website (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) and supplemented by adding the sequences of known Six proteins that are not annotated in the public database. To identify the plant proteins, all spectra were also searched against a custom Solanaceae EST database from plant-assembled transcripts (http://plantta.jcvi.org/).

YEAST TWO-HYBRID

The matchmaker GAL4 two-hybrid system and yeast strain PJ694a were used for analyzing protein interactions. Yeast transformation was performed using lithium-acetate and polyethylene glycol 3350 as described (Gietz and Woods, 2002). Eight colonies were picked and transferred from the MM-WL plates, lacking Trp and Leu, to a fresh MM-WL plate and incubated for 5 days at 30°C. Next, one colony per combination was re-suspended in 25 μl 0.9 % NaCl and 6 μl was spotted on MM-WL, MM-HWL, MM-AWL, and MM-HWL plates containing 3 mM 3-amino-1,2,4-triazole. After 5 days incubation at 30°C, the plates were checked for growth and photographed.

CO-IMMUNOPRECIPICATION

For Co-IP experiments, total proteins were extracted from *N. benthamiana* leaves, as described above, 36 h after infiltrating with *A. tumefaciens* GV3101 containing either SLD::Δsp*AVR2*-HASBP or pGWB451::Δsp*AVR2* or a mixture of both *A. tumefaciens* strains. Immunoprecipication was performed as described above. A portion of the supernatant was reserved as input sample. 20 μl immunoprecipicated samples and 40 μl input samples were resuspended in 1× SDS-PAGE loading buffer and loaded on 12% SDS-PAGE gels. Next, the gels were subjected to immunoblotting using anti-HA peroxidase at dilution ratio 1:3000 (clone 3F10; Roche), and anti-GFP at dilution ratio 1:3000 (Invitrogen, VXA6455).

CONFOCAL MICROSCOPY

Confocal microscopical analysis was performed with an LSM510 (Zeiss, Germany). Excitation of GFP was done at 488 nm with an Ar-ion laser and emission was captured with a 505–530 nm pass filter. Excitation of RFP occurred at 543 nm with a HeNe laser. The 590–620 nm filter captured emission. To monitor co-localization RFP was excited at 543 nm and GFP at 488 nm or YFP at 514 nm.

GFP and YFP emission was captured with a 505–530 nm filter and RFP with a 565–615 nm filter. Images were scanned eight times.

Agrobacterium-MEDIATED TRANSIENT TRANSFORMATION OF *Nicotiana benthamiana*

A. tumefaciens strain GV3101(pMP90) (Koncz and Schell, 1986) was transformed with binary constructs as described previously (Takken et al., 2004). Agrobacterium-mediated transient transformation was performed according to methods described by Ma et al. (2012). Briefly, the agrobacteria were grown to an absorbance of 0.8 at 600 nm in LB-mannitol medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 2.5 g l^{-1} NaCl, 10 g l^{-1} mannitol) supplemented with 20 μm acetosyringone and 10 mm MES pH 5.6. Cells were pelleted by centrifugation at 4000 g at 20°C for 20 min and then resuspended in infiltration medium (1× MS salts, 10 mm MES pH 5.6, 2% w/v sucrose, 200 μm acetosyringone). Infiltration was done in *N. benthamiana* leaves at an absorbance of 0.1 (for *I-2* constructs) or 0.5 (for *AVR2* constructs) of 4–5-weeks-old plants.

PLASMOLYSIS

For plasmolysis, a plasma membrane marker labeled with YFP (ZmHVR-YFP) (Ma et al., 2012) and Avr2-RFP were co-infiltated in *N. benthamiana* leaves. Two days after infiltration, small infiltrated leaf pieces were collected and treated with 800 mM mannitol for 30 min to induce plasmolysis. Subsequently, the pieces were mounted in 30% glycerol on a glass slide for microscopy.

RESULTS

AVR2 IS PREDOMINANTLY EXPRESSED IN XYLEM-COLONIZING FUNGAL HYPHAE

To determine at which stage of infection *AVR2* is expressed, a *Fol* strain carrying an *AVR2*-promoter-reporter construct was created. mRFP was used as reporter and the p*AVR2:RFP* construct was transformed into a *Fol*-p*AVR3:GFP* strain. In this strain the coding sequence for Avr3 has been replaced by *GFP* encoding Green Fluorescent Protein (Van Der Does et al., 2008). The advantage of employing the *Fol*-p*AVR3:GFP* strain is that GFP can be used to monitor the growth of *Fol* in roots, as the *AVR3* gene is specifically expressed inside roots (Van Der Does et al., 2008). The p*AVR2:RFP* construct was designed to facilitate its integration into the *AVR2* locus by homologous recombination (see Materials and Methods) to ensure expression from the native locus, avoiding position effects. The double transformant was used to inoculate ten-days-old tomato seedlings grown hydroponically (Van Der Does et al., 2008). Expression of both reporter genes was studied in a time-course analysis by visualizing the RFP and GFP signals in inoculated roots using confocal microscopy. **Figure 1** shows that, at one-day post inoculation, only few of the germinated spores penetrating the roots display RFP florescence (*AVR2* promoter activity), whereas a GFP signal (*AVR3* promoter activity) is present in many germinating spores penetrating the roots. This observation confirms the earlier finding that expression of *AVR3* is induced upon contact with tomato roots (Van Der Does et al., 2008), and shows that in the majority of hyphae that colonize the root cortex *AVR2* is not expressed. Two days after inoculation an RFP signal was still only detectable in a very limited number of hyphae or spores (**Figure 1**). At three days after inoculation red fluorescence was visible in some of the hyphae growing between the cortical cells (**Figure 1**), but the majority of the green fluorescent hyphae did not show red fluorescence. At stages later than three days after inoculation, RFP and GFP double fluorescent fungal hyphae were frequently found growing inside xylem vessels (**Figure 1**), demonstrating that *AVR2* is highly expressed at this stage of infection. However, still not all hyphae express both genes at this stage and frequently hyphae were observed that contained only GFP (or, sometimes, RFP). From these observations we conclude that upon contact with tomato roots and during early stages of infection, expression of *AVR3* precedes that of *AVR2*. From three days after inoculation and onward high expression of both *AVR3* and *AVR2*

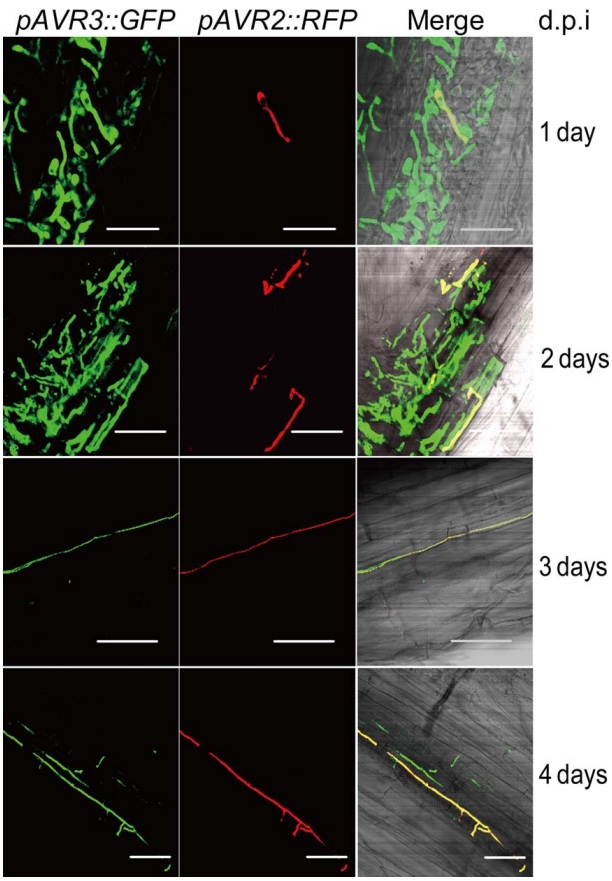

FIGURE 1 | Expression of p*AVR2:RFP* p*AVR3:GFP* in *Fol* colonizing tomato roots. RFP and GFP fluorescence was visualized using confocal microscopy. Imagines are depicted as separate red and green channels and as a merged figure. Ten-days-old tomato seedlings were inoculated with a *Fol* spore suspension and roots were analyzed at different time points post inoculation. One day after inoculation germinating spores can be found on the surface of tomato roots. Two days after inoculation hyphae have penetrated the epidermis and start to grow between the cortical cells. Three days after inoculation hyphae are growing between cortical cells. After three days, hyphae grow inside the xylem vessels. White scale bars represent 25 μm.

was found in hyphae growing in the xylem vessels of tomato roots.

Avr2 LOCALIZES TO THE CYTOSOL AND NUCLEUS OF PLANT CELLS

To examine the localization of Avr2 in plant cells, *A. tumefaciens* harboring either full length *AVR2* C-terminally fused to *RFP* or *AVR2* lacking its signal peptide (Δsp*AVR2*) fused to RFP, was infiltrated in *N. benthamiana* leaves. The localization of Avr2-RFP and ΔspAvr2-RFP was then examined by confocal microscopy. The red fluorescence originating from the wild-type Avr2-RFP fusion was mainly found in the apoplastic space (**Figure 2A**, left panel, arrows). To confirm an apoplastic localization, and to exclude the possibility that Avr2 was tethered to the plasma membrane or cell wall, the *AVR2-RFP* construct was co-expressed with a plasma membrane marker (ZmHVR-YFP) and the plant cells were plasmolysed before microscopical analysis. As shown in **Figure 2B**, the yellow fluorescence from the ZmHVR-YFP protein is specifically localized at the plasma membrane flanking the diffuse red signal from Avr2-RFP, confirming the apoplastic localization of the latter (**Figure 2B**, right panel, arrows). The dispersed RFP signal suggests that Avr2 is secreted into the apoplast and diffuses between the plant cells. In contrast, the ΔspAvr2-RFP protein lacking its signal peptide localized inside the plant cells. Here the fusion protein was found in the cytosol and the nucleus (**Figure 2A**, right panel, arrow). The nuclear localization of Avr2 can clearly be seen by the exclusion of the fluorescent protein from the nucleolus—more easily visible using a GFP-tagged Avr2 protein.

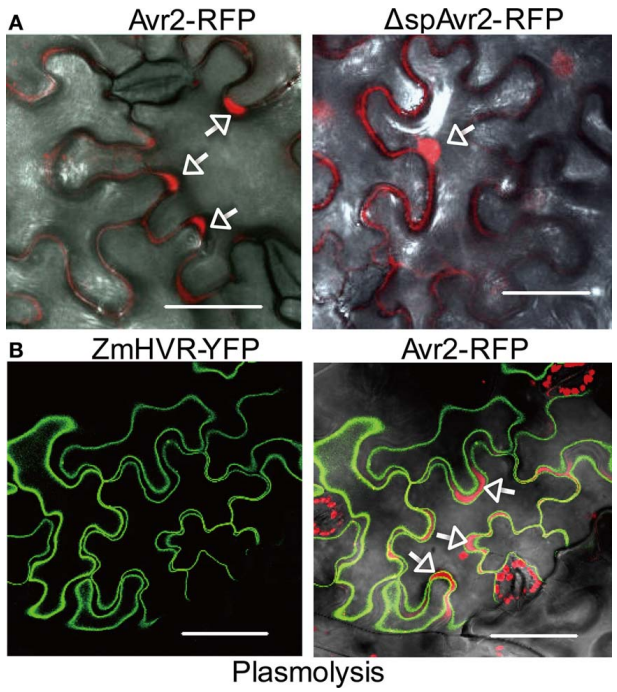

FIGURE 2 | Avr2 localizes in the cytosol and nucleus of *N. benthamiana* cells after agroinfiltration. (A) Confocal images of mesophyll cells in *N. benthamiana* leaves 36 h after agroinfiltration with Avr2-RFP or ΔspAvr2-RFP lacking its signal peptide for secretion. White arrows indicate the apoplastic spaces. **(B)** Transient co-expression of Avr2-RFP and the plasma membrane marker ZmHVR-YFP in epidermal cells of *N. benthamiana* after plasmolysis. Avr2-RFP is clearly visible in the apoplastic spaces (arrows) that are enlarged due to plasmolysis. The white scale bars represent 25 μm.

NUCLEAR LOCALIZED Avr2 IS REQUIRED TO TRIGGER I-2-DEPENDENT CELL DEATH IN *N. benthamiana*

To determine at which subcellular localization Avr2 is recognized by I-2, Δ*spAVR2* was fused to either a nuclear import signal (NLS) at its N-terminus, or a NES at its C-terminus. A NLS targets the protein to the nucleus whereas the NES translocates Avr2 from the nucleus to the cytoplasm, reducing its nuclear concentration (Kalderon et al., 1984). To examine whether the localization signals are functional, the Δ*spAVR2* variants were C-terminally fused to *GFP* and expressed in *N. benthamiana* leaves using agroinfiltration. At 36 h after infiltration, green fluorescence was imaged using confocal microscopy. As observed before, wild-type Avr2, lacking its signal peptide (Δsp) and fused to GFP, localized in both cytosol and nucleus (**Figure 3A**, arrow). NLS tagged Avr2 (NLS-ΔspAvr2-GFP) was only detected in the nucleus and not in the cytoplasm (**Figure 3A**, arrows). In contrast, the NES tagged Avr2 (ΔspAvr2-NES-GFP) protein was found in both the cytoplasm and the nucleus albeit at a lower concentration as the ΔspAvr2-GFP control (**Figure 3A**). So, although the NES translocates Avr2 from the nucleus it does not exclude nuclear entry. To further reduce the amount of Avr2 in the nucleus the NES-containing Avr2 protein was fused to a CBL1 (Myristoylation signal) motif at its N-terminus to tether it to the plasma membrane, preventing nuclear entry. As a negative control an Avr2 fusion with both a mutated CBL1 (cbl1) and a mutated NES was made. Both constructs were expressed in *N. benthamiana* leaves using agroinfiltration and green fluorescence was imaged using confocal microscopy. The CBL1 and NES tagged Avr2 (CBL1-ΔspAvr2-NES-GFP) protein was found exclusively at the plasma membrane and not in the nucleus (**Figure 3A**). The Avr2 variant carrying the cbl1 and nes signals (cbl1-ΔspAvr2-nes-GFP) displayed a distribution similar as ΔspAvr2. Immunoblotting showed that all ΔspAvr2-GFP fusion proteins accumulate at similar levels (**Figure 3B**). The majority of the proteins are intact, as bands were found at the expected size of ∼43 kDa. For the CBL1-Avr2-NES-GFP and cbl1-Avr2-nes-GFP extracts also some smaller bands were observed, which could be the consequence of limited proteolytic cleavage (**Figure 3B**).

We next utilized the above-described constructs to assess whether the enforced relocalization of Avr2 affected its ability to trigger I-2-mediated cell death. Thereto *I-2* and the *AVR2* constructs carrying the various translocation signals were co-expressed in *N. benthamiana* leaves using agroinfiltration. **Figure 3C** shows that at approximately 36 h after co-infiltration nuclear localized NLS-ΔspAvr2-GFP triggered an *I-2*-dependent cell death response equivalent to that of ΔspAvr2. In contrast, CBL1-Avr2-NES-GFP, which is retained in the plasma membrane, was unable to activate *I-2* and induce cell death. Avr2 fused to the mutated (inactive) CBL1 and NES motifs triggered an *I-2* specific cell death response similar to that of the ΔspAvr2 protein, showing that the mere extension of Avr2 with these

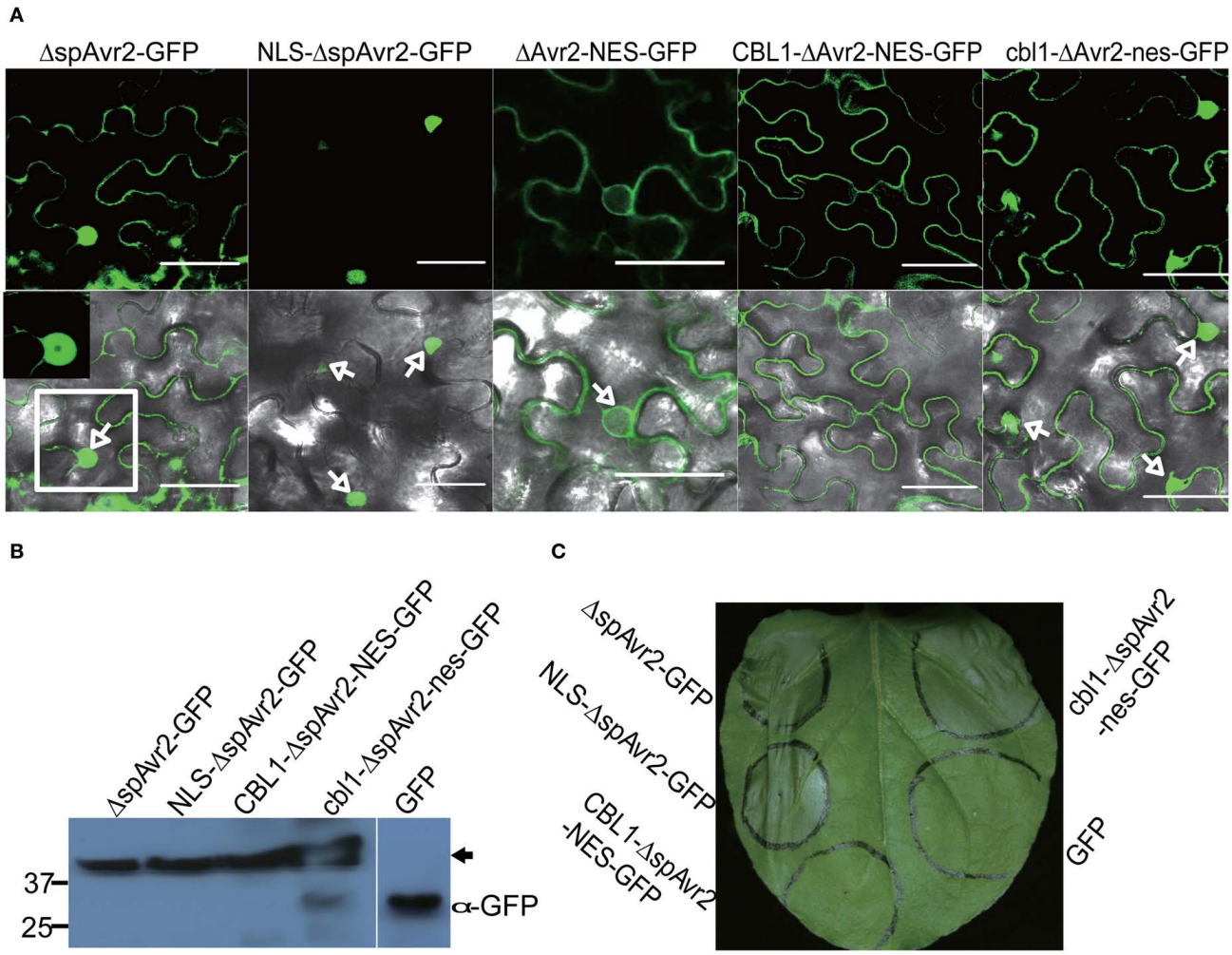

FIGURE 3 | Nuclear localization of Avr2 is required to trigger *I-2*-dependent cell death in *N. benthamiana* leaves. (A) Confocal image of ΔspAvr2-GFP, NLS-ΔspAvr2-GFP, ΔspAvr2-NES-GFP, CBL1-ΔspAvr2-NES-GFP, and cbl1-ΔspAvr2-nes-GFP in *N. benthamiana* leaves 36 h after agroinfiltration. Arrows indicate the nucleus, top GFP channel, bottom GFP and bright field channels merged. **(B)** Immunoblot analysis of GFP-fusion proteins accumulating *in planta* 36 h after infiltration. Blots were probed with an α-GFP antibody. The GFP alone control was used to assess the specificity of the antibody. Sizes in kDa are indicated on the left and an arrow indicates the 43 kDa band of the full-length protein. **(C)** Nuclear localization is required for effector-triggered cell death. *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* carrying ΔspAvr2:GFP, NLS-ΔspAvr2:GFP, CBL1-ΔspAvr2-NES:GFP, cbl1-ΔspAvr2-nes:GFP, or GFP alone with a strain carrying an *I-2* containing vector. A representative picture was taken three days after infiltration. Cell death is visible by tissue collapse of the infiltrated region. The white scale bars represent 25 μm.

sequences did not interfere with its cell death inducing activity (**Figure 3C**). Co-expression of *I-2* with the *NES*-tagged *Avr2* induced a cell death response comparable to that of co-expression with *cbl1-Avr2-nes-GFP* protein and wildtype Avr2 (data not shown) consistent with the similar subcellular distribution pattern of the latter proteins. In summary, these results indicate that a nuclear localization of Avr2 is required to trigger *I-2*-dependent cell death.

THE N-TERMINAL REGION OF Avr2 IS DISPENSABLE TO TRIGGER *I-2*-MEDIATED CELL DEATH

To define the minimal region of Avr2 required to trigger *I-2*-dependent cell death, two N-terminally truncated and one C-terminally truncated Avr2 protein was generated. To assist demarcation of the Avr2 truncations, PSIPRED (Buchan et al., 2010) was used to predict the secondary structure of the Avr2 protein (**Figure 4A**). Based on this prediction, the following variants were constructed: Avr2Δ37, in which the first predicted random coil downstream of the signal peptide was deleted; Avr2Δ40, a slightly extended deletion that includes the first cysteine; and Avr2CTΔ11, which lacks the last predicted β-strand at its C-terminus (**Figure 4B**). Both wild type and the three variants were equipped with a C-terminal TAP-tag.

A. tumefaciens strains containing plasmids encoding these truncated *AVR2-TAP* constructs were co-infiltrated with an *A. tumefaciens* strain harboring *I-2* into *N. benthamiana* leaves. Expression of Δ*spAVR2-TAP* together with *I-2* served as a positive control, and *I-2* together with an *GFP* containing vector as

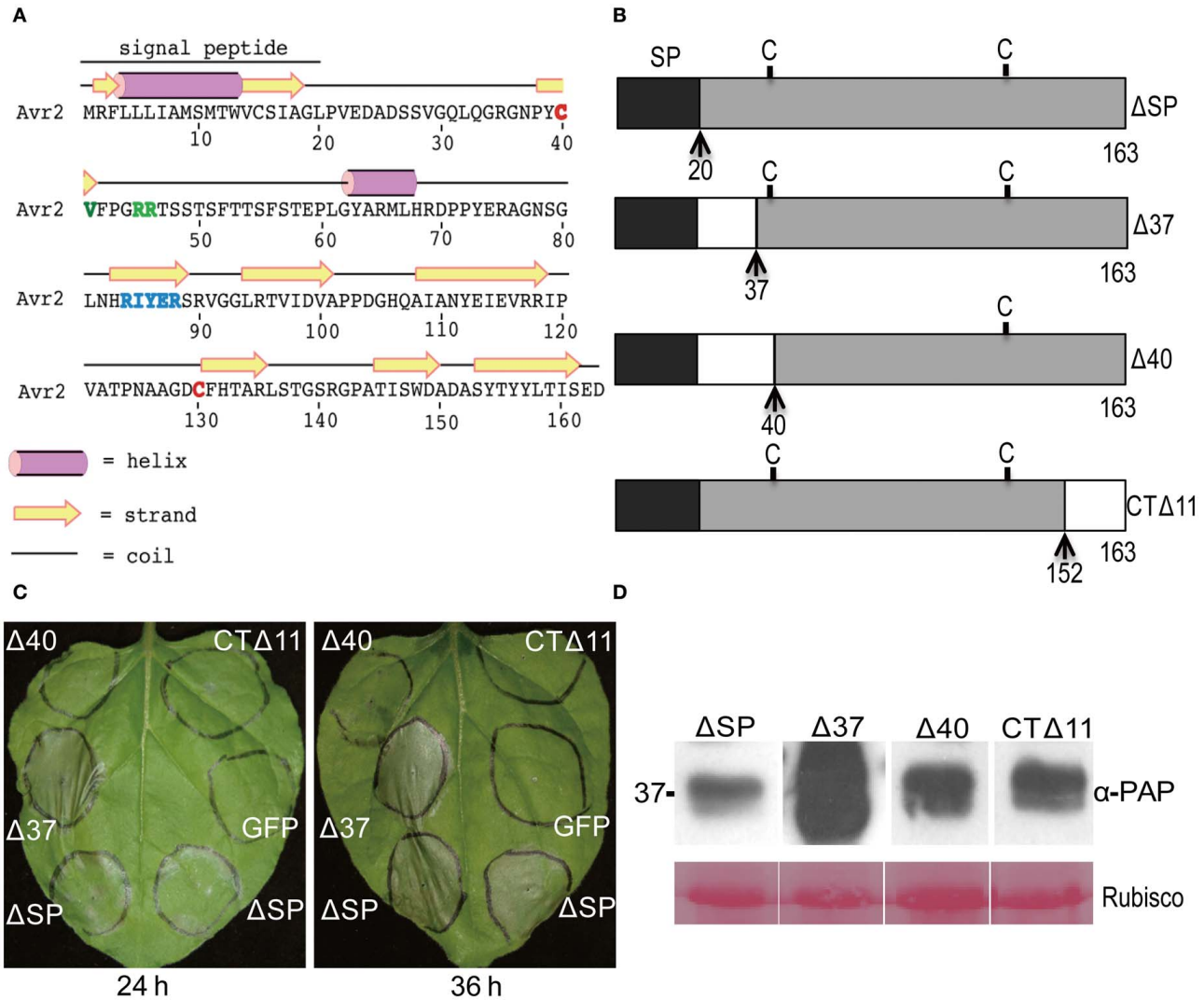

FIGURE 4 | A small N-terminal region of Avr2 is dispensable for *I-2*-dependent cell death. (A) Secondary structure prediction of Avr2. The two cysteine residues are marked red. The three polymorphisms in *I-2*-breaking (*Fol* race 3) Avr2 variants are marked green. **(B)** Schematic diagram showing the Avr2 truncations. The signal peptide is shaded dark, C indicates a cysteine residue; N- and C-terminal truncation sites are indicated by dashed lines and arrows along with the corresponding amino acid number. **(C)** *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* cultures containing *AVR2* truncations and *I-2*. The left panel shows a leaf photographed 24 h after infiltration and the right panel a leaf photographed 36 h after infiltration. **(D)** Immunoblot of proteins extracted from agroinfiltrated *N. benthamiana* leaves expressing Avr2 truncations fused C-terminally to a TAP tag. Leaves were harvested 36 h after infiltration and analyzed by immunoblotting using an anti-PAP antibody recognizing the TAP tag (α-PAP). Ponceau S staining of Rubisco (lower panel) is shown as a measure of the amount of protein loaded in each lane. Sizes in kDa are indicated on the left.

a negative control. Compared to ΔspAvr2, Avr2Δ37 induced a much faster and stronger cell death response (**Figure 4C**). I-2-dependent cell death triggered by Avr2Δ37 was observed as early as 20–24 h after infiltration, whereas cell death induced by Avr2 did not appear until 10–12 h later. Expression of the two other truncated variants, Avr2Δ40 and Avr2CTΔ11, did not induce *I-2*-dependent cell death (**Figure 4C**). Immunoblotting analysis demonstrated that all truncated proteins accumulated at similar levels, except the Avr2Δ37 truncation that accumulated in much higher amounts (**Figure 4D**). Hence, the inability of Avr2Δ40 and CTΔ11 to trigger *I-2*-mediated cell death is not due to a lack of protein accumulation. The high accumulation of Avr2Δ37 might be correlated with its ability to trigger a faster and stronger cell death response. Based on these observations, we concluded that Avr2 can be functionally divided into two parts, a small N-terminal part that is not required for I-2-mediated cell death and a large C-terminal region that includes the two cysteines and is indispensable for this activity.

Avr2 HOMODIMERIZES *in vivo*

Immunobloting of agroinfiltrated *N. benthamiana* leaves expressing a C-terminally human influenza HA and streptavidin-binding

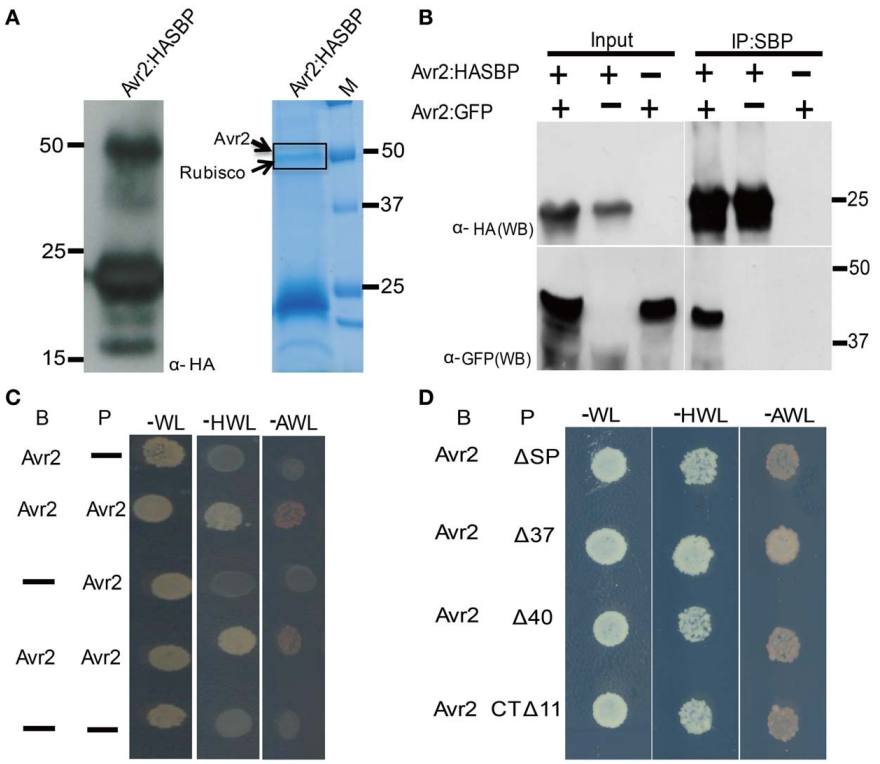

FIGURE 5 | Avr2 forms homodimers *in planta* and in yeast.
(A) Immunoblot probed with anti-HA showing that Avr2-HA-SBP is detected at the expected apparent molecular weight of ±25 kDa as well as in a ±50 kDa band. Avr2-HA-SBP was expressed in *Agrobacterium*-infiltrated *N. benthamiana* leaves and subsequently affinity-purified using the SBP tag. The purified protein was size separated using SDS-PAGE and the gel was stained with Colloidal Coomassie. The dashed rectangle indicates the section used for mass spectrometric analysis and the identified proteins are indicated on the left. Positions and sizes of the molecular weight marker are shown. **(B)** Immunoprecipitation of Avr2 from total plant protein extracts. Proteins extracted from *N. benthamiana* leaves expressing pairwise combinations of Avr2 C-terminally tagged with either HASBP or GFP. The fusion proteins were immunoprecipitated using streptavidin beads. Total extracted proteins (input) and immunoprecipitated proteins (IPs) were analyzed by immunoblotting by probing with either anti-HA (α-HA; upper) or anti-GFP (α-GFP; lower). Positions and sizes of protein mass makers are shown. **(C)** Growth of yeast strain pJ694a transformed with prey (P) constructs containing *AVR2* or empty vector (–) and bait (B) constructs containing *AVR2* or empty vector (–). All transformed yeasts could grow on minimal media lacking tryptophan and leucine (–WL) due to presence of the bait and prey plasmids. Only yeast containing both Avr2 as prey and bait was able to grow on selection plates lacking histidine, tryptophan and leucine (–HWL), and the more stringent selection medium lacking alanine, tryptophan and leucine (–AWL). Neither empty bait nor prey or Avr2 alone in combination with an empty vector could grow on the selection plates. **(D)** All Avr2 truncations interacted with wild-type Avr2 in yeast.

peptide (SBP) double-tagged Avr2 fusion protein (Avr2-HASBP), frequently revealed an additional ∼50 kDa band, i.e., twice the molecular mass of the Avr2-HASBP protein (**Figure 5A**). Since this larger product cross-reacted with the HA antibody it likely contains the Avr2-HASBP protein incorporated in a larger complex. To identify the constituents of this complex mass spectrometric analysis was performed. Avr2-HASBP containing complexes were affinity purified using SBP beads from a protein extract isolated from agroinfiltrated *N. benthamiana* leaves transiently expressing *AVR2-HASBP*. Next, the purified Avr2 protein complexes were size-separated on SDS-PAGE and the region corresponding to the ∼50 kDa product was cut out from the gel (**Figure 5A**). The proteins in the slice were in-gel digested and subjected to mass spectrometric analysis. Four peptides matching to Avr2 and ten peptides corresponding to Rubisco were identified in the peptide list (data not shown). Since the latter is a likely contaminant, the absence of other proteins raised the possibility that Avr2-HASBP might homodimerize, giving rise to the 50 kDa product.

To confirm the ability of Avr2 to physically self-interact *in planta*, co-immunoprecipitation experiments were performed. Two different *AVR2* constructs were used that each carry a different epitope tag: HASBP or GFP. Following co-agroinfiltration of these constructs into *N. benthamiana* leaves, the Avr2-HASBP protein was pulled down using SBP affinity beads and co-purification of the other was assessed using its GFP tag. **Figure 5B** shows that GFP-tagged Avr2 co-precipitates with HASBP-tagged Avr2 when both genes were co-expressed in *N. benthamiana* leaves, demonstrating that Avr2 has the ability to multimerize. To further test this, a yeast-two hybrid experiment was conducted using Avr2 both as bait and prey. As shown in **Figure 5C**, Avr2 interacts with itself, as yeast transformed both with bait and prey plasmids harboring Avr2 grew on the selective-HWL and also on the more stringent-AWL medium. Yeast co-transformed with Avr2 and empty bait or prey plasmid

was unable to grow on the selection plates (**Figure 5C**). Together, these data strongly suggest that Avr2 can form dimers *in planta* and in yeast.

To determine whether dimerization is correlated with the ability of Avr2 to activate I-2, the dimerization capacity of the three truncated Avr2 variants (**Figure 4**) was examined using yeast two-hybrid assays. As shown in **Figure 5D** all constructs supported growth on selective-HWL and -AWL medium, suggesting that the central region of Avr2 of 104 amino acids (aa 40-144) is sufficient for dimerization. Since Avr2Δ40 and Avr2CTΔ11 are not capable of activating I-2 it can be concluded that dimerization of the central region alone is not sufficient to induce *I-2*-mediated cell death.

DISCUSSION

Expression of *AVR2* was rarely observed during early stages of infection when the fungus colonizes the epidermis and invades the roots to grow between the cortical cells (**Figure 1**). However, during later stages of infection when the fungus colonizes the xylem vessels *AVR2* expression could readily be detected (**Figure 1**). This expression pattern differs from that of *AVR3* (*SIX1*), which was found to be expressed early upon infection and green fluorescence can be visualized already one-day post inoculation. The *AVR3* gene continues to be expressed during later stages of infection [(Van Der Does et al., 2008) and **Figure 1**]. Notably, at these later stages many fungal hyphae could be observed that express both genes, but also hyphae were found that express only one of the two genes. The latter is surprising since previous studies showed that expression of most *Six* genes, including *AVR3* (*SIX1*), and *AVR2* (*SIX3*), depends on the presence of the same transcription factor, Sge1 (Six Genes Expression 1) (Michielse et al., 2009). The dissimilar expression of *AVR2* with *AVR3* indicates that expression of these genes is not solely regulated by Sge1, but is likely also controlled by other factors. It will be interesting to analyse the expression profile of other effector genes during infection and to compare these to *AVR3* and *AVR2* to identify whether they are controlled in similar fashion. The relatively late expression of *AVR2* suggests that *I-2*-mediated resistance occurs relative late in infection, e.g., when the fungus colonizes the xylem vessels. *I-2*-mediated resistance acting in the xylem tissues is in agreement with (1) the expression of *I-2* in the vasculature and its lack of expression in cortical root cells (Mes et al., 2000), (2) the presence of the Avr2 protein in the xylem sap of tomato (Houterman et al., 2009), and (3) the observation that in an *I-2* plant *Fol* is able to colonize the cortical root cells and to reach the xylem vessels which it can colonize to an extent (Rep, pers. communication).

Deletion of the N-terminal 17 amino acids (Δ37) of Avr2 did not impair its ability to trigger *I-2*-dependent cell death. Actually, upon agrotransformation the truncated protein induced a faster and stronger cell death response than full length Avr2 protein, which correlated with an increased accumulation of the truncated protein (**Figures 4C** and **D**). The mechanism underlying the higher accumulation of this truncated protein is unknown, but the truncated form resembles the shorter forms found in the xylem sap. On 2D protein gels of xylem sap from infected tomato plants Avr2 localizes in at least three spots ranging in size from 11 to 14 kDa. Mass spectrometric analysis of these spots revealed that the smallest form of Avr2 in xylem sap has a N-terminal deletion similar to that of Δ37 (Houterman et al., 2007). The removal of these 17 aa might be due to N-terminal processing by plant proteases in xylem sap. An extended deletion removing 20 amino acids at the N-terminus encompassing the first cysteine after the signal peptide (Δ40) completely abolished the ability of Avr2 to trigger *I-2*-dependent cell death (**Figure 4C**). Since there are two cysteine residues present in Avr2 (**Figure 4A**), it is possible that a disulfide bond is formed in the mature Avr2 protein. Deletion of the cysteine would disrupt this bond, potentially affecting protein structure. However, the protein apparently retains at least part of its fold, as the mutant is still able to interact with wild-type Avr2 in yeast. Alternatively, the cysteine at position 40 might be part of a motif that is required for I-2-mediated recognition. Support for this hypothesis is the observation that Avr2 variants from race 3 strains of *Fol* that overcome I-2-mediated resistance carry a mutation in one of three nearby residues; valine 41, arginine 45, or arginine 46 (Houterman et al., 2009). Possibly, these residues together form an epitope that is recognized by I-2. A C-terminal deletion also abolished *I-2*-mediated recognition, but retained the proteins' ability to interact with wild-type Avr2 in yeast (**Figure 5D**). Together, these data show that dimerization alone is insufficient to activate I-2, and that also the C-terminus contains sequences required for I-2-mediated recognition (**Figure 5D**). The central part that can dimerize contains the "RIYER" sequence motif that was identified by Kale and co-workers as an "RXLR-like" motif that could be involved in entry of this effector in plant cells (Kale et al., 2010). A truncated Avr2 protein, consisting of the N-terminal half of the protein containing this domain, was taken up by soybean root cells whereas various RIYER mutants were not (Kale et al., 2010). In many oomycete effectors mutations in the conserved RxLR motif abolish their uptake by host cells (Grouffaud et al., 2008). One proposed function for the RxLR motif is binding to phosphatidylinositol-3-phosphate (PI3P) present on the outer surface of the plant plasma membrane, enabling vesicle-mediated endocytosis (Kale et al., 2010). An alternative function for the RXLR motif of an oomycete effector was recently proposed for AVR3a from *Phythophthora infestans* in which this region is required for homodimerization (Boutemy et al., 2011; Wawra et al., 2012). Whether the RIYER motif in Avr2 is also required for dimerization awaits elucidation of its 3D protein structure. Solving the structure will not only aid identification of surface localized residues involved in homodimerization, but could also reveal residues that mediate interaction with plant proteins.

Using a heterologous expression system, we demonstrated that nuclear localization of Avr2 is required to trigger an *I-2*-dependent cell death response (**Figure 3**). Unfortunately, the subcellular localization of I-2 is unknown and its determination is hampered by the lack of sensitive antibodies and the loss of function of tagged I-2 variants (Tameling et al., 2002). A truncated I-2 protein, lacking its LRR domain, localizes in both the nucleus and the cytosol when expressed via agroinfiltration in *N. benthamiana* (manuscript in preparation). In addition, a potential NLS (RKHK) has been predicted in the CC domain of

I-2 (Simons et al., 1998). These observations imply that I-2 could also be localized in the nucleus.

Proximity of an NLR to its recognized effector(s) is a likely prerequisite for its activation. Recent examples are Arabidopsis RPM1 that initiates signaling at the plasma membrane where its effectors AvrRpm1 and AvrB reside (Gao et al., 2011). Likewise, the potato R3a protein is activated only when it colocalizes with its cognate *Phytophtora infestans* effector $Avr3a^{KI}$ at endosomal compartments (Engelhardt et al., 2012). Finally, the tobacco Rx protein needs to co-localize with the viral effector in the cytoplasm to become activated (Slootweg et al., 2010). The different sites for NLR activation could reflect the surveillance of diverse effector activities, which would imply a nuclear function for Avr2. Whether I-2 resistance signaling also requires its nuclear location remains to be investigated. A growing body of evidence suggest that nuclear or a nucleocytoplasmic localization for at least some R proteins, such as tobacco N, potato Rx, and barley Mla10 is essential for proper immune signaling (Burch-Smith et al., 2007; Slootweg et al., 2010; Tameling et al., 2010; Bai et al., 2012; Heidrich et al., 2012) [reviewed by Deslandes and Rivas (2011), Rivas (2012)]. Interestingly, the *P. syringae* effector AvrRps4 was found to trigger compartment-specific immune responses in which nuclear localized AvrRps4 triggers RPS4-dependent resistance and cytoplasmic AvrRps4 induces cell death, implying that cell death and resistance signaling are independent processes (Heidrich et al., 2011). The barley resistance protein MLA10 also displays compartment-specific immunity; the nuclear pool being involved in resistance and the cytoplasmic pool in triggering cell death (Bai et al., 2012). It will be interesting to determine whether Avr2-mediated cell death and resistance also require different locations for I-2 or whether both immune responses originate from the plant nucleus.

ACKNOWLEDGMENTS

We are gratefully to Harold Lemereis, Thijs Hendrix, and Ludek Tikovsky for plant care, and to Martijn Rep for stimulating discussions and critical reading of the manuscript. This work was financially supported by the CBSG, a NGI initiative, and the University of Amsterdam.

REFERENCES

Alfano, J. R. (2009). Roadmap for future research on plant pathogen effectors. *Mol. Plant Pathol.* 10, 805–813.

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

Batistic, O., Sorek, N., Schultke, S., Yalovsky, S., and Kudla, J. (2008). Dual fatty acyl modification determines the localization and plasma membrane targeting of CBL/CIPK Ca2+ signaling complexes in Arabidopsis. *Plant Cell* 20, 1346–1362.

Beckman, C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiol. Mol. Plant P* 57, 101–110.

Boutemy, L. S., King, S. R., Win, J., Hughes, R. K., Clarke, T. A., Blumenschein, T. M., et al. (2011). Structures of Phytophthora RXLR effector proteins: a conserved but adaptable fold underpins functional diversity. *J. Biol. Chem.* 286, 35834–35842.

Buchan, D. W., Ward, S. M., Lobley, A. E., Nugent, T. C., Bryson, K., and Jones, D. T. (2010). Protein annotation and modelling servers at University College London. *Nucleic Acids Res.* 38, W563–W568.

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

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.

De Jonge, R., Bolton, M. D., and Thomma, B. P. H. J. (2011). How filamentous pathogens co-opt plants: the ins and outs of fungal effectors. *Curr. Opin. Plant Biol.* 14, 400–406.

Deslandes, L., and Rivas, S. (2011). The plant cell nucleus: a true arena for the fight between plants and pathogens. *Plant Signal. Behav.* 6, 42–48.

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11, 539–548.

Dou, D., Kale, S. D., Wang, X., Jiang, R. H., Bruce, N. A., Arredondo, F. D., et al. (2008). RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* 20, 1930–1947.

Ellis, J. G., Rafiqi, M., Gan, P., Chakrabarti, A., and Dodds, P. N. (2009). Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens. *Curr. Opin. Plant Biol.* 12, 399–405.

Engelhardt, S., Boevink, P. C., Armstrong, M. R., Ramos, M. B., Hein, I., and Birch, P. R. (2012). Relocalization of late blight resistance protein r3a to endosomal compartments is associated with effector recognition and required for the immune response. *Plant Cell* 24, 5142–5158.

Gao, Z., Chung, E. H., Eitas, T. K., and Dangl, J. L. (2011). Plant intracellular innate immune receptor resistance to *Pseudomonas syringae pv.* maculicola 1 (RPM1) is activated at, and functions on, the plasma membrane. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7619–7624.

Gietz, R. D., and Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* 350, 87–96.

Grouffaud, S., Van West, P., Avrova, A. O., Birch, P. R., and Whisson, S. C. (2008). *Plasmodium falciparum* and *Hyaloperonospora parasitica* effector translocation motifs are functional in Phytophthora infestans. *Microbiology* 154, 3743–3751.

Heidrich, K., Blanvillain-Baufume, S., and Parker, J. E. (2012). Molecular and spatial constraints on NB-LRR receptor signaling. *Curr. Opin. Plant Biol.* 15, 385–391.

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.

Houterman, P. M., Ma, L., Van Ooijen, G., De Vroomen, M. J., Cornelissen, B. J. C., Takken, F. L. W., et al. (2009). The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant J.* 58, 970–978.

Houterman, P. M., Speijer, D., Dekker, H. L., De Koster, C. G., Cornelissen, B. J. C., and Rep, M. (2007). The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. *Mol. Plant Pathol.* 8, 215–221.

Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. *Cell* 39, 499–509.

Kale, S. D., Gu, B., Capelluto, D. G., Dou, D., Feldman, E., Rumore, A., et al. (2010). External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell* 142, 284–295.

Kamoun, S. (2006). A catalogue of the effector secretome of plant pathogenic oomycetes. *Annu. Rev. Phytopathol.* 44, 41–60.

Koncz, C., and Schell, J. (1986). The promoter of Tl-DNA gene 5 controls the tissue-specific expression of Chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* 204, 383–396.

Krasikov, V., Dekker, H. L., Rep, M., and Takken, F. L. W. (2011). The tomato xylem sap protein XSP10 is required for full susceptibility to Fusarium wilt disease. *J. Exp. Bot.* 62, 963–973.

Ma, L., Lukasik, E., Gawehns, F., and Takken, F. L. (2012). The use of agroinfiltration for transient expression of plant resistance and fungal effector proteins in *Nicotiana benthamiana* leaves. *Methods Mol. Biol.* 835, 61–74.

Maekawa, T., Kufer, T. A., and Schulze-Lefert, P. (2011). NLR functions in plant and animal immune systems: so far and yet so close. *Nat. Immunol.* 12, 818–826.

Mes, J. J., Van Doorn, A. A., Wijbrandi, J., Simons, G., Cornelissen, B. J. C., and Haring, M. A. (2000). Expression of the Fusarium resistance gene *I-2* colocalizes with the site of fungal containment. *Plant J.* 23, 183–193.

Michielse, C. B., and Rep, M. (2009). Pathogen profile update: *Fusarium oxysporum*. *Mol. Plant Pathol.* 10, 311–324.

Michielse, C. B., Van Wijk, R., Reijnen, L., Manders, E. M., Boas, S., Olivain, C., et al. (2009). The nuclear protein Sge1 of *Fusarium oxysporum* is required for parasitic growth. *PLoS Pathog.* 5:e1000637. doi: 10.1371/journal.ppat.1000637

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., et al. (2007). Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci. Biotechnol. Biochem.* 71, 2095–2100.

Rafiqi, M., Gan, P. H., Ravensdale, M., Lawrence, G. J., Ellis, J. G., Jones, D. A., et al. (2010). Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. *Plant Cell* 22, 2017–2032.

Rep, M., Dekker, H. L., Vossen, J. H., De Boer, A. D., Houterman, P. M., Speijer, D., et al. (2002). Mass spectrometric identification of Isoforms of PR proteins in xylem sap of fungus-infected tomato. *Plant Physiol.* 130, 904–917.

Rep, M., Van Der Does, H. C., Meijer, M., Van Wijk, R., Houterman, P. M., Dekker, H. L., et al. (2004). A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. *Mol. Microbiol.* 53, 1373–1383.

Rivas, S. (2012). Nuclear dynamics during plant innate immunity. *Plant Physiol.* 158, 87–94.

Rohila, J. S., Chen, M., Cerny, R., and Fromm, M. E. (2004). Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. *Plant J.* 38, 172–181.

Schornack, S., Van Damme, M., Bozkurt, T. O., Cano, L. M., Smoker, M., Thines, M., et al. (2010). Ancient class of translocated oomycete effectors targets the host nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17421–17426.

Shames, S. R., and Finlay, B. B. (2012). Bacterial effector interplay: a new way to view effector function. *Trends Microbiol.* 20, 214–219.

Simons, G., Groenendijk, J., Wijbrandi, J., Reijans, M., Groenen, J., Diergaarde, P., et al. (1998). Dissection of the fusarium *I2* gene cluster in tomato reveals six homologs and one active gene copy. *Plant Cell* 10, 1055–1068.

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.

Spoel, S. H., and Dong, X. N. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Genet.* 12, 89–100.

Takken, F., and Rep, M. (2010). The arms race between tomato and *Fusarium oxysporum*. *Mol. Plant Pathol.* 11, 309–314.

Takken, F. L., Van Wijk, R., Michielse, C. B., Houterman, P. M., Ram, A. F., and Cornelissen, B. J. (2004). A one-step method to convert vectors into binary vectors suited for Agrobacterium-mediated transformation. *Curr. Genet.* 45, 242–248.

Tameling, W. I. L., Elzinga, S. D. J., Darmin, P. S., Vossen, J. H., Takken, F. L. W., Haring, M. A., et al. (2002). The tomato R gene products I-2 and Mi-1 are functional ATP binding proteins with ATPase activity. *Plant Cell* 14, 2929–2939.

Tameling, W. I. L., 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.

Thomma, B. P., Nurnberger, T., and Joosten, M. H. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *Plant Cell* 23, 4–15.

Tyler, B. M. (2009). Entering and breaking: virulence effector proteins of oomycete plant pathogens. *Cell Microbiol.* 11, 13–20.

Van Der Does, H. C., Duyvesteijn, R. G. E., Goltstein, P. M., Van Schie, C. C. N., Manders, E. M. M., Cornelissen, B. J. C., et al. (2008). Expression of effector gene *SIX1* of *Fusarium oxysporum* requires living plant cells. *Fungal Genet. Biol.* 45, 1257–1264.

Wawra, S., Agacan, M., Boddey, J. A., Davidson, I., Gachon, C. M., Zanda, M., et al. (2012). Avirulence protein 3a (AVR3a) from the potato pathogen *Phytophthora infestans* forms homodimers through its predicted translocation region and does not specifically bind phospholipids. *J. Biol. Chem.* 287, 38101–38109.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., et al. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450, 115–118.

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: 07 February 2013; paper pending published: 28 February 2013; accepted: 27 March 2013; published online: 11 April 2013.

Citation: Ma L, Cornelissen BJC and Takken FLW (2013) A nuclear localization for Avr2 from Fusarium oxysporum is required to activate the tomato resistance protein I-2. Front. Plant Sci. ***4****:94. doi: 10.3389/fpls.2013.00094*

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

APPENDIX

Table A1 | Primers used in this study.

Number	Sequences
FP2708	GCGGTACCACTAGTTTCTGTGGCAGTTCCCCT
FP2663	CCCGGTACCCAGTCCCCACACAGTATTCTTTC
FP1074	CCAGCCAGAAGGCCAGTTT
FP2706	CCACTAGTATGGCCTCCTCCGAGGAC
FP2707	CGACTAGTAGATCTTTAGGCGCCGGTGGAGTGGCGG
FP3478	CAAAGGCAGCAAAAGAATTTCCATATTGCGTGTTTCCCGGCCGCCGCACGT
FP3482	AGAGGAGGAAGTTGAAGATCCTCTGAGATAGTAAGATAGTAGGTATAACT
FP3481	GCGTCTAGAATGGCCAGCTTCCACTCAAAGGCAGCAAAAGAATTTCCATA
FP3485	GCGTCTAGAAAGAGTGGCTCTTTCAGCAGGAGGGGCTTGAAGATCCTCTG
FP3480	CAAAGGCAGCAAAAGAATTTCCATATTGCGTGTTTCCCGGCCGCCGCACGT
FP3484	GCAGGAGGGGCTTGAAGATCCTCTGAGATAGTAAGATAGTAGGTATAACT
FP2959	CCTCTAGATTGCTCACCTAAGAAGAGAAAGGTTGGAGGAC
FP2222	CGCGCGAGCTCTTATTTGTATAGTTCATCCA
FP3479	GCGTCTAGAATGGGCTGCTTCCACTCAAAGCAGCAAAAGAATTT
FP3483	GCGTCTAGAAAGAGTAAGTCTTTCAAGAGGAGGAAGTTGAAGATC
FP1749	AAAAAGCAGGCTGGATGCCTGTGGAAGATGCCGATTCATC
FP1751	AGAAAGCTGGGTATCCATCCTCTGAGATAGTAAGATAG
FP2684	AAAAAGCAGGCTCTATGCCATATTGCGTGTTTCCCGGCCG
FP2699	AAAAAGCAGGCTCTATGGTGTTTCCCGGCCGCCGCACG
FP2685	AGAAAGCTGGGTAAGCGTCGGCATCCCAACTGATTGTG
FP872	GGGGACAAGTTTGTACAAAAAAGCAGGCT
FP873	GGGGACCACTTTGTACAAGAAAGCTGGGT
FP1873	AAACCATGGAAGATGCCGATTCATC
FP1874	AAAGAATTCAATCCTCTGAGATAGTAAG
FP2525	CGCTCTAGAATGCCTGTGGAAGATGCCGAT
FP2274	GCGGGATCCTCCATCCTCTGAGATAGTAAG

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PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 21 October 2013
doi: 10.3389/fpls.2013.00387

Characterization of cell death inducing *Phytophthora capsici* CRN effectors suggests diverse activities in the host nucleus

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Plant-Microbe interactions are complex associations that feature recognition of Pathogen Associated Molecular Patterns by the plant immune system and dampening of subsequent responses by pathogen encoded secreted effectors. With large effector repertoires now identified in a range of sequenced microbial genomes, much attention centers on understanding their roles in immunity or disease. These studies not only allow identification of pathogen virulence factors and strategies, they also provide an important molecular toolset suited for studying immunity in plants. The *Phytophthora* intracellular effector repertoire encodes a large class of proteins that translocate into host cells and exclusively target the host nucleus. Recent functional studies have implicated the CRN protein family as an important class of diverse effectors that target distinct subnuclear compartments and modify host cell signaling. Here, we characterized three necrosis inducing CRNs and show that there are differences in the levels of cell death. We show that only expression of CRN20_624 has an additive effect on PAMP induced cell death but not AVR3a induced ETI. Given their distinctive phenotypes, we assessed localization of each CRN with a set of nuclear markers and found clear differences in CRN subnuclear distribution patterns. These assays also revealed that expression of CRN83_152 leads to a distinct change in nuclear chromatin organization, suggesting a distinct series of events that leads to cell death upon over-expression. Taken together, our results suggest diverse functions carried by CRN C-termini, which can be exploited to identify novel processes that take place in the host nucleus and are required for immunity or susceptibility.

Keywords: ***Phytophthora capsici*, effector, CRN, nucleus, cell death, immunity**

INTRODUCTION

Within the natural environment, plants are continuously challenged by a diverse array of microbes that can cause disease, including bacteria, fungi, and oomycetes. In order to counteract infection, plants have evolved the ability to recognize Microbe or Pathogen Associated Molecular Patterns (MAMPs or PAMPs, respectively) through Pattern Recognition Receptors (PRRs) localized in the host cell membrane. This recognition of PAMPs in turn activates PAMP-Triggered Immunity (PTI), preventing establishment of disease (Zipfel, 2008; Monaghan and Zipfel, 2012). In a select few cases, pathogens successfully infect plants by either limiting PAMP perception or perturbing PTI by interfering with signal transduction or associated cellular processes required for effective host immune responses (Jones and Dangl, 2006; Zipfel, 2008). This implies that pathogens have evolved molecular strategies to evade or circumvent host immunity. Consequently, host-pathogen interactions are considered dynamic associations featuring specialized pathogen machineries that aim to suppress (inducible) immune responses.

Key to understanding the mechanisms by which pathogens evade or suppress plant immune responses has been the identification of secreted proteins, termed effectors, which have been found in virtually all pathogen genomes studied to date (Hogenhout et al., 2009; Stergiopoulos and de Wit, 2009; Hann et al., 2010; Oliva et al., 2010). In some cases, effector activities toward virulence have been demonstrated and linked to host susceptibility, supporting the notion that effectors can trigger susceptibility on their hosts [Effector Triggered Susceptibility (ETS)] (Bos et al., 2010; Yeam et al., 2010). Consequently, models have now emerged which describe secreted effector proteins that upon delivery to host cellular compartments, modify their targets and trigger susceptibility (Howden and Huitema, 2012). Besides PRR mediated responses, plants have acquired another layer of immunity. Most plants carry another class of receptors (termed Nucleotide Binding-Leucine Rich Repeat proteins or NB-LRRs), which reside inside host cells and upon recognition of cytoplasmic effectors, trigger immunity (Effector Triggered Immunity, ETI). With an increasing number of PRRs, PAMPs, effectors and

NB-LRRs identified and characterized, observations suggest that both secreted pathogen proteins together with host receptors and signaling protein repertoires, determine interaction outcomes at the early stages of infection.

In recent years, a body of evidence has emerged which implicates the nucleus as a key cellular compartment in which the fate of host-pathogen interactions is determined (Liu and Coaker, 2008; Deslandes and Rivas, 2011; Rivas, 2012). In agreement with this, host protein classes with diverse functions have been shown to function in the nucleus toward immunity. These include plant disease resistance proteins, mitogen-associated protein (MAP) kinase signaling components, and transcription factors that collectively operate to regulate defence response genes following pathogen perception (Kinkema et al., 2000; Pandey and Somssich, 2009; Deslandes and Rivas, 2011; Park and Ronald, 2012; Rasmussen et al., 2012). In some cases, the mechanisms of activation are known and a major emerging theme is the exchange of key regulators and cellular signals between the cytosol and host nucleus (Shen and Schulze-Lefert, 2007). These processes generally result in the activation of defence responses and initiation of transcriptional programmes that elevate resistance. Given the role of the nucleus in plant defences and the ability of pathogens to suppress immunity, the view has emerged that perturbation of nuclear signaling by means of secreted pathogen effectors, may form an important virulence strategy to achieve disease.

Plant pathogenic oomycetes form a distinct lineage of eukaryotes that cause devastating diseases on a wide range of plants important to agriculture, forestry and natural ecosystems. For example, *Phytophthora infestans*, the causal agent of late blight on potato and tomato continues to cause hardship throughout the world with multi-billion dollar losses each year (Lamour et al., 2007). Other economically devastating pathogens include *P. sojae* and *P. capsici*, the major disease-causing agents on soybean and pepper, respectively. The shear economic impact that this group of pathogens incites has been, and continues to be, a driving force in our quest to understand *Phytophthora* parasitism.

Plant pathogenic oomycetes harbor a diverse class of effectors, termed the Crinklers (CRNs). All CRN proteins feature a conserved N-terminal domain specifying translocation and diverse C-terminal regions carrying distinct effector functions (Schornack et al., 2010). Crucially, a considerable number of CRN proteins have been identified in the genomes of all plant pathogenic oomycetes examined to date (Tyler et al., 2006; Gaulin et al., 2008; Haas et al., 2009; Lévesque et al., 2010; Schornack et al., 2010; Links et al., 2011; Lamour et al., 2012; Stam et al., 2013), suggesting that they have important roles in oomycete pathogenesis on plants.

Localization studies on diverse sets of CRN effectors from divergent oomycete species revealed they all accumulate in the host nucleus upon ectopic expression in plants (Schornack et al., 2010; Stam et al., 2013). These observations combined with the identification of (cytoplasmic) RXLR effector proteins that target the nucleus (Dou et al., 2008; Caillaud et al., 2012; Qiao et al., 2013) suggest that plant nuclear processes must present an important target for filamentous pathogens to achieve virulence (Birch et al., 2006; Morgan and Kamoun, 2007; Schornack et al., 2009). If true, nuclear effectors would carry the activities that allow modification of nuclear signaling networks and suppression of plant defences, providing useful tools for understanding the role of the plant nucleus during immunity.

CRN proteins were initially identified through their ability to cause crinkling and necrosis upon expression in plant tissue, and consequently this protein family is generally considered as a class of cell death inducing effectors (Torto et al., 2003). Recent studies, however, show that this is not a universal feature of either CRN proteins or their C-terminal effector domains. Expression of CRN effector domains leads to cell death in only a select few cases, suggesting diverse activities underpinning effector function (Haas et al., 2009; Schornack et al., 2010; Stam et al., 2013). Importantly, despite inducing cell death upon ectopic expression, infection assays revealed that only one CRN effector promotes virulence. Localization studies revealed distinct subnuclear localization patterns, further suggesting diverse functions in plants leading to cell death (Stam et al., 2013). In this paper, we expand on our work on CRN effectors and provide evidence suggesting diverse molecular events leading to cell death in plants. Comparative analyses between three necrosis-inducing CRN effector domains (DN17, D2, and DXZ) revealed differences in the timing and occurrence of cell death in *N. benthamiana*. Consistent with diverse effector activities, we show that expression of only one CRN domain has an additive effect on PAMP-induced cell death, suggestive of distinct effector induced perturbations affecting different nuclear processes. Confocal and OMX 3D-SIM microscopy on living cells substantiated these observations by showing distinct subnuclear localization patterns for each cell death inducing effector and crucially, specific effector induced changes in nuclear morphology, possibly leading to cell death. Taken together, our results suggest diverse functions carried out by CRN C-termini in the host nucleus that lead to cell death. We conclude that although cell death induction may not be a direct virulence function, it may represent an important phenotypic outcome, suited to study effector and target functions. A firm understanding of the molecular basis of CRN-induced changes to plant cells and nuclei in particular, will not only help understand CRN effector function, but also unveil novel nuclear processes that impact on cell death and immunity. We anticipate that ultimately, the study of nuclear effectors is pivotal to appreciate the nuclear processes that help determine infection outcomes.

MATERIALS AND METHODS

BACTERIAL CULTURE GROWTH, CULTURE FILTRATE PREPARATION PROCEDURES, PLANT GROWTH CONDITIONS, AND PHENOTYPE SCORING

For all experiments, *Agrobacterium tumefaciens* strain AGL1 was used as recipient strain for all constructs. AGL1 strains carrying respective constructs were grown in liquid cultures at 28°C (shaking at 225 rpm) until mid-log phase. Optical Density (OD) was measured (at 600 nm) and cells adjusted to relevant densities using infiltration media (described below). *P. capsici* culture filtrates (CFs) were prepared by inoculating liquid pea broth (PB) with mycelial plugs of strain LT1534. Cultures were incubated at 25°C in the dark without agitation for 5 days. CF was prepared by removing the mycelial mat after which the resulting liquid culture was filter sterilized. PB used as negative

controls was prepared simultaneously and sterilized before use in PTI assays. *Nicotiana benthamiana* plants were grown in a greenhouse under 16 h of light and maintained at a temperature of ∼25/22°C (day/night). For all experiments, 5-week old plants were used and kept under these conditions during the course of the experiment, unless otherwise stated. The level of cell death observed in plants during experiments was visually scored using a scale of 0–6, with a score of 0 indicating no symptoms, and a score of 6 indicating severe black necrotic lesions. This scale was used as described previously (Stam et al., 2013).

PREPARATION OF FUSION CONSTRUCTS

For construction of a GFP fusion construct containing the CRN N-terminus, corresponding gene fragments were amplified using primers 168080-F_BHI (5′-aaaaaggatccccGTGAAAGTGGA CGAAGGCGC-3′) and 168080_R_EcoRI (5′-aaaacgaattctaCG GAACCACCACCAGCACGTG-3′). For cloning of the mature gene coding fragment, primers 168080-F_BHI together with 20_624-R (5′-AAAAAGGCGCGCCTTATTGCAGCATCGCG TAAATTTTCCC-3′) and ASC-I-STREPII-TAG (5′-aaaaagcg gccGCTCACTTCTCGAACTGCGGGTGCGACCACCGGCGCG CC-3′) were used. *Bam*HI/*Eco*RI and *Bam*HI/*Asc*I digestions were performed for CRN-N terminal and mature protein constructs before ligation into pre-digested pENTR1a vector. Preparation of CRN C-terminal constructs has been described in Stam et al. (2013). pENTR1A-CRN constructs were sequence verified and used for recombination into the binary vector pB7WGF2 (Karimi et al., 2002), carrying a 35S promoter element and N-terminal GFP-fusion, using Gateway LR reactions (Life Technologies). Constructs were sequence verified before transformation into *A. tumefaciens* strain AGL1.

CRN INDUCED CELL DEATH ASSAYS

All EGFP-CRN effector domain fusion and control constructs were generated previously and prepared for infiltration as described in Stam et al. (2013). For cell death assays with CRN20_624 N-terminus, C-terminus and mature fusion proteins, all relevant cultures were adjusted to an OD of 1.0. Cultures were then mixed 1:1 with *A. tumefaciens* AGL1 cells carrying the silencing suppressor P19 at an OD of 1.0, giving a final OD of 0.5 for each CRN and 0.5 for P19. For experiments aimed to compare the kinetics of cell death induction upon ectopic expression of CRN20_624 (DN17), CRN83_152 (DXZ), and CRN79_188 (D2), ODs were adjusted to 0.5 for each culture and mixed with P19 in a 1:1 ratio (giving a final OD of 0.25). This OD proved to be optimal for monitoring cell death simultaneously for all of the CRNs. Plants were infiltrated with the bacterial suspensions and the level of cell death scored up to 7 days post-infiltration (dpi) as described above. Ten to twenty-five individual spot infiltrations were used per construct and all experiments were repeated at least three times. Means for the three CRN constructs were compared for each time point using One-Way ANOVA with SPSS Statistics 21. Graphs show average values for one representative experiment. In a complementary experiment, ion leakage measurements were taken during the time course. For each measurement, 8 leaf disks were harvested from *N. benthamiana* plants infiltrated as described above, and placed together in 10 ml of Milli Q H_2O and shaken at room temperature at 75 rpm for 2 h. After this time, total dissolved solids (TDS) were measured in the solution using a Primo pocket TDS tester (Hanna Instruments). For each time point and treatment, 6 individual measurements were taken from plants grown in 2 separate greenhouses.

PTI ASSAYS

A. tumefaciens AGL1 cells carrying EGFP-CRN fusion constructs were prepared and used for infiltrations as described above using a final OD of 0.25 for each effector. After 48 h, leaf panels were infiltrated with either CF generated from *P. capsici* liquid cultures or a control solution of PB media prepared as described above. Development of symptoms was recorded and the level of cell death was scored 48 h after CF treatment. We infiltrated and scored ten leaves for each construct as described above. The experiment was conducted three times. Statistical analysis was done using SPSS Statistics 21. Equality of the means was tested for each relevant pair of treatments, using the *t*-test with independent samples.

PTI MARKER GENE EXPRESSION ANALYSES

For qRT-PCR analyses, leaf panels expressing EGFP prepared as above, were treated with CF or a control solution of PB. After this second infiltration, 3 leaf discs (around 75 mg of tissue) were collected from individual plants at three different time points (1, 3, and 12 h post CF/PB infiltration). Tissues were then used for RNA extraction using the RNeasy plant mini kit (Qiagen). RNA was treated using the DNA-free kit (Ambion) following the manufacturers protocol. cDNA was synthesized using superscript III reverse transcriptase kit (Invitrogen). qPCR was performed using the Power SYBR Green kit (Applied Biosystems) following manufacturer's instructions. The primer pairs used are described in Nguyen et al. (2010) and have previously been used successfully for *P. infestans* CF (McLellan et al., Accepted): NbEF1α-F (5′-TGGACACAGGGACTTCATCA-3′) and NbEF1α-R (5′-CAAGGGTGAAAGCAAGCAAT-3′), NbPti5-F (5′-CCTCCAAG TTTGAGCTCGGATAGT-3′) and NbPti5-R (5′-CCAAGAAA TTCTCCATGCACTCTGTC-3′), NbAcre31-F (5′-AATTCGGC CATCGTGATCTTGGTC-3′) and NbAcre31-R (5′-GAGAAACT GGGATTGCCTGAAGGA-3′), and NbGras2-F (5′-TACCTAGC ACCAAGCAGATGCAGA-3′) and NbGras2-R (5′-TCATGAGG CGTTACTCGGAGCATT-3′).

The following cycle conditions were used for all primers: initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, with a plate read after each cycle. Melt curve reads were performed every 1°C between 60 and 95°C and held for 5 s. Expression levels of each gene induced by CF were calculated relative to expression in leaves mock-infiltrated with PB. Expression of marker genes was normalized to the *NbEF*1α endogenous control gene.

ETI ASSAYS

A. tumefaciens AGL1 cells carrying GFP-CRN fusion constructs, empty vector (EV), dexamethasone-inducible $Avr3a^{KI}$ (in pBAV105), R3a and the silencing suppressor P19 were prepared for infiltration as described above. Cultures carrying CRN, EV,

and P19 constructs were diluted to a final OD of 0.25, and those harboring Avr3a^{KI} and R3a were adjusted to a final OD of 0.1 before infiltration of plants. An OD of 0.1 was chosen for Avr3a^{KI} and R3a since higher ODs prevented an accurate comparison of the level of cell death between the three CRNs. For conditional expression of Avr3a^{KI}, 30 mM dexamethasone (DEX) in 0.1% Tween 20 was infiltrated into leaves 48 h after initial *Agrobacterium* infection as described by Engelhardt et al. (2012). As a negative control, we co-expressed R3a with the allelic variant Avr3a^{EM}, which is not recognized by R3a (Bos et al., 2009). Development of Avr3a^{KI}-R3a dependent cell death on CRN expressing leaves was assessed 24 h after DEX treatment, scored and tested for significance as described above.

WESTERN BLOTTING

Plant tissue was harvested 2, 3, and 4 dpi from infiltrated sites and frozen in liquid nitrogen. Protein extractions were performed on ground tissue using GTEN buffer (10% Glycerol, 25 mM Tris, 1 mM EDTA, 150 mM NaCl) supplemented with 2% PVPP, 10 mM DTT, and 1X Complete protease inhibitor cocktail (Thermo Scientific). Samples were run on Biorad TGX gels before transfer to PVDF membranes using the Biorad Trans Blot Turbo Transfer System. Blots were blocked for 30 min with 5% milk in TBS-T (0.1% Tween 20), probed with StrepII-HRP antibody (1:5000) (Genscript) to detect CRNs, and then washed three times in TBS-T for 5 min before incubation with Millipore Luminata Forte substrate. Images were collected on a Syngene GBox TX4 Imager. Blots were then re-probed with GFP antibody (Cambio) followed by anti Mouse-HRP antibodies (Santa Cruz) (1:20000), to detect free EGFP, and washed three times in TBS-T for 5 min before being imaged as before.

CONFOCAL IMAGING

For confocal microscopy, *A. tumefaciens* cells were resuspended in infiltration buffer (25 mM MgCl2 and 150 μM acetosyringone) to a final OD of 0.05—0.1 enabling CRN visualization while reducing the risk of observing over-expression artifacts. Control localizations with free EGFP were carried out using plants infiltrated with EV. For nucleolar imaging, *A. tumefaciens* GFP-CRN suspensions were combined 1:1 with *A. tumefaciens* cells carrying a RFP-Fibrillarin expression construct (Goodin et al., 2007) to give a final OD of 0.05 for the CRN and 0.05 for RFP-Fibrillarin. Confocal imaging was carried out 48 h post-infiltration. For DAPI staining, leaves were infiltrated with 4′,6-Diamidino-2-Phenylindole dilactate (Invitrogen) at a final concentration of 5 μg/ml. Subnuclear localization was examined on a Zeiss LSM 710 confocal microscope with a W Plan-Apochromat 40× /1.0 DIC M27 water dipping lens and using the following settings: GFP (488 nm excitation and 495–534 nm emission), mRFP (561 nm excitation and 592–631 nm emission) and DAPI (405 nm excitation and 415–481 nm emission). Cell viability was monitored during CRN localization using transmitted light detection. Confocal imaging for localization of the N- and C-terminus and mature protein was carried using *A. tumefaciens* at an OD of 0.1 and using a Leica SP2 with HCX APO L U-V-I 63.0× water dipping lens with 488 nm excitation wave length.

OMX 3D-SIM IMAGING

For OMX imaging, *A. tumefaciens* AGL1 cells transformed with GFP-CRN fusion constructs (CRN20_624, CRN83_152, and CRN79_188) were grown and prepared as described above to a final OD of 0.05. The bacterial suspensions were infiltrated into leaves of 5 week old *N. benthamiana* H2B-RFP transgenic plants (Martin et al., 2009) and *N. tabacum* plants grown and kept in the greenhouse as described above. OMX imaging was carried out 48 h post-infiltration. Epidermal peels were harvested from infiltrated leaf panels and placed immediately into an agarose pad (*N. benthamiana*) or in 70% glycerol (for *N. tabacum*) for imaging. OMX 3D-SIM was performed as described in Posch et al. (2010).

RESULTS

CRN20_624 INDUCED CELL DEATH AND LOCALIZATION ONLY REQUIRES THE C-TERMINAL EFFECTOR DOMAIN

CRN effectors are modular proteins harboring a conserved N-terminus required for translocation and C-terminal regions carrying effector activities (Haas et al., 2009; Schornack et al., 2010; Liu et al., 2011; Stam et al., 2013). Given their modularity and a possible impact of CRN N-termini on effector function, we assessed whether the N-terminus of CRN20_624 alters localization or cell death inducing activity. To assess and compare localization of the CRN20_624 N-terminus, the C-terminal effector domain as well as the mature protein were fused to EGFP, expressed in *N. benthamiana* leaves and localized by confocal microscopy in epidermal cells (**Figure 1**). Both mature protein and the C-terminal domain exclusively localized to the nucleus, suggesting that the CRN C-terminus drives nuclear localization of mature CRN protein (**Figure 1A**). Consistent with this, expression of the EGFP-tagged N-terminal domain contrasted

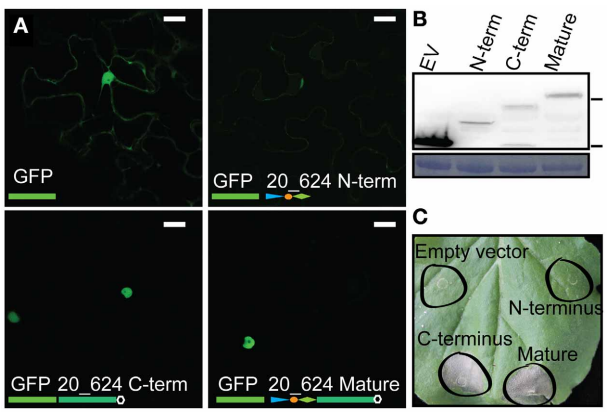

FIGURE 1 | CRN induced cell death and nuclear localization is conferred by the C-terminus. (A) Localization of ectopically expressed CRN-GFP fusion products. The panels show the localization for free eGFP, CRN20_624 N-terminus, CRN20_624 C-terminus, and mature CRN20_624 protein, 2 days post infiltration at OD 0.1 Scale bar = 25 μm. **(B)** Immunoblot analysis of CRN20_624 upon over-expression in plant tissue. The blot was probed with anti-GFP antibody. 70 and 25 kDa markers are indicated on the right hand side. Lower panel shows coomassie brilliant blue staining loading control. **(C)** Cell death inducing activity of CRN-GFP fusion products 7 days after infiltration at an OD of 1.0.

specific nuclear accumulation as this domain was found distributed throughout the cell, resembling distribution of free EGFP in the cytosol (**Figure 1A**). We used Western blot analysis to confirm that all EGFP-CRN domain fusions were expressed to similar levels *in planta*. Besides protein levels, these analyses revealed that resultant proteins were largely stable in plant cells as only low levels of EGFP cleavage was observed (**Figure 1B**). To test whether the presence of the N-terminus affects CRN induced cell death, we infiltrated *N. benthamiana* leaves with all CRN20_624 fusion constructs and the EV control. These experiments showed that both the mature protein and the C-terminus of CRN20_624 induce cell death at similar levels (**Figure 1C**). Consistent with our localization experiments, expression of the CRN N-terminus and GFP control only resulted in mild chlorosis. These data confirm that the CRN C-terminus is sufficient for nuclear accumulation and cell death inducing activity. Furthermore, these results suggest that the CRN N-terminus does not contribute to or impede effector activity once inside host cells.

ECTOPIC EXPRESSION OF CRN EFFECTOR DOMAINS LEADS TO DIFFERENT LEVELS OF CELL DEATH

Previously, we have shown that CRN20_624, CRN79_188, and CRN83_152 C-termini, classified as DN17, D2, and DXZ domains, respectively, induce cell death upon ectopic overexpression in *N. benthamiana* (Stam et al., 2013). Given that these three CRNs induce cell death but differentially affect *P. capsici* virulence in infection assays (Stam et al., 2013) we elected to compare and contrast CRN induced cell death phenotypes in more detail. To assess whether there are differences in cell death inducing activity, we expressed each CRN effector domain in *N. benthamiana* and scored for cell death across different time points (**Figure 2**). Assessment of cell death occurring from 1–7 days showed significant differences in the timing and level of cell death between the CRNs from day 2 to day 7 (ANOVA $p < 0.01$) (**Figure 2A**). Expression of CRN83_152 led to a fast cell death response, reaching maximum levels (6) within 4 days of agro-infiltration, whereas CRN79_188 only induced marginal levels of cell death in the course of this experiment. Compared to CRN83_152 and CRN79_188, CRN20_624 exhibited an intermediate phenotype in these assays. *Post-hoc* Bonferroni tests show that cell death scores for all three CRN proteins were significantly different ($p < 0.05$) on almost all days except for day 2, when CRN20_624 and CRN79_188 show no activity yet and day 7, where CRN20_624 and CRN83_152 both reached maximum cell death scores. We excluded the possibility of variation between leaves by expressing all CRNs and the EV on the same leaf (**Figure 2C**) and using multiple leaves in multiple experiments. To independently verify the levels of CRN induced cell death, we repeated these experiments and measured levels of ion leakage at 3 and 5 days (**Figure 2B**). Levels of ion leakage in infiltrated leaves differed significantly between CRN and EV constructs at both days and was consistent with macroscopic evaluation of CRN induced cell death (**Figure 2A**). CRN83_152 caused the greatest level of ion leakage determined by measuring TDS, while CRN79_188 caused ion leakage at levels just above those for the EV control. CRN20_624 expression led to ion leakage at levels between those seen for CRN83_152 and

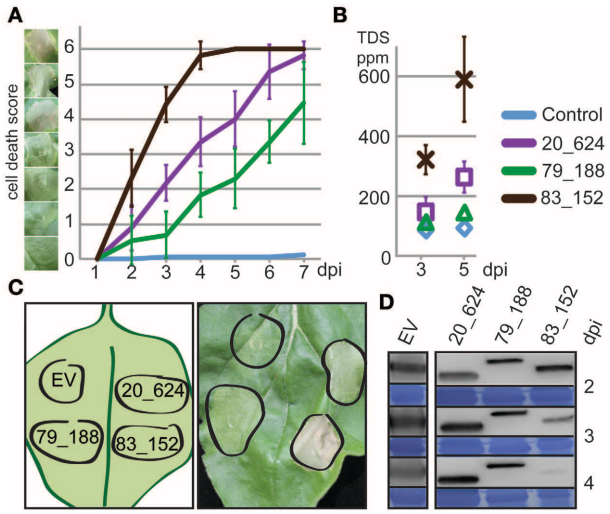

FIGURE 2 | Necrosis inducing CRNs show distinct cell death inducing dynamics. (A) Progression of cell death in *N. benthamiana* leaves infiltrated with CRNs. Cell death was scored every 24 h on a scale of 0–6. Example lesions for each score are shown on the left. The graph shows average values ± standard deviation for one representative experiment. **(B)** Ion leakage measurements confirming differences in cell death response. Each data point is an average of 6 measurements ± standard deviation. TDS ppm: total dissolved solids in parts per million. **(C)** Graphical representation of the experimental set-up (left) and a typical leaf 4 days post inoculation. **(D)** Western blots and loading control for CRNs and control samples showing protein levels up to 4 dpi. EV: GFP antibodies, CRNs: strepII antibodies.

79_188. Beyond 5 days it was not possible to measure ion leakage accurately, due to the advanced state of tissue necrosis. Given the possibility that differences in cell death induction are due to levels of CRN proteins, we measured and compared EGFP-CRN levels in a typical experiment at day 2, 3, and 4. Western blots (**Figure 2D**) showed slight variation in expression levels between CRN constructs, which was not correlated to cell death levels. Given the differences in levels of cell death induction and the similar levels of EGFP-CRN found accumulating in our experiments, we conclude that CRN induced cell death phenotypes are distinct and may reflect different effector activities.

CRN20_624 EXPRESSION HAS AN ADDITIVE EFFECT ON PAMP BUT NOT EFFECTOR INDUCED CELL DEATH

Given their proposed roles as virulence factors and the distinct differences in CRN sequence, cell death induction and subnuclear localization, we asked whether CRN effector activity leads to perturbation of host PTI or ETI signaling pathways. To test for effects on PAMP induced cell death, *N. benthamiana* leaf panels expressing EGFP-CRN fusion proteins and EGFP were infiltrated with *P. capsici* derived CFs and PB as negative control (**Figure 3**). Treatment of agro-infiltrated leaf panels with CF leads to PTI induction as qRT-PCR analyses on cDNA derived from EGFP-expressing leaf panels, treated with PB or CF, showed significant induction of PTI marker genes *NbPti5*, *NbAcre31*, and *NbGras2* when compared to expression in PB treated tissues at 1 and 12 h,

respectively (**Figure 3A**). Moreover, leaf panels expressing EGFP showed a specific cell death response to CF since infiltration of PB did not result in visible cell death (**Figures 3B,D**). Control experiments in which leaf panels expressing the *P. infestans* effector AVR3a were treated with CF, led to reduced cell death, suggesting suppression of CF induced response to PAMPs (data not shown). Interestingly, expression of CRN20_624 was found to have an additive effect on cell death induced by CF treatments in our experiments (**Figure 3B**). Direct comparisons of cell death between EV and CRN20_624 expressing leaf panels showed a significant increase of cell death ($p < 0.01$), which contrasted results obtained with CRN79_188. Although CRN79_188 induced some cell death without CF treatment, the combination of CRN79_188 with CF did not result in a stronger cell death response when compared to the EV control ($p = 0.8$) (**Figure 3B**). In these assays, we were not able to assess the effect of CRN83_152 on PTI since we could not find significant differences in the levels of cell death between CF treatment and the PB control (*t*-test for equality of means, $p = 1$) (**Figure 3B**). These results indicate that the CRN effector activities leading to cell death are distinct and in the case of CRN20_624, intersect with other cell death pathways in plants.

Given that CRN20_624 has an additive effect on cell death upon CF treatment, we asked whether any of our effectors affect ETI mediated cell death (**Figure 4**). To test this, we over-expressed CRN20_624, CRN83_152, and CRN79_188 in *N. benthamiana* leaves with R3a whilst also introducing *P. infestans* Avr3a^{KI} and Avr3a^{EM} coding genes under a DEX inducible promoter. In these assays, Avr3a^{EM} served as a negative control, as it is not recognized by R3a (Bos et al., 2009). Co-infiltration of CRN fusion proteins and EGFP in combination with R3a and AVR3a constructs, allowed us to express CRN fusion proteins with R3a first before activating Avr3a^{KI} induced ETI with DEX treatment. Phenotypic assessment of leaf panels 24 h after DEX induction revealed robust HR development. In these assays, there was no evidence of either enhanced or reduced ETI responses in CRN expressing leaves based on direct comparisons to our EV controls (ANOVA, $p = 1$). These results suggest that the presence of these CRN effectors does not affect ETI induced cell death. As expected, induction of AVR3a^{EM} expression in the presence of R3a did not lead to HR demonstrating that the observed cell death was due to specific recognition of AVR3a^{KI}. From these results, we conclude that CRN20_624 specifically promotes PAMP induced cell death. We suggest that the contrasting observations between CRN proteins reflect differences in effector functions, each of which leads to cell death upon ectopic expression.

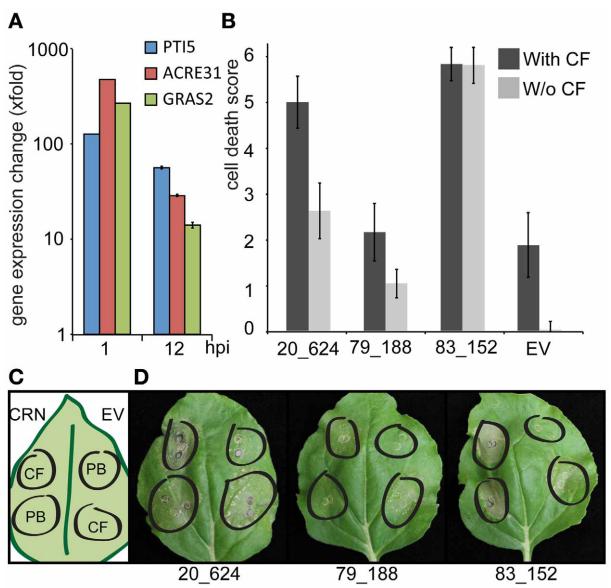

FIGURE 3 | Expression of CRN20_624, but not CRN79_188 and CRN83_152 has an additive effect on PAMP induced cell death. (A) qRT-PCR analyses on cDNA derived from leaf panels transiently expressing EGFP and treated with PB and CF. Each bar represents the fold change in gene expression upon CF treatment relative to PB ± standard deviation. Expression was examined for known PTI marker genes *NbPti5*, *NbAcre31*, and *NbGras2* at 1 and 12 h post infiltration (hpi). **(B)** Graph showing average necrosis scores ± standard deviation for three independent experiments. **(C)** Graphical representation of the experimental set-up. CRN-GFP fusion constructs were infiltrated into *N. benthamiana* plants and after 48 h, leaves were infiltrated with either a PAMP cocktail (*Phytophthora capsici* culture filtrate) or a control solution of pea broth. Cell death was scored on a scale of 0–6, 48 h after CF treatment. **(D)** Examples of representative leaves for each treatment on day of scoring.

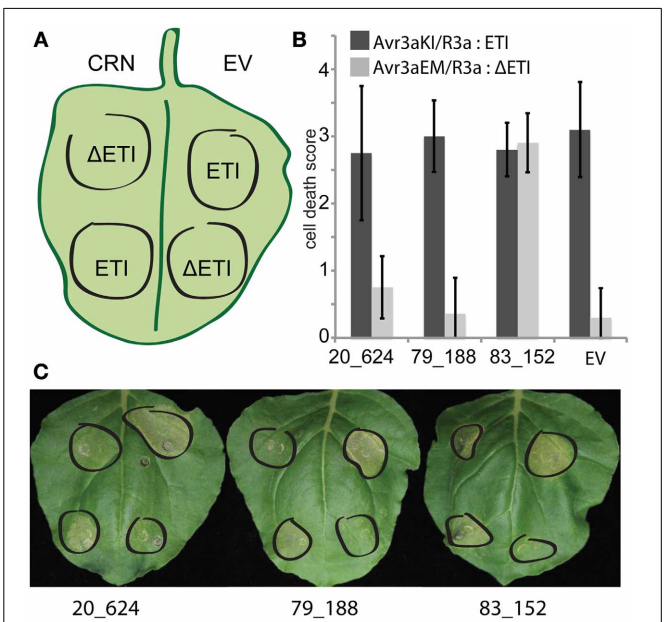

FIGURE 4 | Necrosis inducing CRNs do not cause altered ETI responses within the plant. (A) Graphical representation of the experimental set-up. CRN-GFP fusion and EV constructs were co-infiltrated into *N. benthamiana* leaves with Avr3a^{KI} and R3a (ETI), and Avr3a^{EM} and R3a (ΔETI) to monitor ETI responses. After 48 h, leaves were infiltrated with dexamethasone and incubated for a further 24 h for induction of Avr3a expression. **(B)** Cell death in response to CRNs in ETI and non-ETI induced leaves scored on a scale of 0–6. Graph shows average necrosis scores ± standard deviation for one representative experiment. **(C)** Examples of representative leaves for each treatment on day of scoring.

CRN EFFECTOR DOMAINS FEATURE DISTINCT SUBNUCLEAR LOCALIZATION AND THEIR ECTOPIC EXPRESSION CAUSES DISTINCT CHANGES IN HOST NUCLEAR MORPHOLOGY

We have presented evidence suggesting that CRN83_152, CRN20_624, and CRN79_188 feature distinct cell death phenotypes and differentially affect cell death pathways. Given their distinct localization patterns upon over-expression (Stam et al., 2013) we asked whether localization of nuclear markers during CRN expression would allow further insights into the onset of cell death in plants. Confocal microscopy was used to determine the nuclear localization of EGFP-CRN proteins as well as the nucleolar marker Fibrillarin and nuclear DNA. CRN20_624 showed a clustered distribution pattern confined to the nucleoplasm that contrasted localization of CRN79_188. Expression of CRN79_188 consistently led to the detection of filament-like structures in the nucleus. In contrast, CRN83_152 was present in patches within the nucleus, with clear areas of nuclear space in which EGFP-CRN83_152 protein appeared absent (**Figure 5**). These patterns were observed in living cells as cytoplasmic streaming was evident in cells expressing all EGFP-CRN fusions (**Supplementary videos 1–4**). Interestingly, distribution of DAPI stained nuclear DNA appeared altered in cells expressing CRN83_152 (**Figure 5A**), suggesting re-localization of host chromatin.

To confirm this observation, we expressed EGFP-CRN83_152 in transgenic *N. benthamiana* plants carrying histone-RFP (**Figure 5B**). These experiments revealed that consistent with our observation on DAPI stained DNA, over-expression of CRN83_152 caused Histone 2B-RFP labeled DNA to accumulate in distinct patches within the nucleus. In these assays, CRN83_152 was found to accumulate in areas in the nucleus from which DNA had been excluded. Consequently, CRN83_152 and DAPI/Histone 2B-RFP signal did not co-localize in both of our experiments (**Figures 5A,B**). In contrast to CRN83_152, over-expression of CRN20_624 and CRN79_188 did not alter the distribution of DNA. DAPI and histone-RFP signal were detected evenly within the nuclear space, with only some small patches where DNA was absent, similar to the pattern observed for cells expressing free GFP (**Figures 5A,B**). To exclude the possibility of changes in nuclear morphology after cell death, we repeated these assays whilst confirming cell viability by assessing cytoplasmic streaming and vesicle movement within the cytoplasm during CRN and EGFP expression. In these experiments, nuclear reorganization caused by expression of CRN83_152 did not appear to affect cell viability within the time scale of these experiments (**Supplementary video 3**).

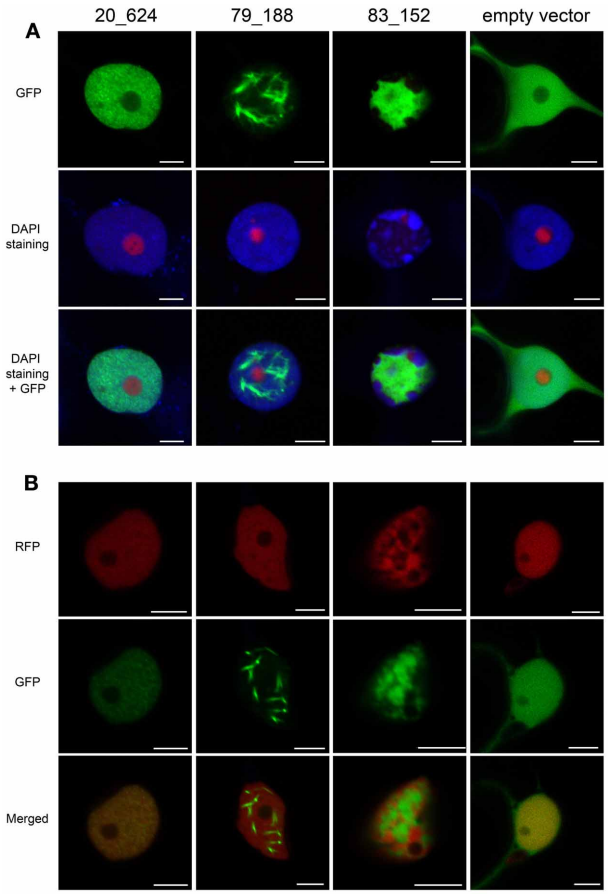

FIGURE 5 | CRN 83_152 causes re-localization of DNA within the nucleus. CRN-GFP fusion constructs were over expressed in *N. benthamiana* plants and imaged by confocal microscopy 48 h post-infiltration. **(A)** Leaves were co-infiltrated with RFP-fibrillarin and were DAPI stained by infiltrating with 4′,6-Diamidino-2-Phenylindole dilactate at a final concentration of 5 μg/ml. **(B)** *N. benthamiana* plants stably expressing histone RFP were infiltrated with CRN-GFP fusion constructs as described above. Scale bars = 5 μm.

3D-SIM IMAGING OF CRN EFFECTORS REVEALS DISTINCT LOCALIZATION WITHIN THE NUCLEUS

Using confocal microscopy, we observed distinct subnuclear localization and structures upon expression of the three CRN effectors characterized in this study (**Figure 5**). To gain a better understanding of these results, we used super-resolution 3D structured illumination microscopy (3D-SIM) to visualize the possible structures CRN proteins form or interact with at the subnuclear level (**Figure 6**). 3D-SIM imaging of *N. benthamiana* leaves expressing Histone 2B-RFP and EGFP-CRN fusions confirmed localization patterns observed in our confocal microscopy experiments for CRN83_152 and CRN79_188 (**Figure 5**). CRN20_624 was found distributed in clusters throughout the nucleoplasm in close proximity to Histone 2B-RFP labeled chromatin (**Figure 6A**). CRN79_188 was found to form regular and evenly distributed fibril-like structures interspersed with chromatin (**Figure 6B**). In both cases, distribution of chromatin in the nucleus is not impaired. High-resolution images, however, shines a different light on CRN83_152 localization. Whereas confocal images suggest that CRN83_152 localizes in a uniform manner in patches within the nucleoplasm, OMX microscopy reveals that these patches consist of long and undulating strands, surrounding areas of re-localized chromatin. This is particularly evident in single plane images (**Figure 6B**) and could not be observed with confocal microscopy.

Although the mechanism of chromatin exclusion or degradation in the presence of CRN83_152 is yet elusive, our results suggest that one mechanism of cell death induction could rely

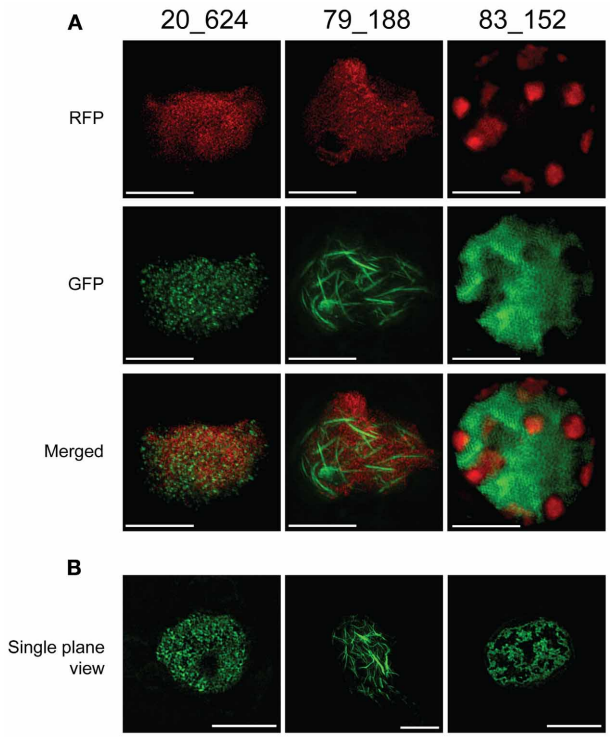

FIGURE 6 | 3D-SIM imagining of CRN C-terminal constructs on OMX confirms distinct localization within the nucleus. (A) Projection view of 3D-SIM images on epidermal peels of *N. benthamiana* H2B-RFP transgenic lines infiltrated with CRN-GFP fusion constructs. Histone 2B distribution is shown in RFP channel whereas CRN protein distribution is in the GFP channel. Merged channel confirms impairment of chromatin distribution in CRN83_152 expressing cells. **(B)** Single plane views of 3D-SIM images on epidermal peels of *N. tabacum* infiltrated with CRN-GFP fusion constructs show distinct nuclear distribution patterns for CRN effector proteins. All images were taken 48 h after infiltration. Scale bars = 5 μm.

on the modification of chromatin affecting its integrity and consequently, disrupting important host cell processes.

DISCUSSION

CRN effectors are considered a diverse and ubiquitous class of effectors found in all plant pathogenic oomycetes sequenced to date. Consequently, various studies have hinted at a role in virulence, suppression of PTI and more recently, ETI (Liu et al., 2011; Van Damme et al., 2012; Shen et al., 2013; Stam et al., 2013). Here we provide further evidence of functional diversity amongst *P. capsici* CRN proteins by studying the activity of three necrosis-inducing effectors. Consistent with previous studies, we show that for CRN20_624, the N-terminal region does not affect cell death induction or localization, suggesting that only the C-terminal effector domain is required for function in the nucleus. Although only shown for CRN20_624, this work further supports the observation that nuclear localization is required for cell death induction as shown for *P. infestans* CRN8 (Schornack et al., 2010) and that CRN C-termini carry the cell death inducing activity (Torto et al., 2003; Haas et al., 2009). We demonstrate that based on timing and intensity of cell death as well as their effects on CF triggered cell death, CRN20_624, CRN79_188, and CRN83_152 have distinct activities *in planta*.

Macroscopic evaluation of cell death as well as ion leakage measurements upon CRN expression revealed that CRN83_152 expression causes rapid cell death and tissue collapse, whereas CRN79_188 causes delayed cell death and CRN20_624 features an intermediate phenotype. Western blot analyses revealed that all EGFP-CRN fusions accumulated to similar levels, suggesting that differences in cell death reflect distinct activities rather than effector abundance. This observation is further illustrated by the diverse CRN localization patterns as well as distinct changes in nuclear morphology and DNA distribution upon CRN83_152 accumulation.

Besides cell death induction, we have presented evidence that CRN20_624, but not CRN83_152 and CRN79_188, has an additive effect on PAMP induced cell death. Treatment of CRN expressing leaves with either PB or CFs showed a marked increase in cell death on CF treated panels, suggesting modification of PAMP induced cell death signaling. These results contrasted with cell death induced by recognition of the *P. infestans* effector AVR3a by R3a. Importantly, these results could suggest that CRN20_624 activity induces specific cell death pathways, excluding those associated with ETI. If true, this would mean that CRN proteins could be used to classify and study PAMP triggered nuclear signaling pathways. CRN20_624 mediated promotion of PTI is counter-intuitive as effectors are generally thought of as suppressors of PTI. It is possible, however, that PTI stimulation represents a virulence function in the late stages of a hemi-biotrophic lifecycle, when cell death and tissue collapse is apparent (Jupe et al., 2013). Interestingly, CRN20_624, which contains the DN17 C-terminal domain, is expressed at later stages during infection, coinciding with the switch of *P. capsici* from a biotrophic to a necrotrophic lifestyle (Stam et al., 2013). This adds additional weight to a model in which *P. capsici* deploys effectors to co-opt host PTI signaling pathways and promote cell death. If true, the identification and engineering of CRN20_624 host targets may allow reduction of cell death during *P. capsici* infection and slow disease progression.

Consistent with diverse functions, we reveal distinct subnuclear localization patterns for the CRN effectors studied here. Detailed co-localization studies of CRN83_152, CRN20_624, and CRN79_188 together with DAPI staining as well as nucleolar and chromatin markers, not only confirmed the organization of EGFP-CRN proteins in distinct patterns, but unveiled unexpected changes in the organization of nuclear chromatin upon expression of CRN83_152. Multiple localization experiments showed that CRN83_152 occupies the nuclear space around DAPI and H2B-RFP labeled patches of DNA. 3D-SIM high resolution microscopy not only confirmed these observations but added additional detail, showing organization of CRN83_152 in intricately organized convoluted structures, wrapping around or in close proximity to nuclear chromatin. At this stage, we do not know the molecular basis or function of CRN83_152. Although we have previously shown that CRN83_152 enhances *P. capsici* virulence, we do not know the relevance of chromatin reorganization toward immunity or susceptibility. Studies currently on the way in our group will aim to identify the principal targets for CRN83_152 and study their role in immunity. It is likely that these studies will help unveil novel processes underpinning *Phytophthora* virulence.

In contrast to CRN83_152, microscopy revealed that CRN79_188 is distributed in long thin filamentous strands. Importantly, we found that these filaments are uniform and evenly distributed throughout the nucleoplasm. These results suggest that CRN79_188 either forms these structures by itself or interacts with yet unknown structures in the nucleus. In this regard, the recent identification of F-actin containing structures in plant cells containing the Turnip Vein Clearing Virus movement protein MP-TVCV (Levy et al., 2013), raises this possibility.

Based on our results, we question as to whether cell death induction is a direct virulence function or rather, is a feature that is an indirect consequence of (distinct) effector activities. Ectopic expression in plant cells led to rapid accumulation of CRN proteins in *N. benthamiana* cells to levels that are unlikely to occur *in vivo* during infection. We also cannot exclude that perception of bacterial PAMPs has an impact on our results in the case of our cell death assays. However, leaf panels expressing EGFP remained healthy, showing low levels of ion leakage and were responsive to CF treatment as evidenced by induction of PTI marker genes and occurrence of CF-specific cell death in our experiments. Whether priming of defence responses affect levels of cell death or not, the differences in cell death kinetics for the CRN effectors tested were consistent and significant across our experiments. Because of the necessity for both an epitope tag and fluorescent reporter, we have used EGFP-CRN protein fusions for this work. We can therefore not formally exclude the possibility that the presence of N-terminal EGFP affects CRN function or activity levels. Given the observations that CRN proteins are modular in nature and mature CRN proteins also feature sizeable N-terminal region that does not appear to affect function or localization for CRN20_624 (**Figure 1**), this is not a likely scenario.

Taken together, our work suggests distinct differences in cell death mediated by diverse CRN effector activities. These findings are thus consistent and build on previous work, which showed differential effects of CRN over-expression on *P. capsici* virulence (Stam et al., 2013). This study further supports the emerging view that through yet unknown mechanisms this ancient class of effector proteins act on processes required for plant immunity. With an increasing number of nuclear host defence signaling components identified in plants together with pathogen effectors that target the nucleus, there is a critical need to understand the nuclear processes that drive plant immunity and ETS. Our results strongly suggest that exploring the functions of CRN effectors, including those that induce cell death, will uncover immunity-associated nuclear processes in the host. Given the enormous diversity of nuclear effectors now identified in the oomycetes, these proteins form a rich source of molecular probes suited to study nuclear biology. CRN effectors and other nuclear effectors will thus emerge as valuable tools to unravel nuclear processes involved in plant immunity.

ACKNOWLEDGMENTS

The Authors are grateful to the referees for their constructive comments. We would like to thank Prof. Paul Birch for providing Dexamethasone inducible Avr3a and R3a binary vectors. We are grateful to Dr. Markus Posch for his technical assistance and expertise offered during OMX microscopy experiments. Use of the OMX microscope was funded by an MRC Next Generation Optical Microscopy Award (Ref: MR/K015869/1). The Huitema lab is funded by the Biotechnology and Biological Sciences Research Council, the European Research Council and the Royal Society of Edinburgh.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://figshare.com/articles/Characterisation_of_cell_death_inducing_Phytophthora_capsici_CRN_effectors_suggests_diverse_activities_in_the_host_nucleus/787690

Supplementary video 1 | Time-lapse video of cells expressing CRN 20_624. *N. benthamiana* plants were infiltrated with CRN 20_624 at a final OD of 0.05 and video images captured 48 h post-infiltration using transmitted light detection. Videos show vesicle movement within the cytoplasm during CRN expression. Video was taken using a 40× water dipping lens and a 3.2× zoom (Scale bar = 20 μm).

Supplementary video 2 | Time-lapse video of cells expressing CRN 79_188. *N. benthamiana* plants were infiltrated with CRN 79_188 at a final OD of 0.05 and video images captured 48 h post-infiltration using transmitted light detection. Videos show vesicle movement within the cytoplasm during CRN expression. Video was taken using a 40× water dipping lens and a 3.3× zoom (Scale bar = 20 μm).

Supplementary video 3 | Time-lapse video of cells expressing CRN 83_152. *N. benthamiana* plants were infiltrated with CRN 83_152 at a final OD of 0.05 and video images captured 48 hours post-infiltration using transmitted light detection. Videos show vesicle movement within the cytoplasm during CRN expression. Video was taken using a 40× water dipping lens and a 1.2× zoom (Scale bar = 20 μm).

Supplementary video 4 | Time-lapse video of cells expressing pB7WGF2 empty vector (EV). *N. benthamiana* plants were infiltrated with EV at a final OD of 0.05 and video images captured 48 h post-infiltration using transmitted light detection. Videos show vesicle movement within the cytoplasm during free GFP expression. Video was taken using a 40× water dipping lens and a 4.6× zoom (Scale bar = 20 μm).

REFERENCES

Birch, P. R. J., Rehmany, A. P., Pritchard, L., Kamoun, S., and Beynon, J. L. (2006). Trafficking arms: oomycete effectors enter host plant cells. *Trends Microbiol.* 14, 8–11. doi: 10.1016/j.tim.2005.11.007

Bos, J. I. B., Armstrong, M. R., Gilroy, E. M., Boevink, P. C., Hein, I., Taylor, R. M., et al. (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. doi: 10.1073/pnas.0914408107

Bos, J. I. B., Chaparro-Garcia, A., Quesada-Ocampo, L. M., McSpadden Gardener, B. B., and Kamoun, S. (2009). Distinct amino acids of the *Phytophthora infestans* effector AVR3a condition activation of R3a hypersensitivity and suppression of cell death. *Mol. Plant Microbe Interact.* 22, 269–281. doi: 10.1094/MPMI-22-3-0269

Caillaud, M.-C., Piquerez, S. J. M., Fabro, G., Steinbrenner, J., Ishaque, N., Beynon, J., et al. (2012). Subcellular localization of the Hpa RxLR effector repertoire identifies a tonoplast-associated protein HaRxL17 that confers enhanced plant susceptibility. *Plant J.* 69, 252–265.

doi: 10.1111/j.1365-313X.2011.04787.x

Deslandes, L., and Rivas, S. (2011). The plant cell nucleus: a true arena for the fight between plants and pathogens. *Plant Signal. Behav.* 6, 42–48. doi: 10.4161/psb.6.1.13978

Dou, D., Kale, S. D., Wang, X., Jiang, R. H. Y., Bruce, N. A., Arredondo, F. D., et al. (2008). RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. *Plant Cell* 20, 1930–1947. doi: 10.1105/tpc.107.056093

Engelhardt, S., Boevink, P. C., Armstrong, M. R., Ramos, M. B., Hein, I., and Birch, P. R. J. (2012). Relocalization of late blight resistance protein R3a to endosomal compartments is associated with effector recognition and required for the immune response. *Plant Cell* 24, 5142–5158. doi: 10.1105/tpc.112.104992

Gaulin, E., Madoui, M.-A., Bottin, A., Jacquet, C., Mathé, C., Couloux, A., et al. (2008). Transcriptome of *Aphanomyces euteiches*: new oomycete putative pathogenicity factors and metabolic pathways. *PLoS ONE* 3:e1723. doi: 10.1371/journal.pone.0001723

Goodin, M. M., Chakrabarty, R., Banerjee, R., Yelton, S., and DeBolt, S. (2007). New gateways to discovery. *Plant Physiol.* 145, 1100–1109. doi: 10.1104/pp.107.106641

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., et al. (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461, 393–398. doi: 10.1038/nature08358

Hann, D. R., Gimenez-Ibanez, S., and Rathjen, J. P. (2010). Bacterial virulence effectors and their activities. *Curr. Opin. Plant Biol.* 13, 388–393. doi: 10.1016/j.pbi.2010.04.003

Hogenhout, S. A., Van der Hoorn, R. A., Terauchi, R., and Kamoun, S. (2009). Emerging concepts in effector biology of plant-associated organisms. *Mol. Plant Microbe Interact.* 22, 115–122. doi: 10.1094/MPMI-22-2-0115

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

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

Jupe, J., Stam, R., Howden, A. J. M., Morris, J. A., Zhang, R., Hedley, P. E., et al. (2013). *Phytophthora capsici*-tomato interaction features dramatic shifts in gene expression associated with a hemi-biotrophic lifestyle. *Genome Biol.* 14:R63. doi: 10.1186/gb-2013-14-6-r63

Karimi, M., Inzé, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.* 7, 193–195. doi: 10.1016/S1360-1385(02)02251-3

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12, 2339–2350. doi: 10.2307/3871233

Lamour, K. H., Mudge, J., Gobena, D., Hurtado-Gonzales, O. P., Schmutz, J., Kuo, A., et al. (2012). Genome sequencing and mapping reveal loss of heterozygosity as a mechanism for rapid adaptation in the vegetable pathogen *Phytophthora capsici*. *Mol. Plant Microbe Interact.* 25, 1350–1360. doi: 10.1094/MPMI-02-12-0028-R

Lamour, K. H., Win, J., and Kamoun, S. (2007). Oomycete genomics: new insights and future directions. *FEMS Microbiol. Lett.* 274, 1–8. doi: 10.1111/j.1574-6968.2007.00786.x

Lévesque, C. A., Brouwer, H., Cano, L., Hamilton, J. P., Holt, C., Huitema, E., et al. (2010). Genome sequence of the necrotrophic plant pathogen *Pythium ultimum* reveals original pathogenicity mechanisms and effector repertoire. *Genome Biol.* 11:R73. doi: 10.1186/gb-2010-11-7-r73

Levy, A., Zheng, J. Y., and Lazarowitz, S. G. (2013). The tobamovirus turnip vein clearing virus 30-kilodalton movement protein localizes to novel nuclear filaments to enhance virus infection. *J. Virol.* 87, 6428–6440. doi: 10.1128/JVI.03390-12

Links, M. G., Holub, E., Jiang, R. H. Y., Sharpe, A. G., Hegedus, D., Beynon, E., et al. (2011). De novo sequence assembly of *Albugo candida* reveals a small genome relative to other biotrophic oomycetes. *BMC Genomics* 12:503. doi: 10.1186/1471-2164-12-503

Liu, J., and Coaker, G. (2008). Nuclear trafficking during plant innate immunity. *Mol. Plant* 1, 411–422. doi: 10.1093/mp/ssn010

Liu, T., Ye, W., Ru, Y., Yang, X., Gu, B., Tao, K., et al. (2011). Two host cytoplasmic effectors are required for pathogenesis of *Phytophthora sojae* by suppression of host defenses. *Plant Physiol.* 155, 490–501. doi: 10.1104/pp.110.166470

Martin, K., Kopperud, K., Chakrabarty, R., Banerjee, R., Brooks, R., and Goodin, M. M. (2009). Transient expression in *Nicotiana benthamiana* fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta. *Plant J.* 59, 150–162. doi: 10.1111/j.1365-313X.2009.03850.x

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15, 349–357. doi: 10.1016/j.pbi.2012.05.006

Morgan, W., and Kamoun, S. (2007). RXLR effectors of plant pathogenic oomycetes. *Curr. Opin. Microbiol.* 10, 332–338. doi: 10.1016/j.mib.2007.04.005

Nguyen, H. P., Chakravarthy, S., Velásquez, A. C., McLane, H. L., Zeng, L., Nakayashiki, H., et al. (2010). Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol. Plant Microbe Interact.* 23, 991–999. doi: 10.1094/MPMI-23-8-0991

Oliva, R., Win, J., Raffaele, S., Boutemy, L., Bozkurt, T. O., Chaparro-Garcia, A., et al. (2010). Recent developments in effector biology of filamentous plant pathogens. *Cell. Microbiol.* 12, 705–715. doi: 10.1111/j.1462-5822.2010.01471.x

Pandey, S. P., and Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* 150, 1648–1655. doi: 10.1104/pp.109.138990

Park, C.-J., and Ronald, P. C. (2012). Cleavage and nuclear localization of the rice XA21 immune receptor. *Nat. Commun.* 3, 920. doi: 10.1038/ncomms1932

Posch, M., Khoudoli, G. A., Swift, S., King, E. M., Deluca, J. G., and Swedlow, J. R. (2010). Sds22 regulates aurora B activity and microtubule-kinetochore interactions at mitosis. *J. Cell Biol.* 191, 61–74. doi: 10.1083/jcb.200912046

Qiao, Y., Liu, L., Xiong, Q., Flores, C., Wong, J., Shi, J., et al. (2013). Oomycete pathogens encode RNA silencing suppressors. *Nat. Genetics* 45, 330–333. doi: 10.1038/ng.2525

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

Rivas, S. (2012). Nuclear dynamics during plant innate immunity. *Plant Physiol.* 158, 87–94. doi: 10.1104/pp.111.186163

Schornack, S., Huitema, E., Cano, L. M., Bozkurt, T. O., Oliva, R., Damme, M. V. A. N., et al. (2009). Ten things to know about oomycete effectors. *Mol. Plant Pathol.* 10, 795–803. doi: 10.1111/j.1364-3703.2009.00593.x

Schornack, S., van Damme, M., Bozkurt, T. O., Cano, L. M., Smoker, M., Thines, M., et al. (2010). Ancient class of translocated oomycete effectors targets the host nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17421–17426. doi: 10.1073/pnas.1008491107

Shen, D., Liu, T., Ye, W., Liu, L., Liu, P., Wu, Y., et al. (2013). Gene duplication and fragment recombination drive functional diversification of a superfamily of cytoplasmic effectors in phytophthora sojae. *PLoS ONE* 8:e70036. doi: 10.1371/journal.pone.0070036

Shen, Q.-H., and Schulze-Lefert, P. (2007). Rumble in the nuclear jungle: compartmentalization, trafficking, and nuclear action of plant immune receptors. *EMBO J.* 26, 4293–4301. doi: 10.1038/sj.emboj.7601854

Stam, R., Jupe, J., Howden, A. J. M., Morris, J. A., Boevink, P. C., Hedley, P. E., et al. (2013). Identification and characterisation of CRN effectors in *Phytophthora capsici* shows modularity and functional diversity. *PLoS ONE* 8:e59517. doi: 10.1371/journal.pone.0059517

Stergiopoulos, I., and de Wit, P. J. (2009). Fungal effector proteins. *Annu. Rev. Phytopathol.* 47, 233–263. doi: 10.1146/annurev.phyto.112408.132637

Torto, T. A., Li, S., Styer, A., Huitema, E., Testa, A., Gow, N. A., et al. (2003). EST mining and functional expression assays identify extracellular effector proteins from the plant pathogen Phytophthora. *Genome Res.* 13, 1675–1685. doi: 10.1101/gr.910003

Tyler, B. M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R. H. Y., Aerts, A., et al. (2006). Phytophthora genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* 313, 1261–1266. doi: 10.1126/science.1128796

Van Damme, M., Bozkurt, T. O., Cakir, C., Schornack, S., Sklenar, J., Jones, A. M. E., et al. (2012). The irish potato famine pathogen *Phytophthora infestans* translocates the CRN8 kinase into host plant cells. *PLoS Pathog.*

8:e1002875. doi: 10.1371/journal.ppat.1002875

Yeam, I., Nguyen, H. P., and Martin, G. B. (2010). Phosphorylation of the *Pseudomonas syringae* effector AvrPto is required for FLS2/BAK1-independent virulence activity and recognition by tobacco. *Plant J.* 61, 16–24. doi: 10.1111/j.1365-313X.2009.04028.x

Zipfel, C. (2008). Pattern-recognition receptors in plant innate immunity. *Curr. Opin. Immunol.* 20, 10–16. doi: 10.1016/j.coi.2007.11.003

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: 31 May 2013; accepted: 11 September 2013; published online: 21 October 2013.

*Citation: Stam R, Howden AJM, Delgado-Cerezo M, M M Amaro TM, Motion GB, Pham J and Huitema E (2013) Characterization of cell death inducing Phytophthora capsici CRN effectors suggests diverse activities in the host nucleus. Front. Plant Sci. **4**:387. doi: 10.3389/fpls.2013.00387*

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

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 03 September 2013
doi: 10.3389/fpls.2013.00333

Predicting promoters targeted by TAL effectors in plant genomes: from dream to reality

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Reviewed by:
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Keywords: TAL effectors, targets, prediction, *Xanthomonas*, susceptibility genes

INTRODUCTION

Transcription Activator-Like (TAL) effectors from the plant pathogenic bacteria of the genus *Xanthomonas* are molecular weapons injected into eukaryotic cells to modulate the host transcriptome. Upon delivery, TAL effectors localize into the host cell nucleus and bind to the promoter of plant susceptibility (S) genes to activate their expression and thereby facilitate bacterial multiplication (Boch and Bonas, 2010; Schornack et al., 2013). In resistant plants, a few TAL effectors have been shown to bind to promoters of executor resistance (*R*) genes, resulting in localized cell death and preventing pathogen spread (reviewed in Doyle et al., 2013). Remarkably, TAL effectors harbor a novel type of DNA-binding domain with a unique modular architecture composed of 1.5–33.5 almost identical tandem repeats of 33–35 amino acids. Each repeat type specifies one or more bases through direct interaction with the second amino acid in a centrally located "Repeat Variable Diresidue" (RVD). The number and sequence of the RVDs across the whole repeat region of the TAL protein defines the DNA target. The code of DNA-binding specificity of *Xanthomonas* TAL effectors was inferred from experimental, computational and later on structural approaches (Boch et al., 2009; Moscou and Bogdanove, 2009; Deng et al., 2012; Mak et al., 2012). This new paradigm for protein-DNA interaction is now revolutionizing our perspectives for the understanding of TAL effectors roles during plant disease and defense since the identification of their plant targets is largely facilitated. A few algorithms are now available to predict *in silico* candidate genes of a given TAL effector. This *Opinion* gives an overview of the current tools and strategies that may be applied for finding targets of TAL effectors. We also raise limitations and pitfalls and emphasize what may be improved to gain in prediction accuracy. Finally, we also highlight several perspectives offered by these new tools.

In silico PREDICTION OF TAL EFFECTORS TARGETS

One major output of the modular TAL effector–DNA recognition code discovery is the possibility to predict through computer programs, the DNA binding sites of a TAL effector within a whole plant genome or promoterome (i.e., the sequences immediately upstream of the transcriptional start sites) of any sequenced organism. Four bio-informatic tools are currently available and enable to scan genomes for TAL effectors binding sites, rapidly providing users with lists of potential *S* or *R* targets. *Target Finder* from the TALE-NT 2.0 suite (https://tale-nt.cac.cornell.edu/, Doyle et al., 2012), *Talvez* (http://bioinfo.mpl.ird.fr/cgi-bin/talvez/talvez.cgi, Pérez-Quintero et al., 2013) and *Storyteller* (http://bioinfo-prod.mpl.ird.fr/xantho/tales, Pérez-Quintero et al., 2013) algorithms are available as web interface and/or standalone software. For these three examples, predictions rely on the use of a RVD-nucleotide association matrix based on known TAL effector–target pairs, to convert a sequence of RVDs of a given TAL effector into a positional weight matrix (PWM). These PWM are regularly updated based on novel experimental insights into TAL-DNA binding or the availability of experimentally confirmed TAL target sequences. *Target Finder* and *Talvez* both use the PWM to scan and score all possible binding sites in a promoter region with a log-likelihood function. In contrast, *Storyteller* uses this matrix to generate a set of possible binding sequences and takes the advantage of a faster pattern-search algorithm based on Hidden Markov models. Moreover, *Talvez* incorporates a position correction parameter, which enables to tolerate RVD-nucleotide mismatches toward the C-terminal end of RVD sequences and improves target sites prediction. Finally, *TALgetter* (http://galaxy.informatik.uni-halle.de; web interface or standalone) differs from the above-mentioned programs as it is based on a statistical model which parameters are estimated from training data computationally (Grau et al., 2013). Furthermore, *TALgetter* decodes the RVDs according to their binding specificity, but takes into account RVD "efficiency" or affinity, as reported by Streubel et al. (2012). Though these predictions yield a number of validated targets, we are still at early days. As an example, we used the Hax4 RVDs from *Xanthomonas campestris* pv. *campestris* strain Xca5 (Kay et al., 2005; Bolot et al., 2013) to mine for Arabidopsis targets in Col-0 promoterome using all 4 algorithms mentioned above. Among the 98 top targets identified by each algorithm, only 17 targets were predicted by all 4 algorithms and 51 by at least 3 algorithms (**Figure 1**). Although our knowledge on the efficiency (i.e., the percentage of validation of the predictions) of these bioinformatics tools remains poorly documented, significant differences exist between the four algorithms. Thus, combining predictions might help to reduce numbers of true

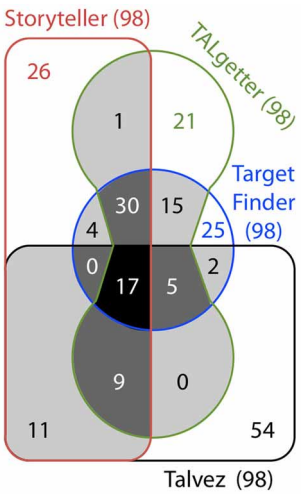

FIGURE 1 | Venn diagram representing the shared targets of Hax4 (GenBank AY993939; RVDs = NI HD HD NG NS NS NI HD NG NI NS NI NG NI NG) predicted in the Arabidopsis promoterome (1 kb upstream of the translation start site from the Col-0 ecotype genes in the TAIR annotation release 10) with the 4 currently available algorithms for TAL target prediction: *Storyteller* (parameters: rounds "10^5", noise "0.5", noise-shape "hvaa-dependent," max e-value "700," minscore "2," gap probability "10^{-3}"), *TALgetter* (default parameters: "TALgetter long"), *Talvez* (version 3.1, parameters: pseudocounts "10^{-5}", minimum score "9", number of reported TALEs "100" and position correction "19") and *Target Finder* (TALE-NT 2.0, default parameters). Shaded areas in light gray, dark gray and black indicate putative targets identified with 2, 3 or 4 prediction algorithms, respectively. Numbers in bracket indicate the total number of predicted targets for each prediction tool.

targets for subsequent experimental validation. Yet, false negatives appear as the greatest threats in such approach since true biological targets could be missed this way. Further experimentally validation of TAL targets in different plant genomes are needed to improve the quality of the algorithms and move toward a higher confidence in the predictions.

PREDICTIONS: CAVEATS AND POSSIBLE IMPROVEMENTS

Yet, our incomplete knowledge of the molecular mechanisms underlying the TAL-DNA interaction and subsequent transcriptional activation is a major limit of these predictions. Since the RVD-DNA code is somewhat degenerate, predictions for TALs with fewer repeats or rich in unspecific RVDs will yield significant amounts of false positive/false negative target sites especially when scrutinizing large plant genomes. Besides, our understanding of the relative contribution of each individual RVDs to the general protein affinity is still very scarce. Recently, it was shown both by reporter gene expression-based *in vivo* assays and/or biochemical studies that RVDs display different affinity to their favored nucleotides (Christian et al., 2012; Streubel et al., 2012; Meckler et al., 2013). These pioneer studies clearly point to the necessity of systematically evaluating the affinity of each individual most frequent but also rare RVDs for a given nucleotide. Other uncertainties include our incapacity to predict the effect of neighboring RVDs over the binding of a particular RVD, as well as the influence of the binding-site direct environment and the status of epigenetic marks. Finally, another source of inaccuracy in DNA-binding sites prediction deals with our difficulty to estimate TAL effectors tolerance for imperfect pairings which may vary depending on the type, position and context of the mismatch (Doyle et al., 2013). In the same line of idea, Meckler et al. (2013) recently showed that N-terminal RVDs contribute more to the overall DNA affinity than C-terminal RVDs. This result is corroborated by the analysis of Pérez-Quintero et al. (2013), showing from a set of well-characterized RVD-DNA interactions that perfect RVD-nucleotide pairing in TAL effectors N-terminal region (first 15–19 RVDs) probably determines for the most part the target DNA recognition and activity. Thus, mismatches in the C-terminal end of the repeat region generally appear to be better tolerated than in the N-terminal end. Altogether, this illustrates the fact that additional systematic experiments of both the binding affinity and specificity of each RVDs for their preferred nucleotides are required to optimize current predictive models, which would also gain in accuracy if trained with additional experimentally validated pairs of TALs and targets.

Independently of DNA-binding itself, transcriptional activation was strongly enhanced for TAL target sites in the −300 to +200 region relative to the transcriptional start site (TSS, Grau et al., 2013). Thus, proper structural annotation of genomes including RNAseq-based or EST based annotation of TSS should greatly enhance the quality of the predictions. Though not formally included in the current algorithms, filtering manually for putative target sites close to transcriptional or translational start sites is advisable.

In silico OR WET LAB?: PROBABLY BOTH!

What comes up as an obvious and promising strategy is the use of experimental data to identify new targets. Recently, *Bs4C* executor target was identified solely based on a thoroughly designed RNAseq approach in pepper. The *X. axonopodis* pv. *vesicatoria* AvrBs4 TAL effector target was pinned down to a single promoter to which direct binding was demonstrated (Strauss et al., 2012). Yet, Q-RT-PCR can also be used to confirm predicted targets and may be a cheap shortcut when whole transcriptome profiling (micro-arrays or RNAseq) is not an option: prediction algorithms yield a number of true positives. For instance, 21 TAL targets predicted by *Target Finder* in the rice genome for 14 presumably *X. oryzae* TALs predicted could be verified experimentally (Doyle et al., 2012). As already shown in several studies, comparing the transcriptome of plants challenged with *Xanthomonas* strains carrying a TAL effector of interest vs. a strain defective for that particular *tal* gene or mock inoculation, produces lists of up-regulated genes which are enriched for direct *S* or *R* targets of the TAL effector under study (Yang et al., 2006; Sugio et al., 2007; Yu et al., 2011). Hence, one strategy for evaluating the validity of computationally predicted virulence targets is certainly to benchmark them against TAL effector-dependent profiling experiments, as successfully applied to assess the validity of *TALgetter* (Grau et al., 2013) and *Talvez* (Pérez-Quintero et al., 2013). Nevertheless, a main concern in the overall process is due to the difficulty of discriminating direct and biologically relevant TAL targets from direct and biologically irrelevant TAL targets or secondary/indirect targets. Indeed, off-targets can be found predicted and induced, inherently to the degeneracy of the TAL effector—DNA recognition code and as exemplified by the *X. oryzae* pv. *oryzae* TAL effector AvrXa7 which induces both the

expression of the well-characterized susceptibility gene *OsSWEET14* and a gene coding for a retrotransposon (Li et al., 2012). Secondary indirect targets might be induced independently of the presence of the *tal* gene or as a result of the induction of a TAL direct target. The use of a cycloheximide treatment can help to identify genes which expression does not directly result from TAL activity. One alternative way to counter select off-targets may be to favor candidate targets subjected to functional convergence events, as illustrated for the rice susceptibility gene *OsSWEET14*, which was found to be activated by 4 different TAL effectors originating from 4 different strains of *Xoo* and binding to 3 different target sites in the *OsSWEET14* promoter. Upon the analysis of the *Xoo* TAL repertoire for which targets where predicted and compared to publicly available expression data, several instances of functional convergence between different strains could be demonstrated (Pérez-Quintero et al., 2013).

PERSPECTIVES: TAL *S* TARGETS AS NEW TOOLS TO DECIPHER HOST SPECIALIZATION OF *Xanthomonas* SPECIES?

Despite recent breakthrough in TAL effectors biology, the contribution of TAL targets in promoting susceptibility is yet poorly understood. This is particularly true considering the diversity of the *Xanthomonas* genus (27 species and more than 100 pathovars), of the diseases caused on more than 400 different host plants and of the corresponding TAL repertoire (none to 26 TAL copies per strain). The discovery of the *Xanthomonas* TALome is a major task which is seriously hindered by the fact that current sequencing technologies and genome assembly pipelines cannot properly assemble the highly repetitive TAL DNA sequences from whole genome shotgun sequencing data. Also, our knowledge about the relative contribution of TAL effectors to pathogenicity in strains containing multiple *tal* genes is limited to a "happy few" pathosystems such as *X. axonopodis* pv. *vesicatoria*, *X. citri* pv. *citri*, *X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *malvacearum* and *X. axonopodis* pv. *manihotis*. Revealing the susceptibility genes involved in these processes will be key to deciphering as many potentially unique disease scenarios and represent unprecedented means to access a wealth of information and dissecting the molecular executors of susceptibility. In fact, identifying major virulence TAL effectors of well-studied and more exotic *Xanthomonas* pathovars and fishing their targets offers a unique strategy to understand what may drive host specialization in a species level.

CONCLUDING REMARKS

As time passes, experimental data will accumulate and help to refine the prediction algorithms. Yet, the most challenging aspect remains the biology of the *Xanthomonas*/plant interaction. During the co-evolution process, bacteria have selected a TAL repertoire to adapt to the diversity of natural hosts and the selection of novel crop species by humans. The latter might be the reason why some strains of the rice pathogens *Xoo* and *Xoc* have so many TALs (up to 26) (Schornack et al., 2013). Therefore, the choice of the right host plant genotype to find the genuine TAL targets is critical. One will always find a target for a TAL in any plant or even animal genome. The experimental validation of target gene induction or direct TAL-binding to the promoter still does not indicate that the right biological system was studied. If the *tal* gene studied contributes significantly to the pathogenicity on the selected plant genotype, one has the chance to find important *S* genes. Yet, in nature, the contribution of many TAL effectors to disease development will be subtle and dependent on the plant genotype. This means that in the future, *Xanthomonas* and diseased hosts should be sampled together in epidemics to advance in the identification of genuine TAL targets and in our understanding of *Xanthomonas* virulence strategies. Combining pathosystems isolated from natural epidemics with *in silico*, genomic and transcriptomic approaches are certainly the way to go in the next decade. These approaches should yield a large number of targets which contribute quantitatively to susceptibility and resistance for marker-assisted breeding in important crop species.

ACKNOWLEDGMENTS

We are grateful to A. Pérez-Quintero for providing us with the predicted targets of Hax4 obtained with *Talvez* and *Storyteller* and E. Doyle for providing us with Hax4 targets with *Target Finder*. This work was in part supported by the LABEX TULIP (ANR-10-LABX-41) and a Jeunes Chercheurs grant from the Agence Nationale de la Recherche (Xopaque ANR-10-JCJC-1703–01) to Laurent D. Noël.

REFERENCES

Boch, J., and Bonas, U. (2010). *Xanthomonas* AvrBs3 family-type III effectors: discovery and function. *Annu. Rev. Phytopathol.* 48, 419–436. doi: 10.1146/annurev-phyto-080508-081936

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., et al. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509–1512. doi: 10.1126/science.1178811

Bolot, S., Guy, E., Carrere, S., Barbe, V., Arlat, M., and Noël, L. D. (2013). Genome Sequence of *Xanthomonas campestris* pv. campestris Strain Xca5. *Genome Announc.* 1, e00032–e00012.

Christian, M. L., Demorest, Z. L., Starker, C. G., Osborn, M. J., Nyquist, M. D., Zhang, Y., et al. (2012). Targeting G with TAL effectors: a comparison of activities of TALENs constructed with NN and NK repeat variable di-residues. *PLoS ONE* 7:e45383. doi: 10.1371/journal.pone.0045383

Deng, D., Yan, C., Pan, X., Mahfouz, M., Wang, J., Zhu, J. K., et al. (2012). Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 335, 720–723. doi: 10.1126/science.1215670

Doyle, E. L., Booher, N. J., Standage, D. S., Voytas, D. F., Brendel, V. P., Vandyk, J. K., et al. (2012). TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. *Nucleic Acids Res.* 40, W117–W122. doi: 10.1093/nar/gks608

Doyle, E. L., Stoddard, B. L., Voytas, D.F., and Bogdanove, A. J. (2013). TAL effectors: highly adaptable phytobacterial virulence factors and readily engineered DNA-targeting proteins. *Trends Cell Biol.* 23, 390–398. doi: 10.1016/j.tcb.2013.04.003

Grau, J., Wolf, A., Reschke, M., Bonas, U., Posch, S., and Boch, J. (2013). Computational predictions provide insights into the biology of TAL effector target sites. *PLoS Comput. Biol.* 9:e1002962. doi: 10.1371/journal.pcbi.1002962

Kay, S., Boch, J., and Bonas, U. (2005). Characterization of AvrBs3-like effectors from a Brassicaceae pathogen reveals virulence and avirulence activities and a protein with a novel repeat architecture. *Mol. Plant-Microbe Interact.* 18, 838–848. doi: 10.1094/MPMI-18-0838

Li, T., Liu, B., Spalding, M. H., Weeks, D. P., and Yang, B. (2012). High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* 30, 390–392. doi: 10.1038/nbt.2199

Mak, A. N., Bradley, P., Cernadas, R. A., Bogdanove, A. J., and Stoddard, B. L. (2012). The crystal structure of TAL effector PthXo1 bound to its DNA target. *Science* 335, 716–719. doi: 10.1126/science.1216211

Meckler, J. F., Bhakta, M. S., Kim, M. S., Ovadia, R., Habrian, C. H., Zykovich, A., et al. (2013). Quantitative analysis of TALE-DNA interactions suggests polarity effects. *Nucleic Acids Res.* 41, 4118–4128. doi: 10.1093/nar/gkt085

Moscou, M. J., and Bogdanove, A. J., (2009). A simple cipher governs DNA recognition by TAL effectors. *Science* 326, 1501. doi: 10.1126/science.1178817

Pérez-Quintero, A., Rodriguez-R, L., Dereeper, A., Lopez, C., Koebnik, R., Szurek, B., et al. (2013). An improved method for TAL effectors DNA-binding sites prediction reveals functional convergence in TAL repertoires of *Xanthomonas oryzae* strains. *PLoS ONE* 8:68464. doi: 10.1371/journal.pone.0068464

Schornack, S., Moscou, M. J., Ward, E. R., and Horvath, D. M., (2013). Engineering plant disease resistance based on TAL effectors. *Annu. Rev. Phytopathol.* 51, 383–406. doi: 10.1146/annurev-phyto-082712-102255

Strauss, T., Van Poecke, R. M., Strauss, A., Romer, P., Minsavage, G. V., Singh, S., et al. (2012). RNA-seq pinpoints a *Xanthomonas* TAL-effector activated resistance gene in a large-crop genome. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19480–19485. doi: 10.1073/pnas.1212415109

Streubel, J., Blucher, C., Landgraf, A., and Boch, J. (2012). TAL effector RVD specificities and efficiencies. *Nat. Biotechnol.* 30, 593–595. doi: 10.1038/nbt.2304

Sugio, A., Yang, B., Zhu, T., and White, F. F. (2007). Two type III effector genes of *Xanthomonas oryzae* pv. *oryzae* control the induction of the host genes *OsTFIIAgamma1* and *OsTFX1* during bacterial blight of rice. *Proc. Natl Acad. Sci. U.S.A.* 104, 10720–10725. doi: 10.1073/pnas.0701742104

Yang, B., Sugio, A., and White, F. F. (2006). *Os8N3* is a host disease-susceptibility gene for bacterial blight of rice. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10503–10508. doi: 10.1073/pnas.0604088103

Yu, Y., Streubel, J., Balzergue, S., Champion, A., Boch, J., Koebnik, R., et al. (2011). Colonization of rice leaf blades by an African strain of *Xanthomonas oryzae* pv. *oryzae* depends on a new TAL effector that induces the rice nodulin-3 *Os11N3* gene. *Mol. Plant-Microbe Interact.* 24, 1102–1113. doi: 10.1094/MPMI-11-10-0254

Received: 12 June 2013; accepted: 08 August 2013; published online: 03 September 2013.

*Citation: Noël LD, Denancé N and Szurek B (2013) Predicting promoters targeted by TAL effectors in plant genomes: from dream to reality. Front. Plant Sci. **4**:333. doi: 10.3389/fpls.2013.00333*

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

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 11 April 2013
doi: 10.3389/fpls.2013.00098

Regulate and be regulated: integration of defense and other signals by the AtMYB30 transcription factor

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Transcriptional regulation in host cells plays a crucial role in the establishment of plant defense and associated cell death in response to pathogen attack. Here, we review our current knowledge of the transcriptional control of plant defenses with a focus on the MYB family of transcription factors (TFs). Within this family, the Arabidopsis MYB protein AtMYB30 is a key regulator of plant defenses and one of the best characterized MYB regulators directing defense-related transcriptional responses. The crucial role played by AtMYB30 in the regulation of plant disease resistance is underlined by the finding that AtMYB30 is targeted by the *Xanthomonas* type III effector XopD resulting in suppression of AtMYB30-mediated plant defenses. Moreover, the function of AtMYB30 is also tightly controlled by plant cells through protein-protein interactions and post-translational modifications (PTMs). AtMYB30 studies highlight the importance of cellular dynamics for defense-associated gene regulation in plants. Finally, we discuss how AtMYB30 and other MYB TFs mediate the interplay between disease resistance and other stress responses.

Keywords: Arabidopsis, AtMYB30, hypersensitive response, MYB transcription factor, plant defense, stress responses, transcriptional regulation

INTRODUCTION

As sessile organisms, plants must face the diversity of pathogens that they encounter in their habitat. Unlike mammals, plants rely on cell autonomous innate immunity and on systemic signals originating from infection sites (Jones and Dangl, 2006). Plant immunity is activated by multiple transcriptional regulators that switch cell transcription programs from routine cellular requirements to defense. The arsenal of transcriptional regulators includes DNA-binding transcription factors (TFs) and proteins that regulate these TFs. Plant transcriptional regulators function cooperatively in complex networks to control the speed, intensity, localization, and duration of the immune response (Moore et al., 2011). The rapid and localized programmed death of infected cells is part of a typical plant immune response designated as the Hypersensitive Response (HR) (Mur et al., 2008; Coll et al., 2011). Processes related to the sessile lifestyle of plants have been associated with the expansion of TF families controlling plant-specific functions (Dias et al., 2003; Shiu et al., 2005; Feller et al., 2011). The MYB family of TFs underwent an extensive amplification approximately 500 million years ago due to recent whole-genome duplications and segmental tandem duplication events (Shiu et al., 2005). As a result, the plant MYB family typically comprises hundreds of members, classified based on the number of MYB repeats that they contain (Feller et al., 2011). MYB R2R3 proteins contain two MYB repeats and form the largest group of MYB TFs in plants. Members of the R2R3 MYB family regulate mostly plant-specific functions, including immunity against microbial pathogens (Stracke et al., 2001; Dubos et al., 2010).

In *Nicotiana tabacum*, the expression of the *Ntmyb1* gene is induced during the response to Tobacco Mosaic Virus (TMV) and *Pseudomonas syringae* pv. *syringae* avirulent bacteria. The Ntmyb1 protein binds to the promoter of the defense-related gene *PR-1a* suggesting a role in the regulation of immune responses (Yang and Klessig, 1996). In an independent study, Ntmyb1 was retrieved together with three other R2R3 MYBs as factors binding to the promoter of defense-related genes (Sugimoto et al., 2000). Transgenic *N. tabacum* plants overexpressing the rubber tree *HbMyb1* MYB gene exhibited suppressed HR resulting in enhanced resistance to the necrotrophic fungus *Botrytis cinerea* (Peng et al., 2011). Conversely, overexpression of the wheat *TaPIMP1* MYB gene caused stronger HR and enhanced resistance to the biotrophic bacterial pathogen *Ralstonia solanacearum* in tobacco and to the hemibiotrophic fungal pathogen *Bipolaris sorokiniana* in wheat (Liu et al., 2011; Zhang et al., 2012). In rice, the *OsJaMyb* R2R3 MYB gene is induced during infection by the blast fungus *Magnaporthe oryzae* and in mutants altered in cell death programs suggesting a role in defense responses (Lee et al., 2001). The *Arabidopsis thaliana* genome harbors 137 R2R3 MYB genes some of which have been shown to regulate immunity to microbial pathogens. The *BOTRYTIS-SUSCEPTIBLE1 BOS1/AtMYB108* gene was identified in a screen for mutants altered in their response to the *B. cinerea*. The *bos1* mutant exhibits enhanced susceptibility to *B. cinerea* and *Alternaria brassicicola* necrotrophic pathogens and reduced symptoms but unaltered resistance in response to biotrophic pathogens (Mengiste et al., 2003). Conversely, AtMYB46 negatively regulates resistance to *B. cinerea* likely *via* the regulation of a cell wall-bound

peroxidase (Ramirez et al., 2011). Overexpression and silencing of AtMYB44 demonstrated that it positively regulates resistance to the virulent bacterium *P. syringae* pv. *tomato* (*Pst*) DC3000 but down regulates resistance to *A. brassicicola via* the WRKY70 TF (Shim et al., 2012; Zou et al., 2012). AtMYB96 was first reported as induced upon *Cauliflower Mosaic Virus* infection (Geri et al., 1999). Analysis of plants mis-expressing *AtMYB96* demonstrated that this TF positively controls resistance to *Pst* DC3000 in a salicylic acid-dependent manner (Seo and Park, 2010). Among the closest paralogs of AtMYB96 is AtMYB30, which was the first R2R3 MYB gene to be associated with the regulation of defense response in *Arabidopsis* and one of the best defense-related MYBs characterized to date. Although the mechanisms by which MYB TFs control defense responses are still enigmatic, recent advances in our understanding of AtMYB30 function summarized in this review shed new light on the regulation of plant immunity by this family of TFs.

The *MYB* oncogene homologue *AtMYB30* was first isolated in by differential screening of a cDNA library prepared from *Xanthomonas campestris* pv. *campestris (Xcc)*-inoculated *Arabidopsis* cells (Lacomme and Roby, 1999). Early, transient and specific activation of *AtMYB30*, prior to the onset of the hypersensitive cell death, was observed after treatment with different avirulent bacterial pathogens (Daniel et al., 1999). In addition, overexpression of *AtMYB30* in *Arabidopsis* and tobacco led to acceleration and intensification of the HR, enhanced accumulation of HR molecular markers and increased resistance in response to avirulent pathogens. Conversely, the antisense-mediated downregulation of *AtMYB30* led to a strong decrease or suppression of the HR (Vailleau et al., 2002). These data identify AtMYB30 as a positive regulator of the signaling pathway controlling the establishment of cell death responses to pathogen attack.

During the last few years, the study of AtMYB30 regulatory mechanisms has increased our knowledge about the mode of action of this TF. These studies have uncovered a tight control of the activity of AtMYB30 through protein-protein interactions and post-translational modifications (PTMs). Here, we summarize our current knowledge of the AtMYB30 interaction and regulatory network involved in the control of plant defense responses. Additional roles of AtMYB30 during the integration of other environmental cues are also discussed.

AtMYB30 REGULATES GENES OF THE VLCFA PATHWAY

A transcriptomic analysis revealed that AtMYB30 putative target genes are involved in the lipid biosynthesis pathway that leads to the production of very long chain fatty acids (VLCFAs) (Raffaele et al., 2008). In good agreement, ectopic expression of *AtMYB30* activates genes encoding subunits of the acyl-coA elongase complex and alters the VLCFA content of *Arabidopsis* leaves. Furthermore, defense-related phenotypes of *AtMYB30* transgenic plants are dependent on the VLCFA biosynthesis pathway, supporting the view that AtMYB30 modulates cell death-related lipid signaling by enhancing the synthesis of VLCFAs or VLCFA derivatives (Raffaele et al., 2008) (**Figure 1A**). Downstream products of the VLCFA pathway include sphingolipids, wax and cutin. Wax synthesis was altered by *AtMYB30* over-expression but not by *AtMYB30* silencing, suggesting that sphingolipids could be cell death signals regulated by AtMYB30, and that activators of the wax synthesis pathway could compensate for the lack of AtMYB30 in silenced plants. Interestingly, Seo et al. reported that AtMYB96 activates genes of the wax biosynthesis pathway during drought stress (Seo et al., 2011). AtMYB30 and AtMYB96 belong to the sub-group S1 of *Arabidopsis* R2R3 MYB family (**Figure 2A**) (Dubos et al., 2010). Their N-terminal domain is predicted to mediate DNA-binding through a six alpha-helix domain typical of R2R3 MYBs (**Figure 2B**). AtMYB30 and AtMYB96 share extensive similarity in their N-terminal domain (**Figure 2C**), as expected considering the overlap in their respective lists of target genes. Besides short conserved motifs, the C-termini of sub-group S1 of MYB TFs are highly divergent. In AtMYB30, this C-terminal region harbors numerous putative regulatory sites, including phosphorylation, SUMOylation and ubiquitination sites (**Figure 2C**). As discussed below, modifications of this kind are critical for the regulation of AtMYB30 activity. It is therefore tempting to speculate that the differential activation of the N-termini of MYB TFs of the sub-group S1 may integrate signals arising from multiple stresses to regulate a partially common set of genes. Whether and how the interplay between AtMYB30 and AtMYB96 fine-tunes the activation of VLCFA-mediated responses remains to be investigated. Whether other MYBs of sub-group S1 are able to activate the VLCFA pathway is also unknown. Shared and specific functions of related MYB TFs may explain how expansion and diversification in this family contributed to the emergence of an integrated stress-response machinery in plants.

MANIPULATION OF AtMYB30 ACTIVITY BY BACTERIA

XopD from strain B100 of *Xanthomonas campestris* pv. *campestris* (*Xcc*B100) is a modular type III effector protein of 801 amino acids that presents a modular structure and contains different domains with varied biochemical activities (Canonne et al., 2012). $XopD_{XccB100}$ is targeted to plant cell nuclei (Canonne et al., 2011; Kim et al., 2011) and may interact with chromatin and/or transcriptional units, leading to modulation of host transcription by affecting chromatin remodeling and/or TF activity (Kay and Bonas, 2009).

In agreement with the idea that plant TFs and/or regulators might be direct targets of XopD, $XopD_{XccB100}$ was shown to target AtMYB30. $XopD_{XccB100}$ expression leads to accumulation of AtMYB30 in $XopD_{XccB100}$-containing nuclear foci but the physical interaction between $XopD_{XccB100}$ and AtMYB30 is independent of AtMYB30 relocalization to nuclear foci, as both proteins are also able to interact in the nucleoplasm (Canonne et al., 2011). $XopD_{XccB100}$ targeting of AtMYB30 leads to reduced activation of AtMYB30 VLCFA-related target genes and, therefore, to suppression of plant defense responses during *Xcc*B100 infection (Canonne et al., 2011) (**Figure 1A**). A helix-loop-helix (HLH) domain in $XopD_{XccB100}$ is necessary and sufficient to mediate the interaction with AtMYB30 and repression of AtMYB30 transcriptional activation and plant resistance responses. Consistently, XopD from the 8004 strain of *Xcc* ($XopD_{Xcc8004}$), that does not present the HLH domain and localizes homogenously within

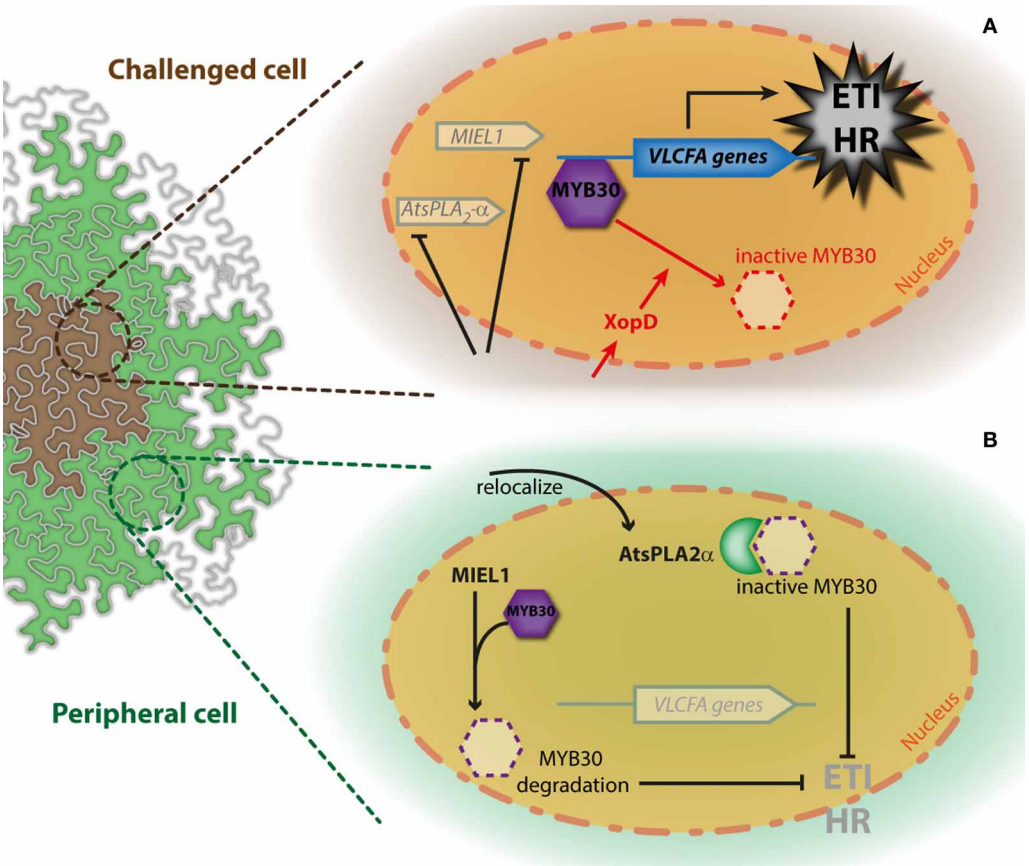

FIGURE 1 | Simplified model for the simultaneous regulation of AtMYB30-mediated HR cell death through interaction with *At*sPLA$_2$-α and MIEL1. The action of with *At*sPLA$_2$-α and MIEL1 on AtMYB30-mediated HR development is presented in cells challenged with bacterial inoculation **(A)** and peripheral cells **(B)**. Activity of the bacterial XopD effector is shown in red. See the text for details.

plant cell nuclei, is not able to interact with AtMYB30 and has no effect on AtMYB30 transcriptional activation. Considering the modular structure of XopD, it is likely that this type III effector mediates multiple molecular (protein-DNA and protein-protein) associations and that, depending on the *Xanthomonas* strain/host plant interaction, XopD is able to target different host components to subvert plant defense. For example, XopD from *Xanthomonas euvesicatoria* (*Xcv*) desumoylates the SlERF4 TF to suppress ethylene responses and promote pathogen growth in tomato (Kim et al., 2013).

REGULATION OF AtMYB30 ACTIVITY THROUGH PROTEIN–PROTEIN INTERACTIONS AND POST-TRANSLATIONAL MODIFICATIONS

Plant resistance to disease involves costly defense responses, closely connected to plant physiological and developmental processes. A typical example is the HR, which includes the development of a form of programmed cell death and needs to be tightly regulated to be not only efficient but also beneficial to the plant. As a result, mutants with constitutively active defense responses often present stunted growth and low fertility (Lorrain et al., 2003). Negative regulatory mechanisms of defense responses are used by the plant to attenuate the activation of defense-related functions and allow a balanced allocation of resources upon pathogen challenge (Journot-Catalino et al., 2006; Mukhtar et al., 2008). AtMYB30 being a positive regulator of plant defense and associated cell death responses, several mechanisms of negative regulation of its activity have been described.

The secretory phospholipase PLA$_2$ protein *At*sPLA$_2$-α controls auxin transport protein trafficking to the plasma membrane (Lee et al., 2010). *At*sPLA$_2$-α localizes to Golgi-associated vesicles and is later secreted to the extracellular space (Froidure et al., 2010; Lee et al., 2010). Translocation of *At*sPLA$_2$-α to the apoplast is enhanced after plant inoculation with avirulent bacteria, suggesting that *At*sPLA$_2$-α may participate to the plant defense response in the apoplast (Jung et al., 2012). Interestingly, intracellular *At*sPLA$_2$-α has also been involved in the non-enzymatic control of plant defense. Indeed, *At*sPLA$_2$-α was identified as interacting with AtMYB30 in yeast (Froidure et al., 2010). In the presence of AtMYB30, *At*sPLA$_2$-α was partially relocalized to the plant cell nucleus where these two proteins interact, leading to repression of the AtMYB30-mediated transcriptional activity. As a result, *Arabidopsis* HR and defense responses are suppressed, supporting the view that AtMYB30 transcriptional activity is

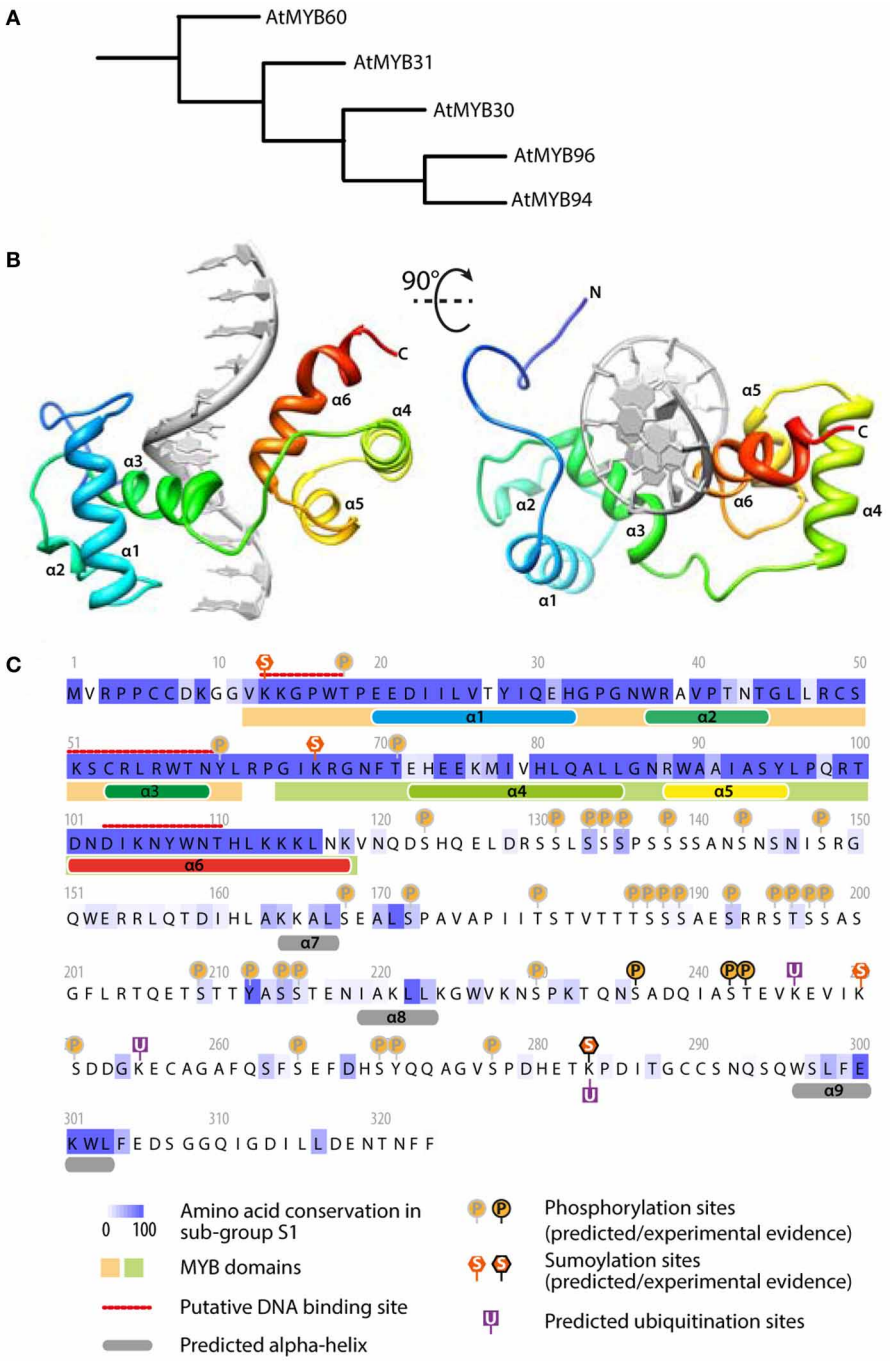

FIGURE 2 | AtMYB30 sequence analysis: relationship with other MYBs, protein motifs and predicted structure. (A) Relationship between MYB TFs of the subgroup S1 (from Dubos et al., 2010). **(B)** Predicted structure of AtMYB30 DNA binding domain bound to DNA (gray). The model was predicted using the I-TASSER server and rendered with UCSF Chimera. **(C)** Sequence analysis of AtMYB30 protein. The conservation between members of subgroup S1 was inferred from a MUSCLE alignment and colored using JALVIEW. Alpha helices and DNA binding sites were predicted using the I-TASSER server. MYB domains were identified using INTERPROSCAN. Phosphorylation, sumoylation and ubiquitation sites were predicted using PhosphAt, Sumoplot and Ubpred respectively.

required to mount an efficient defense response during bacterial infection (Raffaele et al., 2008; Froidure et al., 2010). Notably, *Ats*PLA$_2$-α nuclear targeting, interaction with AtMYB30, repression of AtMYB30 transcriptional activity and HR development appeared to be independent of *Ats*PLA$_2$-α enzymatic activity (Froidure et al., 2010). Therefore, *Ats*PLA$_2$-α was proposed to control AtMYB30-mediated response through interaction with AtMYB30, preventing the activation of its targets, rather than

through a lipid signal produced by *Ats*PLA$_2$-α. Together, these data highlight the importance of dynamic nucleocytoplasmic protein trafficking for the regulation of the transcriptional activation related to defense (Rivas, 2012). Interestingly, *AtMYB30* expression is induced 4 h post-inoculation (hpi) in challenged cells but not in peripheral cells, whereas *AtsPLA$_2$*-α expression peaks 6 hpi in peripheral but not in challenged cells (Froidure et al., 2010). This suggests that *Ats*PLA$_2$-α may contribute to restrict the development of the HR to the inoculated zone, thereby preventing spreading of cell death throughout the leaf (Froidure et al., 2010) (**Figure 1B**).

An additional regulatory mechanism of AtMYB30 action was uncovered by the identification of the *Arabidopsis* RING-type E3-ubiquitin-ligase MIEL1 (AtMYB30-INTERACTING E3 LIGASE1) as an AtMYB30 interactor in yeast (Marino et al., 2013). MIEL1 is able to ubiquitinate AtMYB30 *in vitro*. In *Arabidopsis*, MIEL1 leads to AtMYB30 proteasomal degradation, downregulation of its transcriptional activity and suppression of plant defense responses (Marino et al., 2013). Indeed, Arabidopsis *miel1* mutant plants displayed enhanced HR and resistance after inoculation with avirulent bacteria. These phenotypes are AtMYB30-dependent and correlate with down-regulation of AtMYB30 target genes related to VLCFA metabolism (Marino et al., 2013). *MIEL1* expression is rapidly repressed in challenged cells, indicating that MIEL1 may negatively regulate plant HR and defense activation through degradation of the MYB30 protein in the absence of the pathogen (Marino et al., 2013; **Figure 2B**). Repression of *MIEL1* in challenged cells may release AtMYB30 negative regulation, increasing the intensity of the HR and limiting pathogen growth (Marino et al., 2013; **Figure 2A**). In addition, MIEL1-mediated degradation of AtMYB30 could contribute to the spatial restriction of the HR to inoculated cells since *MIEL1* expression remains constant in peripheral cells (Marino et al., 2013; **Figure 2B**). Work by Marino and co-workers shows the important role played by ubiquitination during the transcriptional control of the HR (Marino et al., 2012) and underlines the sophisticated fine-tuning of plant responses to pathogen attack.

PTM of AtMYB30 by SUMOylation has also been reported. AtMYB30 SUMOylation was first demonstrated after reconstitution of the SUMOylation cascade in *E. coli*, the lysine residue K283 being the major SUMOylation site (Okada et al., 2009; **Figure 2C**). SUMOylation of AtMYB30 K283 by the *Arabidopsis* SUMO E3 ligase SIZ1 was later confirmed in *Arabidopsis* protoplasts and demonstrated to be required for AtMYB30 function during abscisic acid (ABA) signaling (Zheng et al., 2012) (see below). However, whether and how SUMOylation of AtMYB30 affects AtMYB30-mediated defense responses remains to be determined.

Finally, the AtMYB30 C-terminal region is particularly rich in potential phosphorylation sites for several protein kinases (**Figure 2C**). The contribution of these phosphorylation sites to the plant defense response is still unknown but it is tempting to speculate that different combinations of PTMs on AtMYB30 may act as a molecular barcode, which would be important for the regulation of TFs controlling multiple processes (Benayoun and Veitia, 2009). Along these lines, the animal TFs p53 and c-Myc represent excellent paradigms that illustrate the sophistication of transcription regulation with different PTMs providing efficient regulation of TF stability, subcellular localization and activity (Meek and Anderson, 2009; Hammond-Martel et al., 2012; Luscher and Vervoorts, 2012).

AtMYB30, A REGULATOR OF MULTIPLE SIGNALS BEYOND THE RESPONSE TO MICROBES

In addition to its role as a positive regulator of defense responses, AtMYB30 is recruited for the regulation of other signaling processes. The phytohormone ABA plays an essential role during development and in response to abiotic and biotic stress. AtMYB30 SUMOylation by SIZ1 leads to AtMYB30 protein stabilization and affects AtMYB30-mediated transcriptional activation of several ABA-responsive genes (Zheng et al., 2012), underlining the importance of AtMYB30 SUMOylation during the regulation of ABA signaling. As a result, an *atmyb30* mutant is hypersensitive to ABA whereas *AtMYB30*-overexpressing plants are insensitive to ABA (Zheng et al., 2012). Conversely, *AtMYB96* overexpressing plants were found to be hypersensitive to ABA, but an *atmyb96* knockout mutant was still responsive to ABA, possibly due to functional redundancy within the MYB family (Seo et al., 2009). *AtMYB96* expression is induced by ABA and drought and the activation of some ABA-inducible genes is AtMYB96-dependent. Similar to AtMYB30, enhanced disease resistance conferred by AtMYB96 involves salicylic acid synthesis, suggesting that these two MYB TFs regulate cross-talks between hormone signaling pathways and contribute to the integration of signals originating from various stresses (Raffaele et al., 2006; Seo and Park, 2010).

An additional example of the diversity of AtMYB30 functions is the regulation of brassinosteroid (BR) signaling. BRs play important roles in several plant growth and developmental processes as well as during stress/disease resistance. BRs signal through the BES1 (*bri1*-ethylmethane sulphonate suppressor1)/BZR1 (brassinazole-resistant1) family of TFs. BR treatment induces *AtMYB30* gene expression in *Arabidopsis* seedlings and in *bes1-D* plants, that overexpress BES1, *AtMYB30* expression is upregulated, indicating that AtMYB30 may function in the BR signaling pathway (Li et al., 2009). Indeed, chromatin immunoprecipitation (ChIP) experiments showed that BES1 activates *AtMYB30* expression by directly binding to the *AtMYB30* promoter (Li et al., 2009). In agreement with this finding, *atmyb30* knockout mutant plants exhibit reduced BR-related gene expression and phenotypes, indicating that AtMYB30 promotes the expression of a subset of BR target genes (Li et al., 2009). Moreover, the promoters of *AtMYB30* and *BES1* common target genes harbor boxes bound by each TF. Finally, AtMYB30 and BES1 interact with each other. Together, this data shows that AtMYB30 functions to amplify BR signaling through cooperation with BES1 to promote BR target gene expression.

CONCLUSIONS AND PERSPECTIVES

Cellular responses to environmental or physiological cues rely on transduction pathways that must discriminate between different signals and ensure a combinatorial regulation. Thus, combinations of different PTMs and protein-protein interactions provide different layers of information that may allow the

integration of several transduction pathways and warrant highly specific cellular outputs. Accumulating evidence shows that the *Arabidopsis* MYB regulator AtMYB30 is a multi-regulated protein that is involved in the integration of various environmental stimuli, including attack by microbes, abiotic stress and hormone signaling, likely through the activation of shared and specific sets of target genes. How simultaneous and diverse stress signals are integrated into a unified cellular response is a major unknown in cell signaling. The acceleration of large data set acquisition and the development of systems biology approaches promise to offer new insights into the functioning of such complex regulatory networks. The wealth of knowledge gained in recent years on *Arabidopsis* R2R3 MYB TFs provides an excellent framework toward this end.

ACKNOWLEDGMENTS

Our work is performed at the LIPM that is part of the Laboratoire d'Excellence (LABEX) entitled TULIP (ANR-10-LABX-41).

REFERENCES

Benayoun, B. A., and Veitia, R. A. (2009). A post-translational modification code for transcription factors: sorting through a sea of signals. *Trends Cell Biol.* 19, 189–197.

Canonne, J., Marino, D., Jauneau, A., Pouzet, C., Briere, C., Roby, D., et al. (2011). The *Xanthomonas* type III effector XopD targets the *Arabidopsis* transcription factor AtMYB30 to suppress plant defence. *Plant Cell* 23, 3498–3511.

Canonne, J., Pichereaux, C., Marino, D., Roby, D., Rossignol, M., and Rivas, S. (2012). Identification of the protein sequence of the type III effector XopD from the B100 strain of *Xanthomonas campestris* pv. *campestris*. *Plant Signal. Behav.* 7, 184–187.

Coll, N. S., Epple, P., and Dangl, J. L. (2011). Programmed cell death in the plant immune system. *Cell Death Differ.* 18, 1247–1256.

Daniel, X., Lacomme, C., Morel, J.-B., and Roby, D. (1999). A novel *myb* oncogene homolog in *Arabidopsis thaliana* related to the hypersensitive cell death. *Plant J.* 20, 57–66.

Dias, A. P., Braun, E. L., McMullen, M. D., and Grotewold, E. (2003). Recently duplicated maize R2R3 Myb genes provide evidence for distinct mechanisms of evolutionary divergence after duplication. *Plant Physiol.* 131, 610–620.

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., and Lepiniec, L. (2010). MYB transcription factors in Arabidopsis. *Trends Plant Sci.* 15, 573–581.

Feller, A., Machemer, K., Braun, E. L., and Grotewold, E. (2011). Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J.* 66, 94–116.

Froidure, S., Canonne, J., Daniel, X., Jauneau, A., Briere, C., Roby, D., et al. (2010). *Ats*PLA2-alpha nuclear relocalization by the Arabidopsis transcription factor AtMYB30 leads to repression of the plant defense response. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15281–15286.

Geri, C., Cecchini, E., Giannakou, M. E., Covey, S. N., and Milner, J. J. (1999). Altered patterns of gene expression in Arabidopsis elicited by cauliflower mosaic virus (CaMV) infection and by a CaMV gene VI transgene. *Mol. Plant Microbe Interact.* 12, 377–384.

Hammond-Martel, I., Yu, H., and Affar el, B. (2012). Roles of ubiquitin signaling in transcription regulation. *Cell. Signal.* 24, 410–421.

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

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

Jung, J., Kumar, K., Lee, H. Y., Park, Y. I., Cho, H. T., and Ryu, S. B. (2012). Translocation of phospholipase A2alpha to apoplasts is modulated by developmental stages and bacterial infection in Arabidopsis. *Front. Plant Sci.* 3:126. doi: 10.3389/fpls.2012.00126

Kay, S., and Bonas, U. (2009). How *Xanthomonas* type III effectors manipulate the host plant. *Curr. Opin. Microbiol.* 12, 37–43.

Kim, J. G., Stork, W., and Mudgett, M. B. (2013). Xanthomonas type III effector XopD desumoylates tomato transcription factor SlERF4 to suppress ethylene responses and promote pathogen growth. *Cell Host Microbe* 13, 143–154.

Kim, J. G., Taylore, K. W., and Mudgett, M. B. (2011). Comparative analysis of the XopD type III secretion (T3S) effector family in plant pathogenic bacteria. *Mol. Plant Pathol.* 12, 715–730.

Lacomme, C., and Roby, D. (1999). Identification of new early markers of the hypersensitive response in *Arabidopsis thaliana*. *FEBS Lett.* 459, 149–153.

Lee, M. W., Qi, M., and Yang, Y. (2001). A novel jasmonic acid-inducible rice *MYB* gene associates with fungal infection and host cell death. *Mol. Plant Microbe Interact.* 14, 527–535.

Lee, O., Kim, S., Kim, H., Hong, J., Ryu, S., Lee, S., et al. (2010). Phospholipase A2 is required for PIN-FORMED protein trafficking to the plasma membrane in the Arabidopsis root. *Plant Cell* 22, 1812–1825.

Li, L., Yu, X., Thompson, A., Guo, M., Yoshida, S., Asami, T., et al. (2009). Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. *Plant J.* 58, 275–286.

Liu, H., Zhou, X., Dong, N., Liu, X., Zhang, H., and Zhang, Z. (2011). Expression of a wheat MYB gene in transgenic tobacco enhances resistance to *Ralstonia solanacearum*, and to drought and salt stresses. *Funct. Integr. Genomics* 11, 431–443.

Lorrain, S., Vailleau, F., Balagué, C., and Roby, D. (2003). Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends Plant Sci.* 8, 263–271.

Luscher, B., and Vervoorts, J. (2012). Regulation of gene transcription by the oncoprotein MYC. *Gene* 494, 145–160.

Marino, D., Froidure, S., Canonne, J., Ben Khaled, S., Khafif, M., Pouzet, C., et al. (2013). Arabidopsis ubiquitin ligase MIEL1 mediates degradation of the transcription factor MYB30 weakening plant defence. *Nat. Commun.* 4, 1476.

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

Meek, D. W., and Anderson, C. W. (2009). Posttranslational modification of p53: cooperative integrators of function. *Cold Spring Harb. Perspect. Biol.* 1:a000950. doi: 10.1101/cshperspect.a000950

Mengiste, T., Chen, X., Salmeron, J., and Dietrich, R. (2003). The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. *Plant Cell* 15, 2551–2565.

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

Mukhtar, M. S., Deslandes, L., Auriac, M. C., Marco, Y., and Somssich, I. E. (2008). The Arabidopsis transcription factor WRKY27 influences wilt disease symptom development caused by *Ralstonia solanacearum*. *Plant J.* 56, 935–947.

Mur, L. A., Kenton, P., Lloyd, A. J., Ougham, H., and Prats, E. (2008). The hypersensitive response; the centenary is upon us but how much do we know? *J. Exp. Bot.* 59, 501–520.

Okada, S., Nagabuchi, M., Takamura, Y., Nakagawa, T., Shinmyozu, K., Nakayama, J., et al. (2009). Reconstitution of *Arabidopsis thaliana* SUMO pathways in *E. coli*: functional evaluation of SUMO machinery proteins and mapping of SUMOylation sites by mass spectrometry. *Plant Cell Physiol.* 50, 1049–1061.

Peng, S. Q., Wu, K. X., Huang, G. X., and Chen, S. C. (2011). HbMyb1, a Myb transcription factor from *Hevea brasiliensis*, suppresses stress induced cell death in transgenic tobacco. *Plant Physiol. Biochem.* 49, 1429–1435.

Raffaele, S., Rivas, S., and Roby, D. (2006). An essential role for salicylic acid in AtMYB30-mediated control of the hypersensitive cell death program in Arabidopsis. *FEBS Lett.* 580, 3498–3504.

Raffaele, S., Vailleau, F., Leger, A., Joubes, J., Miersch, O., Huard, C., et al. (2008). A MYB transcription factor regulates Very-Long-Chain Fatty Acid biosynthesis for activation of the hypersensitive cell death response in Arabidopsis. *Plant Cell* 20, 752–767.

Ramirez, V., Agorio, A., Coego, A., Garcia-Andrade, J., Hernandez, M. J., Balaguer, B., et al. (2011). MYB46 modulates disease susceptibility to *Botrytis cinerea* in Arabidopsis. *Plant Physiol.* 155, 1920–1935.

Rivas, S. (2012). Nuclear dynamics during plant innate immunity. *Plant Physiol.* 158, 87–94.

Seo, P. J., Lee, S. B., Suh, M. C., Park, M. J., Go, Y. S., and Park, C. M. (2011). The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in Arabidopsis. *Plant Cell* 23, 1138–1152.

Seo, P. J., and Park, C. M. (2010). MYB96-mediated abscisic acid signals induce pathogen resistance response by promoting salicylic acid biosynthesis in Arabidopsis. *New Phytol.* 186, 471–483.

Seo, P. J., Xiang, F., Qiao, M., Park, J. Y., Lee, Y. N., Kim, S. G., et al. (2009). The MYB96 transcription factor mediates abscisic acid signaling during drought stress response in Arabidopsis. *Plant Physiol.* 151, 275–289.

Shim, J. S., Jung, C., Lee, S., Min, K., Lee, Y. W., Choi, Y., et al. (2012). AtMYB44 regulates WRKY70 expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. *Plant J.* 73, 483–495.

Shiu, S. H., Shih, M. C., and Li, W. H. (2005). Transcription factor families have much higher expansion rates in plants than in animals. *Plant Physiol.* 139, 18–26.

Stracke, R., Werber, M., and Weisshaar, B. (2001). The R2R3-*MYB* gene family in *Arabidopsis thaliana*. *Curr. Opin. Plant Biol.* 4, 447–456.

Sugimoto, K., Takeda, S., and Hirochika, H. (2000). MYB-related transcription factor NtMYB2 induced by wounding and elicitors is a regulator of the tobacco retrotransposon *Tto1* and defense-related genes. *Plant Cell* 12, 2511–2527.

Vailleau, F., Daniel, X., Tronchet, M., Montillet, J. L., Triantaphylides, C., and Roby, D. (2002). A R2R3-MYB gene, *AtMYB30*, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10179–10184.

Yang, Y., and Klessig, D. F. (1996). Isolation and characterization of a tobacco mosaic virus-inducible *myb* oncogen homolog from tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14972–14977.

Zhang, Z., Liu, X., Wang, X., Zhou, M., Zhou, X., Ye, X., et al. (2012). An R2R3 MYB transcription factor in wheat, TaPIMP1, mediates host resistance to *Bipolaris sorokiniana* and drought stresses through regulation of defense- and stress-related genes. *New Phytol.* 196, 1155–1170.

Zheng, Y., Schumaker, K. S., and Guo, Y. (2012). Sumoylation of transcription factor MYB30 by the small ubiquitin-like modifier E3 ligase SIZ1 mediates abscisic acid response in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12822–12827.

Zou, B., Jia, Z., Tian, S., Wang, X., Gou, Z., Lü, B., et al. (2012). AtMYB44 positively modulates disease resistance to *Pseudomonas syringae* through the salicylic acid signalling pathway in Arabidopsis. *Funct. Plant Biol.* 40, 304–313.

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

Received: 04 March 2013; paper pending published: 21 March 2013; accepted: 28 March 2013; published online: 11 April 2013.

*Citation: Raffaele S and Rivas S (2013) Regulate and be regulated: integration of defense and other signals by the AtMYB30 transcription factor. Front. Plant Sci. **4**:98. doi: 10.3389/fpls.2013.00098*

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

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 05 April 2013
doi: 10.3389/fpls.2013.00072

Nuclear jasmonate and salicylate signaling and crosstalk in defense against pathogens

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An extraordinary progress has been made over the last two decades on understanding the components and mechanisms governing plant innate immunity. After detection of a pathogen, effective plant resistance depends on the activation of a complex signaling network integrated by small signaling molecules and hormonal pathways, and the balance of these hormone systems determines resistance to particular pathogens. The discovery of new components of hormonal signaling pathways, including plant nuclear hormone receptors, is providing a picture of complex crosstalk and induced hormonal changes that modulate disease and resistance through several protein families that perceive hormones within the nucleus and lead to massive gene induction responses often achieved by de-repression. This review highlights recent advances in our understanding of positive and negative regulators of these hormones signaling pathways that are crucial regulatory targets of hormonal crosstalk in disease and defense. We focus on the most recent discoveries on the jasmonate and salicylate pathway components that explain their crosstalk with other hormonal pathways in the nucleus. We discuss how these components fine-tune defense responses to build a robust plant immune system against a great number of different microbes and, finally, we summarize recent discoveries on specific nuclear hormonal manipulation by microbes which exemplify the ingenious ways by which pathogens can take control over the plant's hormone signaling network to promote disease.

Keywords: jasmonates, salicylic acid, plant hormones, toxins, effector, plant resistance, susceptibility

INTRODUCTION

In nature, plants live in complex environments in which they intimately interact with a broad range of microbial pathogens with different lifestyles and infection strategies. To defend themselves against all these different types of pathogens, plants have evolved sophisticated strategies to perceive their attacker and to translate this perception into an effective immune response. Two tiers of recognition by the innate immune system have been defined (Jones and Dangl, 2006). The first branch is triggered by the recognition of highly conserved microbe-associated molecular patterns (MAMPs) by host cell transmembrane proteins that function as pattern recognition receptors (PRRs), which in turn, activate MAMP-triggered immunity (MTI; Jones and Dangl, 2006). This activates sufficient defense to resist non-pathogenic microbes and probably also, some pathogens. To overcome such line of defenses, adapted pathogens have acquired the ability to introduce virulence effector proteins into the plant cell to promote plant susceptibility (Jones and Dangl, 2006). The second branch recognizes microbial effectors inside the plant cell via nucleotide-binding site-leucine-rich repeat (NB-LRR) resistance (R) proteins (Jones and Dangl, 2006). This leads to activation of effector-triggered immunity (ETI), and is characteristically associated with programmed cell death known as the hypersensitive response (HR; Jones and Dangl, 2006). The HR lesion is a stronger form of defense and limits microbial spread by killing infected plant cells. The final outcome of the battle depends on the balance between the ability of the pathogen to suppress the plant's immune system and the capacity of the plant to recognize the pathogen and to activate effective defenses.

The regulation of the defense network that translates the pathogen-induced early signaling events into activation of effective defense responses depends profoundly on the action of plant phytohormones (Pieterse et al., 2012). These hormones are small signal molecules occurring in low concentrations, essential for the regulation of plant growth, development, reproduction and survival to stresses of biotic and abiotic origin (Robert-Seilaniantz et al., 2011). Upon pathogen attack, the quantity, composition and timing of the phytohormonal blend produced by the plant varies among plant species and depends greatly on the lifestyle and infection strategy of the invading attacker (De Vos et al., 2005). Classic phytohormones are abscisic acid (ABA), auxins, cytokinins (CKs), ethylene (ET), and gibberellins (GAs), but small signaling molecules such as brassinosteroids (BRs), jasmonates (JAs), and salicylic acid (SA) are recognized as phytohormones as well (Pieterse et al., 2012). The importance of JA and SA as primary signals in the regulation of the plant's immune response is well established (Loake and Grant, 2007; Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). The JA pathway is primarily induced by and effective in mediating resistance against herbivores and necrotrophic pathogens, whereas the SA pathway is primarily induced by and effective in mediating resistance against biotrophic pathogens (Glazebrook, 2005). JA and SA defense pathways generally antagonize each other and thus, elevated resistance against necrotrophs is often correlated with increased susceptibility to

biotrophs, and *vice versa* (Grant and Lamb, 2006). This is, however, an overly simplistic view of the complex repertoire of plant hormones that probably play a role in mediating inducible defenses. Indeed, ABA, auxins, BRs, CKs, ET, GAs, and additional oxylipins (other than JA) function as modulators of the plant immune signaling network as well, fine-tuning the hormonal balances to become more resistant to the invading organism (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012; Vicente et al., 2012). The collective contribution and timing of these hormones during plant–pathogen interactions is crucial to the success of the interaction.

Typically, hormone signaling pathways begin with perception of a ligand hormone by a receptor and continue with the propagation of the hormone signal, leading to massive changes in gene expression within the nucleus (Lumba et al., 2010). In some cases, the perception and propagation of the signal initiates in the cytoplasm and then translocates to the nucleus. This is the case for SA, ABA, CK, and ET (Santner and Estelle, 2009; Fu et al., 2012; Pieterse et al., 2012; Wu et al., 2012). However, several plant hormone receptors are located directly in the nucleus. This is the case for the JA, auxins, and GAs hormonal pathways (Fonseca et al., 2009a; Kelley and Estelle, 2012; Pieterse et al., 2012). Although plant nuclear receptors are not transcription factors (TFs) *per se*, as is the case for animal nuclear receptors, they act directly on or just upstream of transcriptional regulators (Chini et al., 2009a; Fonseca et al., 2009a; Lumba et al., 2010). This shortened pathway yields simple and direct control of gene expression that is directly responsive to ligand concentrations. This results in a fast activation of a specific set of defense-related genes that determines the nature and effectiveness of the immune response that is triggered by the attacker (De Vos et al., 2005).

Recent discoveries highlight the importance of the 26S ubiquitin-proteasome system (UPS) in phytohormone signaling (Kelley and Estelle, 2012). In fact, UPS-mediated protein degradation has been demonstrated for every plant hormone, including ABA, auxin, BR, CK, ET, GA, JA, and recently SA (Chini et al., 2009a; Kelley and Estelle, 2012). UPS regulates hormone biosynthesis, transport, and perception and thus provides a simple and direct mechanism to control hormone signaling by the selective destruction of proteins whose concentrations must vary with time and alterations in the state of the cell. Interestingly, most of the hormone-related targets for UPS degradation described to date in plants are nuclear proteins associated with transcriptional repression that contain an ETHYLENE RESPONSE FACTOR-associated amphiphilic repression (EAR) domain (Ohme-Takagi and Shinshi, 1995; Kelley and Estelle, 2012). For example, the auxin/indole-3-acetic acid (Aux/ IAA; auxin), some jasmonate-ZIM domain (JAZ; JA), and the BRASSINAZOLE RESISTANT1 (BZR1; BR) repressors (Kagale et al., 2010). Thus, nuclear protein turnover is an integral and critical component in hormone signaling to ensure a fast and appropriate level of defense responses to a specific pathogen.

Here, we review the most recent and outstanding examples regarding hormonal crosstalk at the molecular level, focusing on the jasmonate and salicylate pathways and how the newly identified nuclear components fine-tune defense responses to build a robust plant immune system against a great number of different microbes. We also describe some of the best characterized molecular examples of specific nuclear hormonal manipulation by microbes, which exemplify the ingenious ways by which pathogens can take control over the plant's hormone signaling network to suppress host immunity.

JASMONATE AND SALICYLATE: MAJOR PLAYERS IN PLANT IMMUNITY

Plant immunity strongly relies on two plant mutually antagonistic hormones, JA and SA (Glazebrook, 2005). Both hormones control defense responses to different types of microbes and thus, they orchestrate a different and complex transcriptional reprogramming that eventually leads to plant resistance. Receptors of both hormones as well as many components of their signaling pathways have been recently identified. These discoveries are facilitating the understanding of the role of these hormones in plant immunity.

THE JASMONATE PATHWAY

Jasmonates are lipid-derived molecules originating from α-linolenic acid from the plastid membrane (Schaller and Stintzi, 2009). Among the plant hormones, JA plays a key role in modulating many physiological processes and is a key cellular signal involved in the activation of immune responses to most insect herbivores and necrotrophic microorganisms (Glazebrook, 2005; Wasternack, 2007). Among all JAs found in nature, (+)-7-iso-JA–L-Ile is the molecularly active form of the hormone (Fonseca et al., 2009b). JA-isoleucine (JA-Ile) is perceived through a co-receptor complex formed by the F-box protein CORONATINE-INSENSITIVE 1 (COI1) and JAZ proteins, a family that comprise 12 members in *Arabidopsis* (Chini et al., 2007, 2009b; Thines et al., 2007; Sheard et al., 2010). COI1 is a nuclear F-box component of an SCF-(Skip-cullin-F-box)-type E3 ubiquitin ligase required for all JA-dependent responses tested so far (Feys et al., 1994; Xie et al., 1998; Katsir et al., 2008; Chini et al., 2009a; Fonseca et al., 2009a). *Arabidopsis* plants lacking the *COI1* gene are more susceptible to necrotrophic pathogens such as *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al., 1998; Lorenzo et al., 2003), whereas these plants are more resistant to biotrophic bacterial pathogens such as *Pseudomonas syringae*, and show elevated SA levels consistently with SA-JA antagonism (Kloek et al., 2001). JAZ co-receptors are COI1 substrates that negatively regulate the JA-signaling pathway by directly interacting with and repressing TFs that control JA-regulated genes (Chini et al., 2007, 2009b; Thines et al., 2007; Sheard et al., 2010; Fernandez-Calvo et al., 2011; Pauwels and Goossens, 2011). In basal conditions, repression of TFs by JAZ proteins require the recruitment of the co-repressor TOPLESS (TPL) and TPL-related proteins (TPR) by the adaptor protein NINJA (Pauwels et al., 2010). Upon elicitation, the hormone-triggered interaction of the COI1-JAZ co-receptor induces the ubiquitination and degradation of JAZ repressors liberating the TFs from NINJA and TPL and activating the transcriptional responses mediated by the hormone (Chini et al., 2007; Maor et al., 2007; Thines et al., 2007; Saracco et al., 2009; Pauwels et al., 2010; Sheard et al., 2010). Recently, a TF-dependent mechanism for nuclear import of cognate JAZs transcriptional repressor has been reported in *Arabidopsis*, supporting the general belief that the JA receptor

complex functions within the nucleus (Withers et al., 2012). Complex formation in the presence of the hormone further requires inositol pentakisphosphate (InsP5) as a COI1 cofactor that potentiates the strength of the COI1–JAZ interaction (Sheard et al., 2010; Mosblech et al., 2011). This sustains previous observations showing that *Arabidopsis* mutants with altered levels of inositol polyphosphates displayed aberrant JA-dependent responses including altered defense capabilities to the insect *Plutella xylostella* (Mosblech et al., 2011).

Several TFs responsible for activation of different JA-mediated responses have been identified and include the basic helix-loop-helix (bHLH) TFs MYC2, MYC3, and MYC4 (Chini et al., 2007; Cheng et al., 2011; Fernandez-Calvo et al., 2011; Niu et al., 2011). Fernandez-Calvo et al. (2011) showed that MYC3 and MYC4 are activators of JA-regulated programs that act additively with MYC2 to regulate specifically different subsets of the JA-dependent transcriptional response. Interestingly, a triple mutant *myc2myc3myc4* in *Arabidopsis* is as impaired as *Arabidopsis* plants lacking the *COI1* gene in the activation of JA-dependent defense responses against insect herbivory by *Spodoptera littoralis* and the bacterial pathogen *Pseudomonas syringae* (Fernandez-Calvo et al., 2011), indicating that JA-dependent defense responses to these pathogens and pests are mostly controlled by these three TFs. Additional JAZ TF targets have been identified in the last 2 years. These include other bHLH TFs such as GL3 (GLABRA3), EGL3 (ENHANCER of GLABRA3) and TT8 (TRANSPARENT TESTA8), and the R2R3 MYB TFs PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT1), GL1, MYB75, MYB21, and MYB24 among others (Qi et al., 2011; Song et al., 2011). However, despite they are known to be involved in several physiological processes, the contribution of most of these TFs to resistant against pathogens remains unknown.

THE SALICYLIC ACID PATHWAY

Salicylic acid is a secondary metabolite produced by a wide range of organisms (An and Mou, 2011). In plants, SA functions as a plant hormone required for innate immunity against biotrophic pathogens such as the bacteria *Pseudomonas syringae* (Vlot et al., 2009). Despite the key role of SA in immunity against microbial infections, how plants detect the hormone has remained unclear until very recently. Using different ligand/receptor-binding methods, two research groups reported that NPR1 (NON-EXPRESSOR OF PR GENES1) or NPR1-related proteins, NPR3 (NPR1-LIKE PROTEIN3) and NPR4 (NPR1-LIKE PROTEIN4), are the long-sought SA receptors in *Arabidopsis* (Fu et al., 2012; Wu et al., 2012). Wu et al. (2012) provided evidence, using a special equilibrium dialysis ligand binding method, that NPR1 itself is a SA receptor. However, Fu et al. (2012) found that the two NPR1-related proteins, NPR3 and NPR4, but not NPR1, bind to SA directly in conventional ligand binding assays and function as truly SA receptors. NPR3 and NPR4 are BTB-CUL3 ligases that direct the degradation of NPR1 via the 26S proteasome (Fu et al., 2012). Consistently, a *npr3npr4* double mutant in *Arabidopsis* exhibits enhanced disease resistance, a phenotype that is opposite to that of the *npr1* mutant (Fu et al., 2012). NPR1 is a master transcriptional positive co-activator of the TGA clade of bZIP (basic region/ leucine zipper motif) transcription factors controlling SA signaling and a large set of defense-related genes such as *PR* (*PATHOGENESIS-RELATED*) genes (Delaney et al., 1995; Dong, 2004). *PR* genes are a diverse group, but several encode proteins with antimicrobial activity (van Loon et al., 2006). NPR1 exists in at least two forms in the cell. When SA levels are low (e.g., in the absence of pathogen infection), NPR1 is sequestered in the cytoplasm as an oligomer through intermolecular disulphide bonds by *S*-nitrosylation of NPR1 via *S*-nitrosoglutathione activity (Tada et al., 2008). However, when the SA levels are high (e.g., after pathogen infection), redox changes in the cytosol trigger the monomerization of NPR1 by the activity of the thioredoxinsTRX-H3 and TRX-H5 (Tada et al., 2008). NPR1 monomers enter the nucleus via nuclear pore proteins, such as MODIFIER OF *snc1* (MOS) 3, 6, and 7 (Cheng et al., 2009). In the nucleus, NPR1 bind TGA TFs initiating the SA-associated global transcriptional response (Dong, 2004). Strikingly, Fu et al. (2012) made interesting observations with crucial biological consequences for the establishment of plant immunity. NPR3 and NPR4 differ in their affinity for the SA hormone and in their roles in NPR1 degradation. NPR3 mediates NPR1 breakdown via 26S proteasome only in the presence of SA and NPR4 only in its absence (Fu et al., 2012). Thus, in healthy plants where SA is not present, NPR4, as part of the CUL3–NPR4 ubiquitin ligase, interacts with NPR1 to remove the NPR1 protein preventing the activation of energy-consuming defenses. In infected tissue, SA levels increase to high concentrations and promotes interaction of NPR3 with NPR1 to mediate degradation of NPR1, leading to strong defense-associated cell death at the site of attack. Upon infection, SA levels also increase at distal parts of plants. In these tissues, NPR1–NPR3 and NPR1–NPR4 interactions are both weakened, resulting in accumulation of NPR1, expression of defense genes without cell death and establishment of systemic acquired resistance. The recent identification of the SA receptors reveals how the hormone controls cell death and survival during plant immune responses in tissues close to and distant from the site of infection.

ANTAGONISTIC CROSSTALK BETWEEN JA AND SA: SELECTING THE RIGHT PATHWAY

The activation of plant defenses implies allocation and ecological costs (Pieterse et al., 2012). For example, the allocation of resources to defense against one type of attacker can reduce the ability of the plant to respond to the challenge of a different invader. Thus, the antagonistic interplay between SA and JA seems to optimize the immune response against a specific single attacker. Plants infected by SA-inducing biotrophic pathogens often suppress JA-dependent defenses, apparently prioritizing the investment of resources in SA-dependent defense over JA-dependent responses (Spoel et al., 2007). Similarly, the elicitation of the JA pathway by pathogens can repress the SA response (Uppalapati et al., 2007). Recently, Van der Does et al. (2013) showed that SA suppresses JA signaling downstream of the COI1-JAZ receptor complex by targeting GCC-box motifs in JA-responsive promoters via a negative effect on the accumulation of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF)-type transcriptional activator ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS59). Indeed, the GCC-box motif is overrepresented in JA-responsive promoters that are suppressed by SA and this promoter motif is sufficient for SA-mediated suppression of JA-induced gene

expression (Van der Does et al., 2013). Interestingly, SA reduces the accumulation of the GCC-box binding TF ORA59, indicating that the antagonistic effect of SA on JA signaling is controlled at the level of transcriptional regulation, through the modulation of TF levels.

Several other proteins are known to play a role in regulating SA-mediated suppression of the JA pathway including mitogen-activated protein kinases, redox regulators, NPR1, and nuclear TGA and WRKY TFs among others (Pieterse et al., 2012). In *Arabidopsis*, mitogen activated protein kinase 4 (MPK4) acts as a negative regulator of SA signaling and positive regulator of JA signaling (Petersen et al., 2000; Brodersen et al., 2006). The *Arabidopsis mpk4* mutants show elevated SA levels, constitutive expression of SA responsive *PR* genes and increased resistance to *Pseudomonas syringae*. The expression of JA responsive genes and the resistance to *Alternaria brassicicola* is also impaired in *mpk4* mutants (Petersen et al., 2000; Brodersen et al., 2006).

Other important regulators affecting the antagonism between SA and JA-mediated signaling are glutaredoxins (GRXs), including GRX480 and several others of the ROXY class (Ndamukong et al., 2007; Zander et al., 2012). These proteins are central players in mediating redox regulation of protein activity because of their capacity to catalyze disulfide transitions (Meyer, 2008). These GRXs interact with TGA TFs involved in the regulation of SA responsive *PR* genes and antagonize JA-responsible genes such as *PLANT DEFENSIN1.2* (*PDF1.2*) and *ORA59* (Ndamukong et al., 2007; Zander et al., 2012). For example, GRX480 interacts with TGA2 and TGA6 (Ndamukong et al., 2007) and has been implicated in SA-mediated suppression of the JA pathway. Indeed, *tga256* triple and *tga2356* quadruple mutants are impaired in SA-mediated suppression of the JA pathway (Ndamukong et al., 2007; Zander et al., 2009), indicating that TGAs effectively regulate SA–JA crosstalk.

Another important regulatory component is the SA master regulator NPR1 itself, which interacts with TGA TFs that are involved in the activation of SA-responsive PR genes (Dong, 2004). Nuclear localization of NPR1 is essential for SA-responsive defense gene expression, but not for SA-mediated suppression of the JA pathway (Spoel et al., 2003), indicating that SA–JA crosstalk is likely mediated by cytosolic NPR1. Despite this, NPR1 regulates several SA-dependent nuclear TFs or cofactors required for suppression of JA-gene expression such as TGA and WRKY TFs (Robert-Seilaniantz et al., 2011).

WRKY TFs are also an important node of convergence between SA and JA signaling (Pieterse et al., 2012). These include WRKY50, WRKY51, WRKY70, and WRKY62 among others (Pieterse et al., 2012). For example, overexpression of WRKY70 resulted in the constitutive expression of SA-responsive PR genes and enhanced resistance to the biotrophic pathogen *Erysiphe cichoracearum* but repressed the expression of JA-responsive marker gene *PDF1.2* and compromised resistance to the necrotrophic pathogen *Alternaria brassicicola* (Li et al., 2004; Li et al., 2006).

Finally, the JA nuclear TF MYC2 acts as a negative regulator of SA signaling in *Arabidopsis* as *myc2* mutants show increased accumulation of SA, enhance expression of PR genes and increase resistance to *Pseudomonas syringae* compared to wild type plants (Laurie-Berry et al., 2006). Similarly, the triple mutant *myc2myc3myc4* in *Arabidopsis* is as resistant as *Arabidopsis* plants lacking the *COI1* gene to the bacterial pathogen *Pseudomonas syringae* (Fernandez-Calvo et al., 2011). Despite the increasing knowledge of proteins playing a role in SA–JA crosstalk, how crosstalk occurs at molecular level remains largely to be elucidated.

NETWORKING OF HORMONES IN PLANT IMMUNITY

Plants use other hormones to fine-tune immune responses built on the SA and JA defense pathways (Pieterse et al., 2012). Incredible progress has been done in the last 2 years in the understanding of how SA and JA routes interact at the molecular level with other hormonal pathways. Here, we review the most outstanding examples regarding nuclear fine-tuning of hormonal balances in plant immunity.

CROSSTALK JA-ET: FINE-TUNNING SYNERGY OF DEFENSES AGAINST PATHOGENS

Ethylene is another important plant hormone that works together with JA to regulate defense against necrotrophic pathogens (Wang et al., 2002). ET is perceived by a group of membrane-located receptor proteins including ETR1 (ETHYLENE RESPONSE 1), ERS1 (ETHYLENE RESPONSE SENSOR 1), ETR2 (ETHYLENE RESPONSE 2), ERS2 (ETHYLENE RESPONSE SENSOR 2), and EIN4 (ETHYLENE INSENSITIVE 4; Bleecker et al., 1988; Ecker, 1995; Hua et al., 1998). In normal conditions, where the level of ET is usually low, the receptors act to suppress ET response by activating the downstream negative regulator raf-like kinase CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) through direct physical interaction (Clark et al., 1998). Downstream of CTR1 is ETHYLENE INSENSITIVE 2 (EIN2), which is an essential positive regulator of ET signaling (Alonso et al., 2003). CTR1 suppression is relieved upon ET binding to the trans-membrane domain of the receptors, facilitating subsequent activation of a diverse set of ET-responsive TFs downstream of EIN2 including EIN3 (ETHYLENE INSENSITIVE 3) and its nearest homolog EIL1 (EIN3-LIKE 1; Chao et al., 1997; Wang et al., 2006). EIN3 levels are regulated through the action of at least two related F-box proteins, EIN3-Binding F-box 1 (EBF1) and EBF2 which are thought to repress EIN3 levels when ET is low (Guo and Ecker, 2003; Potuschak et al., 2003; Binder et al., 2007). EIN3 and EIL1 belong to a multigene family of TFs including six putative members of this family in *Arabidopsis* (Wang et al., 2002). However, EIN3 and EIL1 TFs are largely responsible for primary gene induction downstream of ET sensing including defense against pathogens (Guo and Ecker, 2004).

The crosstalk between JA and ET can be rather complex and context-dependent. However, JA and ET signaling act synergistically during plant defense against necrotrophic pathogens (Broekaert et al., 2006). In *Arabidopsis*, two major branches of the JA signaling pathway are recognized, the MYC branch and the ERF branch (Lorenzo et al., 2004; Dombrecht et al., 2007). In general, the MYC branch is associated with the wound response and defense against insect herbivores (Lorenzo et al., 2004), whereas the ERF branch is associated with enhanced resistance to necrotrophic pathogens (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). The MYC branch is controlled by MYC-type TFs leading to

expression of JA-responsive marker genes such as *VEGETATIVE STORAGE PROTEIN2* (*VSP2*). The ERF branch is regulated by members of the AP2/ERF family of TFs, such as ERF1 and ORA59 (Lorenzo et al., 2003; McGrath et al., 2005; Dombrecht et al., 2007), that regulate the expression of JA-responsive marker genes such as *PDF1.2*. Overexpression of *ERF1* enhances resistance against *B. cinerea* and other necrothrophic pathogens, and increases susceptibility to the hemibiotroph *Pseudomonas syringe* (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). The ERF branch is synergistically regulated by the ET- and the JA-pathways, whereas they antagonize in the regulation of the MYC branch (Lorenzo and Solano, 2005). Zhou and colleagues recently showed that JAZ proteins interact directly with the ET TFs EIN3 and EIL1, inhibiting the transcriptional activity of these TFs by recruitment of the transcriptional co-repressor HISTONE DEACETYLASE6 (HDA6; Zhu et al., 2011). EIN3 and EIL1 transcriptional regulators are stabilized in the presence of ET, allowing the expression of ET-responsive genes. However, their binding to JAZ proteins partially represses the function of EIN3/EIL1, possibly by suppressing their DNA binding capacity. This provides a second level of transcriptional regulation throught JA (Zhu et al., 2011). In the presence of JA, EIN3/EIL1 TFs are released from the JAZ repression allowing full activity of these TFs. This results in the synergistic activation of *ERF1* and its downstream target genes such as *PDF1.2* (Zhu et al., 2011). In addition, EIN3 and EIL1 are known repressors of *SID2* (SALICYLIC ACID INDUCTION DEFICIENT 2), a gene encoding an isochorismate synthase required for SA biosynthesis (Chen et al., 2009). Thus, it is possible that JAZ repressors also mediate JA–SA antagonistic crosstalk thought the suppression exerted on EIN3 and EIL1.

CROSSTALK JA-GIBBERELLINS: BALANCING DEFENSE AND GROWTH

Gibberellins are plant hormones involved in the regulation of plant growth in response to endogenous and environmental signals (Sun, 2011). DELLA proteins are key components of GA signaling and nuclear localized negative regulators of plant growth-promoting TFs, such as PIFs (PHYTOCHROME INTERACTING FACTORS; de Lucas et al., 2008; Feng et al., 2008). In *Arabidopsis*, there are five DELLAs: GAI (GIBBERELLIC ACID INSENSITIVE), RGA (REPRESSOR OF GA1-3), RGL1 (RGA-like1), RGL2, and RGL3 (Peng et al., 1997; Silverstone et al., 2001; Lee et al., 2002; Tyler et al., 2004). Binding of GA to its receptor GID1 (GA INSENSITIVE DWARF1) promotes the GID1–DELLA interaction, which in turn stimulates the interaction between DELLAs and the specific E3 ubiquitin ligase SLY1/GID2 complex, leading to subsequent degradation of DELLAs by the 26S proteasome and activation of PIFs (Silverstone et al., 2001; Tyler et al., 2004; Harberd et al., 2009). Importantly, DELLAs modulate plant immune response by modulating the balance of JA/SA. For example, DELLA activity promotes plant resistance to necrotrophs by potentiating JA signaling and increases plant susceptibility to virulent biotrophs by attenuating the SA pathway (Navarro et al., 2008). Accordingly, JA mediated pathogen defense is attenuated in DELLA loss-of-function mutants while defense genes are hyperactivated by JA in constitutively active DELLA mutants (Navarro et al., 2008). Moreover, a GA-deficient mutant *ga1* shows upregulated expression of JA-responsive defense genes (Hou et al., 2010), indicating that DELLA proteins interact with JA signaling in a positive manner. Interestingly, it was recently reported that DELLA interact with JAZ proteins and modulate JA signaling via competitive binding with MYC TFs for engaging to JAZ repressors (Chen et al., 2004; Hou et al., 2010; Wild et al., 2012; Yang et al., 2012). Consistent with this model, MYC2-dependent JA-responsive genes are more induced in response to JA treatment in mutant backgrounds accumulating DELLAs such as the GA biosynthetic mutant *ga1-3* compared to mock treatment controls (Hou et al., 2010). Indeed, overexpression of RGL3 activates MYC2-dependent JA-induced gene expression, whereas *rgl3* mutation reduces it (Wild et al., 2012). Consistently, RGL3 positively regulates JA-mediated resistance to the necrotroph *B. cinerea* and susceptibility to the hemibiotroph *Pseudomonas syringae* (Wild et al., 2012). On the other hand, Yang et al. (2012) recently reported that JA prioritizes defense over growth by interfering with GAs signaling cascade through the COI1–JAZ–DELLA–PIF signaling module. This provides an explanation about why activation of defense in plants is often accompanied by a significant inhibition of growth. Thus, DELLAs and JAZs seem to integrate environmental signals that enable plants to adapt their growth and development according to their surrounding environment.

CROSSTALK SA/CK: GROWTH HORMONES REGULATING PLANT IMMUNITY

Cytokinin are growth control hormones, which promote cell division, nutrient mobilization, and leaf longevity (Choi et al., 2011). CK have recently emerged as modulators of plant immunity (Choi et al., 2011). In *Arabidopsis*, hybrid histidine protein kinases (AHKs) serve as CK receptors (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). Histidine phosphotransfer proteins (AHPs) transmit the signal from AHKs to nuclear response regulators (ARRs), which can activate or repress transcription (Hwang et al., 2012). CKs promote resistance of *Arabidopsis* to *Pseudomonas syringae*, which correlates with increased SA biosynthesis and *PR1* expression (Choi et al., 2010). As shown by Choi et al. (2010), this is probably mediated by a direct interaction between ARR2, a TF involved in CK signaling, and the SA response TF TGA3. ARR2 specifically interacts with TGA3 and is recruited to the PR1 promoter, inducing resistance to *Pseudomonas syringae*. In contrast, the alternative ARR1 related factor that cannot interact with TGA3, fails to induce resistance to *Pseudomonas syringae* (Choi et al., 2010, 2011). Moreover, the SA biosynthetic genes, *SID1* and *SID2*, and the SA-responsive genes, PR1 and PR5, are over-induced in 35S:ARR2 plants compared to Col-0 controls inoculated with *Pseudomonas syringae* (Choi et al., 2010). Therefore, crosstalk SA/CK is based on the regulation of a module in the SA-mediated defense response network by the ARR2 transcriptional factor.

HIJACKING NUCLEAR HORMONAL NETWORKS BY MICROBES

Microbial pathogens have also developed the ability to manipulate the defense-related regulatory network of plant hormones to cause hormonal imbalances and inappropriate activation of defense responses for their own benefit (Robert-Seilaniantz et al., 2011). In recent years, there have been a number of examples of

plant pathogens that hijack specific hormone-regulated signaling pathways to redirect the immune response in the nucleus. Microbes do this by producing plant hormones, phytohormone mimics, or virulence effectors that target hormone signaling components. Here, we describe the best characterized examples at the molecular level of specific nuclear hormonal manipulation by microbes, which exemplify the ingenious ways by which pathogens can take control over the plant's hormone signaling network to suppress host immunity.

PRODUCTION OF PLANT HORMONES AND HORMONE MIMICS BY PATHOGENS

Interestingly, many pathogens are capable of synthesizing phytohormones. Different bacterial or fungal species are known to produce JA (Mittal and Davis, 1995), ET (Weingart and Volksch, 1997; Weingart et al., 2001), CK (Kakimoto, 2003), ABA (Kitagawa et al., 1995; Siewers et al., 2006), and auxins (Spaepen et al., 2007). For example, the necrotrophic fungi *B. cinerea* produces ABA, CK and secretes an exopolysaccharide that acts as an elicitor of the SA pathway (El Oirdi et al., 2011; Robert-Seilaniantz et al., 2011). In contrast, *Fusarium oxysporum* produces only ABA (Robert-Seilaniantz et al., 2011). Biotrophic fungi such as *Cladosporium fulvum*, *Ustilago maydis*, *Pyrenopeziza brassicae*, and *Venturia inaequalis* are well known to produce CKs (Robert-Seilaniantz et al., 2011). Moreover, the biotrophic bacterial pathogen *Ralstonia solanacearum* produce ET and indolic compounds related to auxin (Valls et al., 2006), and *Pseudomonas syringae* pv. *syringae* B728a also produces auxin by converting host indole acetonitrile into IAA using a nitrilase (Howden et al., 2009). Therefore, it is not surprising that some pathogens trigger symptoms indicative of hormonal imbalances. This is the case of *Agrobacterium tumefaciens* that induces gall formation following T-DNA transfer and *in planta* production of auxin and CK (Akiyoshi et al., 1983). The increased height of rice seedling infected with *Gibberella fujikuroi* is a consequence of production of GAs by the pathogen (Robert-Seilaniantz et al., 2011). Despite all these examples, the roles of hormones during plant–pathogen interactions are still not fully understood in most cases.

Probably, the best understood example corresponds to the production of coronatine (COR), a mimic of the bioactive jasmonate hormone JA-Ile, by some strains of *Pseudomonas syringae* (Brooks et al., 2004; Fonseca et al., 2009b). COR, as the JA-Ile hormone, is perceived in the nucleus through the COI1/JAZ receptor complex that upon COR binding triggers the degradation of JAZ transcriptional repressors via the 26S proteasome (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). This leads to derepression of JAZ-interacting TFs that initiate the transcription of JA-dependent genes, further inhibiting SA-dependent defenses against the bacteria. Indeed, COR is more active than the own JA-Ile plant hormone itself in triggering the COI1–JAZ complexes formation and subsequent JAZ degradation (Katsir et al., 2008; Fonseca et al., 2009b), indicating than COR acts as a potent virulence factor in plants. COR contributes to disease symptomatology by inducing chlorotic lesions (Kloek et al., 2001; Brooks et al., 2004; Uppalapati et al., 2007), facilitates entry of the bacteria into the plant host by stimulating the opening of stomata (Melotto et al., 2006, 2008) and promotes bacterial growth by inhibiting SA-dependent defenses required for *Pseudomonas syringae* resistance through the activation of its antagonistic JA pathway (Cui et al., 2005; Laurie-Berry et al., 2006). Interestingly, it was recently reported that COR suppresses a SA-independent pathway contributing to callose deposition, a hallmark of plant resistance, by reducing accumulation of an indole glucosinolate in a COI1-independent manner (Geng et al., 2012). This indicates that COR may have additional targets to the COI1/JAZ receptor complex inside plant cells opening novel and interesting areas of research. Thus, acquisition of COR by these *Pseudomonas* pathogens has been of tremendous adaptative importance during host-pathogen evolution because it has allowed bacteria to manipulate the host hormonal network to promote susceptibility.

PATHOGEN EFFECTORS TARGETING NUCLEAR HORMONE SIGNALING COMPONENTS

In addition to producing hormones themselves, many pathogens also introduce into the plant cell an arsenal of virulence effector proteins (Jones and Dangl, 2006). Within the cell, these effectors interact with host proteins to promote pathogenesis (Jones and Dangl, 2006). Several bacterial effectors are known to affect hormonal equilibrium into the plant cell. For instance, the *Pseudomonas* effector AvrPtoB stimulates ABA biosynthesis and ABA responses, which in turn antagonize SA biosynthesis and SA-mediated defenses (de Torres Zabala et al., 2007, 2009). In another example, the *Pseudomonas* effector AvrRpt2 alters the auxin physiology to promote disease (Chen et al., 2007). However, how these effectors are able to impact in the hormonal homeostasis remains a mystery. In contrast to the above examples, the transcription activators-like (TAL) effectors of *Xanthomonas* spp. are a reference in their mode of action showing extreme target specificity (Boch and Bonas, 2010). This class of effectors is exemplified by AvrBs3 which is imported into the plant cell nucleus, and targeted to effector-specific gene promoters by mimicking eukaryotic TFs (Boch and Bonas, 2010). The specificity of these TFs arises from interactions between the DNA binding domain of each effector and a sequence in the target gene promoter called the *UPA* box. In a stunning series of papers, the molecular basis of promoter recognition by TAL effectors was decoded (Kay et al., 2007; Boch et al., 2009). The DNA binding domain comprises a central tandem repeat region (Kay et al., 2007; Boch et al., 2009; Moscou and Bogdanove, 2009). Strikingly, two hypervariable amino acid residues in each repeat specify interaction with a characteristic nucleotide within the effector recognition site (Boch and Bonas, 2010). Thus, the nucleotide sequence of the target DNA can be predicted with complete accuracy based on the amino acid sequence of the tandem repeat domain. Interestingly, auxin-induced genes and α-expansins are among the *UPA* genes regulated by AvrBs3 (Kay et al., 2007). Indeed, AvrBs3 directly targets *UPA20*, a bHLH TF that controls cell enlargement and plant cell hypertrophy phenotype through the activation of putative α-expansin UPA7 which is involved in cell wall softening (Kay et al., 2007). Consistently, AvrBs3 causes tissue hypertrophy, which is due to an enlargement of the mesophyll cells in infected tissue and resemble symptoms indicative of hormonal imbalances. This might help the bacteria to escape from infection sites to facilitate bacterial spreading.

Recently, it has been reported that the *Xanthomonas campestris* effector XopD is able to target MYB30, a TF that positively regulates *Arabidopsis* defense and associated cell death responses to bacteria through transcriptional activation of genes related to very-long-chain fatty acid (VLCFA) metabolism (Canonne et al., 2011). XopD specifically interacts with MYB30, resulting in inhibition of the MYB30-dependent transcriptional activation of VLCFA-related genes and suppression of *Arabidopsis* defense (Canonne et al., 2011; Canonne and Rivas, 2012). Interestingly, it was previously reported that MYB30 is a direct target of BES1, a key regulator of BR signaling, and cooperates with BES1 to regulate BR-induced gene expression (Li et al., 2009). However, whether XopD suppresses immunity by also affecting BR homeostasis is currently unknown.

NUCLEAR MODULATION OF HORMONAL PATHWAYS BY BENEFICIAL MICROBES

Beneficial microbes interacting with plants establish long-term relationships with their hosts to fulfill their life cycles (Zamioudis and Pieterse, 2012). In order to do this, they need to contend with the defense mechanisms of the plant to develop within the host and feed on living cells. Recently, the signals from two beneficial microbes that mediate symbiosis with their host plants have been characterized and results nicely show that in both cases these beneficial effectors hijack hormone signaling at the nucleus (Kloppholz et al., 2011; Plett et al., 2011). The MYCORRHIZAL INDUCED SMALL SECRETED PROTEIN 7 (MiSSP7), the most highly symbiosis-upregulated gene from the ectomycorrhizal fungus *Laccaria bicolor*, encodes an effector protein indispensable for the establishment of mutualism with their host plants. MiSSP7 is secreted by the fungus upon receipt of diffusible signals from plant roots, imported into the plant cell via phosphatidylinositol-3-phosphate-mediated endocytosis, and targeted to the plant nucleus where it alters the transcriptome of the plant cell promoting the expression of auxin-responsible genes (Plett et al., 2011).

The SP7 effector of the fungus *Glomus intraradices* is another example of symbiotic effectors that promote a biotrophic interaction by affecting hormonal signaling pathways (Kloppholz et al., 2011). SP7 possess immune-suppressive function by targeting the ET signaling pathway, which is an important component of plant immune responses in the roots (Boutrot et al., 2010). The role of SP7 in hijacking ET signaling is interesting as recent work indicates that the ET pathway is a key determinant in the colonization of plant tissues by fungus (Splivallo et al., 2009; Camehl et al., 2010). SP7 localizes to the plant nucleus where it interacts with ET response factor 19 (ERF19) to repress plant defense signaling. *ERF19* is highly induced in roots by the fungal pathogen *Colletotrichum trifolii* as well as by several fungal extracts, but only transiently during mycorrhiza colonization. When constitutively expressed in roots, SP7 leads to higher mycorrhization while reducing the levels of *Colletotrichum trifolii*-mediated defense responses (Kloppholz et al., 2011). Thus, beneficial microbes also contain effectors that resemble those of pathogenic fungi, nematodes, and bacteria. These effectors are similarly targeted to the plant nucleus to manipulate hormonal signaling and colonization of the plant tissues, and thus can be considered a mutualism effector.

CONCLUDING REMARKS

These are exciting times for hormonal signaling research as recent major discoveries have been made in the last few years. Recent knowledge regarding the perception of plant hormones and the involvement of specific hormone-related proteins in direct cross-talk between various hormonal and environmental signals has advanced our understanding of the molecular basis of how several hormones control plant immunity to a broad range of different pathogens. The recent findings suggest that several important mediators of hormone cross-talk are transcriptional factors or repressors, indicating that cross-talk predominantly takes place in the nucleus downstream of signal transduction, at the level of gene transcription. JAZs and DELLAs are all repressors of positive transcriptional regulators of hormone signaling. The rapid removal of hormonal transcriptional repressors through 26S proteasome and the ability to interact with each other (and additional TFs associated to other hormonal pathways such as EIN3/EIL1) provides a paradigm to explain the rapid and appropriate level of defense responses to specific signals. Moreover, key hormones regulating growth, such as GA and auxins, are involved in the orchestration of the plant immune response suggesting that developmental and defense signaling networks are closely interconnected. This would explain why activation of costly defenses is often accompanied by significant growth inhibition. Thus, hormone homeostasis and cross-talk seem to be a dominant feature to maximize defenses and to fine-tune growth and protection. Not surprisingly, pathogens and beneficial microbes have learned the necessity of manipulation of plant hormonal pathways to rewire the immune signaling circuitry for their own benefit. Indeed, the importance of hormones in plant immunity is highlighted by the increasing number of pathogens that are predicted to produce phytohormones or phytohormone mimics, and the recent findings indicating that microbial effectors also targets hormonal pathways to promote disease or establish beneficial interactions (Robert-Seilaniantz et al., 2011). Due to the increasing number of effectors known to be targeted to the plant cell nucleus (Schornack et al., 2010; Canonne and Rivas, 2012), it is plausible to expect direct virulence activities on hormonal components in order to subvert host transcription. An improved understanding of the mechanisms by which pathogens use their toxins and effectors to manipulate and target hormonal components controlling immunity in plants will prove invaluable for identifying defensive hubs in plants controlling immunity and developing plant lines with improved resistance. It truly is an exciting time to start understanding how complex hormonal signaling and interactions are translated into a definite coordinated defense response that is effective against the type of pathogen that the plant is encountering.

ACKNOWLEDGMENTS

Selena Gimenez-Ibanez was supported by a "Juan de la Cierva" fellowship from the Spanish Ministry for Science and Innovation. Work in Roberto Solano lab was funded by the Spanish Ministry for Science and Innovation grants BIO2010-21739, CSD2007-00057 and EUI2008- 03666 to Roberto Solano. We thank all members of the lab for helpful discussions and critical reading of the manuscript.

REFERENCES

Akiyoshi, D. E., Morris, R. O., Hinz, R., Mischke, B. S., Kosuge, T., Garfinkel, D. J., et al. (1983). Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. *Proc. Natl. Acad. Sci. U.S.A.* 80, 407–411.

Alonso, J. M., Stepanova, A. N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F. M., et al. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2992–2997.

An, C., and Mou, Z. (2011). Salicylic acid and its function in plant immunity. *J. Integr. Plant Biol.* 53, 412–428.

Berrocal-Lobo, M., and Molina, A. (2004). Ethylene response factor 1 mediates *Arabidopsis* resistance to the soilborne fungus *Fusarium oxysporum*. *Mol. Plant Microbe Interact.* 17, 763–770.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. *Plant J.* 29, 23–32.

Binder, B. M., Walker, J. M., Gagne, J. M., Emborg, T. J., Hemmann, G., Bleecker, A. B., et al. (2007). The *Arabidopsis* EIN3 binding F-Box proteins EBF1 and EBF2 have distinct but overlapping roles in ethylene signaling. *Plant Cell* 19, 509–523.

Bleecker, A. B., Estelle, M. A., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* 241, 1086–1089.

Boch, J., and Bonas, U. (2010). *Xanthomonas* AvrBs3 family-type III effectors: discovery and function. *Annu. Rev. Phytopathol.* 48, 419–436.

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., et al. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509–1512.

Boutrot, F., Segonzac, C., Chang, K. N., Qiao, H., Ecker, J. R., Zipfel, C., et al. (2010). Direct transcriptional control of the *Arabidopsis* immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14502–14507.

Broekaert, W. F., Delaure, S. L., De Bolle, M. F., and Cammue, B. P. (2006). The role of ethylene in host-pathogen interactions. *Annu. Rev. Phytopathol.* 44, 393–416.

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

Brooks, D. M., Hernandez-Guzman, G., Kloek, A. P., Alarcon-Chaidez, F., Sreedharan, A., Rangaswamy, V., et al. (2004). Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. tomato DC3000. *Mol. Plant Microbe Interact.* 17, 162–174.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J., et al. (2010). Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana*. *New Phytol.* 185, 1062–1073.

Canonne, J., Marino, D., Jauneau, A., Pouzet, C., Briere, C., Roby, D., et al. (2011). The *Xanthomonas* type III effector XopD targets the *Arabidopsis* transcription factor MYB30 to suppress plant defense. *Plant Cell* 23, 3498–3511.

Canonne, J., and Rivas, S. (2012). Bacterial effectors target the plant cell nucleus to subvert host transcription. *Plant Signal. Behav.* 7, 217–221.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J. R. (1997). Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 89, 1133–1144.

Chen, H., Mccaig, B. C., Melotto, M., He, S. Y., and Howe, G. A. (2004). Regulation of plant arginase by wounding, jasmonate, and the phytotoxin coronatine. *J. Biol. Chem.* 279, 45998–46007.

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

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.

Cheng, Z., Sun, L., Qi, T., Zhang, B., Peng, W., Liu, Y., et al. (2011). The bHLH transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate jasmonate response in *Arabidopsis*. *Mol. Plant* 4, 279–288.

Chen, Z., Agnew, J. L., Cohen, J. D., He, P., Shan, L., Sheen, J., et al. (2007). *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20131–20136.

Chini, A., Boter, M., and Solano, R. (2009a). Plant oxylipins: COI1/JAZs/MYC2 as the core jasmonic acid-signalling module. *FEBS J.* 276, 4682–4692.

Chini, A., Fonseca, S., Chico, J. M., Fernandez-Calvo, P., and Solano, R. (2009b). The ZIM domain mediates homo- and heteromeric interactions between *Arabidopsis* JAZ proteins. *Plant J.* 59, 77–87.

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

Choi, J., Choi, D., Lee, S., Ryu, C. M., and Hwang, I. (2011). Cytokinins and plant immunity: old foes or new friends? *Trends Plant Sci.* 16, 388–394.

Choi, J., Huh, S. U., Kojima, M., Sakakibara, H., Paek, K. H., and Hwang, I. (2010). The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in *Arabidopsis*. *Dev. Cell* 19, 284–295.

Clark, K. L., Larsen, P. B., Wang, X., and Chang, C. (1998). Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5401–5406.

Cui, J., Bahrami, A. K., Pringle, E. G., Hernandez-Guzman, G., Bender, C. L., Pierce, N. E., et al. (2005). *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1791–1796.

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

de Lucas, M., Daviere, J. M., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J. M., Lorrain, S., et al. (2008). A molecular framework for light and gibberellin control of cell elongation. *Nature* 451, 480–484.

de Torres Zabala, M., Bennett, M. H., Truman, W. H., and Grant, M. R. (2009). Antagonism between salicylic and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. *Plant J.* 59, 375–386.

de Torres Zabala, M., Truman, W., Bennett, M. H., Lafforgue, G., Mansfield, J. W., Rodriguez Egea, P., et al. (2007). *Pseudomonas syringae* pv. tomato hijacks the *Arabidopsis* abscisic acid signalling pathway to cause disease. *EMBO J.* 26, 1434–1443.

De Vos, M., Van Oosten, V. R., Van Poecke, R. M., Van Pelt, J. A., Pozo, M. J., Mueller, M. J., et al. (2005). Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol. Plant Microbe Interact.* 18, 923–937.

Dombrecht, B., Xue, G. P., Sprague, S. J., Kirkegaard, J. A., Ross, J. J., Reid, J. B., et al. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell* 19, 2225–2245.

Dong, X. (2004). NPR1, all things considered. *Curr. Opin. Plant Biol.* 7, 547–552.

Ecker, J. R. (1995). The ethylene signal transduction pathway in plants. *Science* 268, 667–675.

El Oirdi, M., El Rahman, T. A., Rigano, L., El Hadrami, A., Rodriguez, M. C., Daayf, F., et al. (2011). *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *Plant Cell* 23, 2405–2421.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., et al. (2008). Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. *Nature* 451, 475–479.

Fernandez-Calvo, P., Chini, A., Fernandez-Barbero, G., Chico, J. M., Gimenez-Ibanez, S., Geerinck, J., et al. (2011). The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *Plant Cell* 23, 701–715.

Feys, B., Benedetti, C. E., Penfold, C. N., and Turner, J. G. (1994). *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* 6, 751–759.

Fonseca, S., Chico, J. M., and Solano, R. (2009a). The jasmonate pathway: the ligand, the receptor and the core signalling module. *Curr. Opin. Plant Biol.* 12, 539–547.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., et al. (2009b). (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nat. Chem. Biol.* 5, 344–350.

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

Geng, X., Cheng, J., Gangadharan, A., and Mackey, D. (2012). The coronatine toxin of *Pseudomonas syringae* is a multifunctional suppressor of *Arabidopsis* defense. *Plant Cell* 24, 4763–4774.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227.

Grant, M., and Lamb, C. (2006). Systemic immunity. *Curr. Opin. Plant Biol.* 9, 414–420.

Guo, H., and Ecker, J. R. (2003). Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* 115, 667–677.

Guo, H., and Ecker, J. R. (2004). The ethylene signaling pathway: new insights. *Curr. Opin. Plant Biol.* 7, 40–49.

Harberd, N. P., Belfield, E., and Yasumura, Y. (2009). The angiosperm gibberellin-GID1-DELLA growth regulatory mechanism: how an "inhibitor of an inhibitor" enables flexible response to fluctuating environments. *Plant Cell* 21, 1328–1339.

Hou, X., Lee, L. Y., Xia, K., Yan, Y., and Yu, H. (2010). DELLAs modulate jasmonate signaling via competitive binding to JAZs. *Dev. Cell* 19, 884–894.

Howden, A. J., Rico, A., Mentlak, T., Miguet, L., and Preston, G. M. (2009). *Pseudomonas syringae* pv. *syringae* B728a hydrolyses indole-3-acetonitrile to the plant hormone indole-3-acetic acid. *Mol. Plant Pathol.* 10, 857–865.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q. G., Bleecker, A. B., Ecker, J. R., et al. (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in *Arabidopsis*. *Plant Cell* 10, 1321–1332.

Hwang, I., Sheen, J., and Muller, B. (2012). Cytokinin signaling networks. *Annu. Rev. Plant Biol.* 63, 353–380.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., et al. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* 409, 1060–1063.

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

Kagale, S., Links, M. G., and Rozwadowski, K. (2010). Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in *Arabidopsis*. *Plant Physiol.* 152, 1109–1134.

Kakimoto, T. (2003). Biosynthesis of cytokinins. *J. Plant Res.* 116, 233–239.

Katsir, L., Schilmiller, A. L., Staswick, P. E., He, S. Y., and Howe, G. A. (2008). COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7100–7105.

Kay, S., Hahn, S., Marois, E., Hause, G., and Bonas, U. (2007). A bacterial effector acts as a plant transcription factor and induces a cell size regulator. *Science* 318, 648–651.

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

Kitagawa, M., Mukai, H., Shibata, H., and Ono, Y. (1995). Purification and characterization of a fatty acid-activated protein kinase (PKN) from rat testis. *Biochem. J.* 310(Pt 2), 657–664.

Kloek, A. P., Verbsky, M. L., Sharma, S. B., Schoelz, J. E., Vogel, J., Klessig, D. F., et al. (2001). Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. *Plant J.* 26, 509–522.

Kloppholz, S., Kuhn, H., and Requena, N. (2011). A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. *Curr. Biol.* 21, 1204–1209.

Laurie-Berry, N., Joardar, V., Street, I. H., and Kunkel, B. N. (2006). The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. *Mol. Plant Microbe Interact.* 19, 789–800.

Lee, S., Cheng, H., King, K. E., Wang, W., He, Y., Hussain, A., et al. (2002). Gibberellin regulates *Arabidopsis* seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. *Genes Dev.* 16, 646–658.

Li, J., Brader, G., Kariola, T., and Palva, E. T. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J.* 46, 477–491.

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

Li, L., Yu, X., Thompson, A., Guo, M., Yoshida, S., Asami, T., et al. (2009). *Arabidopsis* MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. *Plant J.* 58, 275–286.

Loake, G., and Grant, M. (2007). Salicylic acid in plant defence–the players and protagonists. *Curr. Opin. Plant Biol.* 10, 466–472.

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

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J. J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* 15, 165–178.

Lorenzo, O., and Solano, R. (2005). Molecular players regulating the jasmonate signalling network. *Curr. Opin. Plant Biol.* 8, 532–540.

Lumba, S., Cutler, S., and Mccourt, P. (2010). Plant nuclear hormone receptors: a role for small molecules in protein–protein interactions. *Annu. Rev. Cell Dev. Biol.* 26, 445–469.

Maor, R., Jones, A., Nuhse, T. S., Studholme, D. J., Peck, S. C., and Shirasu, K. (2007). Multidimensional protein identification technology (MudPIT) analysis of ubiquitinated proteins in plants. *Mol. Cell. Proteomics* 6, 601–610.

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

Melotto, M., Underwood, W., and He, S. Y. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu. Rev. Phytopathol.* 46, 101–122.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980.

Meyer, A. J. (2008). The integration of glutathione homeostasis and redox signaling. *J. Plant Physiol.* 165, 1390–1403.

Mittal, S., and Davis, K. R. (1995). Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. *Mol. Plant Microbe Interact.* 8, 165–171.

Mosblech, A., Thurow, C., Gatz, C., Feussner, I., and Heilmann, I. (2011). Jasmonic acid perception by COI1 involves inositol polyphosphates in *Arabidopsis thaliana*. *Plant J.* 65, 949–957.

Moscou, M. J., and Bogdanove, A. J. (2009). A simple cipher governs DNA recognition by TAL effectors. *Science* 326, 1501.

Navarro, L., Bari, R., Achard, P., Lison, P., Nemri, A., Harberd, N. P., et al. (2008). DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Curr. Biol.* 18, 650–655.

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

Niu, Y., Figueroa, P., and Browse, J. (2011). Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in *Arabidopsis*. *J. Exp. Bot.* 62, 2143–2154.

Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* 7, 173–182.

Pauwels, L., Barbero, G. F., Geerinck, J., Tilleman, S., Grunewald, W., Perez, A. C., et al. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* 464, 788–791.

Pauwels, L., and Goossens, A. (2011). The JAZ proteins: a crucial interface in the jasmonate signaling cascade. *Plant Cell* 23, 3089–3100.

Peng, J., Carol, P., Richards, D. E., King, K. E., Cowling, R. J., Murphy, G. P., et al. (1997). The *Arabidopsis* GAI gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes Dev.* 11, 3194–3205.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., et al. (2000). *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell* 103, 1111–1120.

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

Plett, J. M., Kemppainen, M., Kale, S. D., Kohler, A., Legue, V., Brun, A., et al. (2011). A secreted effector protein of *Laccaria bicolor* is required for symbiosis development. *Curr. Biol.* 21, 1197–1203.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., et al. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F box proteins: EBF1 and EBF2. *Cell* 115, 679–689.

Qi, T., Song, S., Ren, Q., Wu, D., Huang, H., Chen, Y., et al. (2011). The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in *Arabidopsis thaliana*. *Plant Cell* 23, 1795–1814.

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

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

Saracco, S. A., Hansson, M., Scalf, M., Walker, J. M., Smith, L. M., and Vierstra, R. D. (2009). Tandem affinity purification and mass spectrometric analysis of ubiquitylated proteins in *Arabidopsis*. *Plant J.* 59, 344–358.

Schaller, A., and Stintzi, A. (2009). Enzymes in jasmonate biosynthesis – structure, function, regulation. *Phytochemistry* 70, 1532–1538.

Schornack, S., Van Damme, M., Bozkurt, T. O., Cano, L. M., Smoker, M., Thines, M., et al. (2010). Ancient class of translocated oomycete effectors targets the host nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17421–17426.

Sheard, L. B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T. R., et al. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature* 468, 400–405.

Siewers, V., Kokkelink, L., Smedsgaard, J., and Tudzynski, P. (2006). Identification of an abscisic acid gene cluster in the grey mold *Botrytis cinerea*. *Appl. Environ. Microbiol.* 72, 4619–4626.

Silverstone, A. L., Jung, H. S., Dill, A., Kawaide, H., Kamiya, Y., and Sun, T. P. (2001). Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in *Arabidopsis*. *Plant Cell* 13, 1555–1566.

Song, S., Qi, T., Huang, H., Ren, Q., Wu, D., Chang, C., et al. (2011). The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect Jasmonate-regulated stamen development in *Arabidopsis*. *Plant Cell* 23, 1000–1013.

Spaepen, S., Vanderleyden, J., and Remans, R. (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* 31, 425–448.

Splivallo, R., Fischer, U., Gobel, C., Feussner, I., and Karlovsky, P. (2009). Truffles regulate plant root morphogenesis via the production of auxin and ethylene. *Plant Physiol.* 150, 2018–2029.

Spoel, S. H., Johnson, J. S., and Dong, X. (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18842–18847.

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

Sun, T. P. (2011). The molecular mechanism and evolution of the GA-GID1-DELLA signaling module in plants. *Curr. Biol.* 21, R338–R345.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., and Mizuno, T. (2001). The *Arabidopsis* sensor His-kinase, AHk4, can respond to cytokinins. *Plant Cell Physiol.* 42, 107–113.

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

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* 448, 661–665.

Thomma, B. P., Eggermont, K., Penninckx, I. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P., et al. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 95, 15107–15111.

Tyler, L., Thomas, S. G., Hu, J., Dill, A., Alonso, J. M., Ecker, J. R., et al. (2004). Della proteins and gibberellin-regulated seed germination and floral development in *Arabidopsis*. *Plant Physiol.* 135, 1008–1019.

Ueguchi, C., Sato, S., Kato, T., and Tabata, S. (2001). The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in *Arabidopsis thaliana*. *Plant Cell Physiol.* 42, 751–755.

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

Valls, M., Genin, S., and Boucher, C. (2006). Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solanacearum*. *PLoS Pathog.* 2:e82. doi: 10.1371/journal.ppat.0020082

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M. C., Rodenburg, N., Pauwels, L., et al. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* doi: 10.1105/tpc.112.108548 [Epub ahead of print].

van Loon, L. C., Rep, M., and Pieterse, C. M. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162.

Vicente, J., Cascon, T., Vicedo, B., Garcia-Agustin, P., Hamberg, M., and Castresana, C. (2012). Role of 9-lipoxygenase and alpha-dioxygenase oxylipin pathways as modulators of local and systemic defense. *Mol. Plant* 5, 914–928.

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

Wang, K. L., Li, H., and Ecker, J. R. (2002). Ethylene biosynthesis and signaling networks. *Plant Cell* 14(Suppl.), S131–S151.

Wang, W., Esch, J. J., Shiu, S. H., Agula, H., Binder, B. M., Chang, C., et al. (2006). Identification of important regions for ethylene binding and signaling in the transmembrane domain of the ETR1 ethylene receptor of *Arabidopsis*. *Plant Cell* 18, 3429–3442.

Wasternack, C. (2007). Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* 100, 681–697.

Weingart, H., Ullrich, H., Geider, K., and Volksch, B. (2001). The role of ethylene production in virulence of *Pseudomonas syringae* pvs. *glycinea* and *phaseolicola*. *Phytopathology* 91, 511–518.

Weingart, H., and Volksch, B. (1997). Ethylene production by *Pseudomonas syringae* Pathovars in vitro and in planta. *Appl. Environ. Microbiol.* 63, 156–161.

Wild, M., Daviere, J. M., Cheminant, S., Regnault, T., Baumberger, N., Heintz, D., et al. (2012). The *Arabidopsis* DELLA RGA-LIKE3 is a direct target of MYC2 and modulates jasmonate signaling responses. *Plant Cell* 24, 3307–3319.

Withers, J., Yao, J., Mecey, C., Howe, G. A., Melotto, M., and He, S. Y. (2012). Transcription factor-dependent nuclear localization of a transcriptional repressor in jasmonate hormone signaling. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20148–20153.

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

Xie, D. X., Feys, B. F., James, S., Nieto-Rostro, M., and Turner, J. G. (1998). COI1: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* 280, 1091–1094.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., et al. (2001). The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol.* 42, 1017–1023.

Yang, D. L., Yao, J., Mei, C. S., Tong, X. H., Zeng, L. J., Li, Q., et al. (2012). Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1192–E1200.

Zamioudis, C., and Pieterse, C. M. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant Microbe Interact.* 25, 139–150.

Zander, M., Chen, S., Imkampe, J., Thurow, C., and Gatz, C. (2012). Repression of the *Arabidopsis thaliana* jasmonic acid/ethylene-induced defense pathway by

TGA-interacting glutaredoxins depends on their C-terminal ALWL motif. *Mol. Plant* 5, 831–840.

Zander, M., La Camera, S., Lamotte, O., Metraux, J. P., and Gatz, C. (2009). *Arabidopsis thaliana* class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. *Plant J.* 61, 200–210.

Zhu, Z., An, F., Feng, Y., Li, P., Xue, L., A, M., et al. (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12539–12544.

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: 26 February 2013; paper pending published: 13 March 2013; accepted: 15 March 2013; published online: 05 April 2013.

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

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

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 13 September 2013
doi: 10.3389/fpls.2013.00364

New clues in the nucleus: transcriptional reprogramming in effector-triggered immunity

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The robustness of plant effector-triggered immunity is correlated with massive alterations of the host transcriptome. Yet the molecular mechanisms that cause and underlie this reprogramming remain obscure. Here we will review recent advances in deciphering nuclear functions of plant immune receptors and of associated proteins. Important open questions remain, such as the identities of the primary transcription factors involved in control of effector-triggered immune responses, and indeed whether this can be generalized or whether particular effector-resistance protein interactions impinge on distinct sectors in the transcriptional response web. Multiple lines of evidence have implicated WRKY transcription factors at the core of responses to microbe-associated molecular patterns and in intersections with effector-triggered immunity. Recent findings from yeast two-hybrid studies suggest that members of the TCP transcription factor family are targets of several effectors from diverse pathogens. Additional transcription factor families that are directly or indirectly involved in effector-triggered immunity are likely to be identified.

Keywords: effector-triggered immunity, transcriptional reprogramming, transcription factors, avirulence genes, resistance proteins

INTRODUCTION

A common and early event in effector-triggered immunity (ETI) is the rapid up- or downregulation of pathogenesis-responsive genes. The advent of genomics and transcriptomics provided a comprehensive description of the magnitude of the transcriptional reprogramming that occurs in cells responding to detected effectors (Tao et al., 2003; Caldo et al., 2004; Adams-Phillips et al., 2008; Moscou et al., 2011). Subsequent findings of resistance proteins in the nucleus led to the suggestion that some resistance proteins directly affect transcriptional changes. A few well-discussed examples exist, but it is also clear that this proposed nuclear role is not a general feature of all resistance proteins. Interestingly, transcriptomics studies also highlighted the fact that transcriptional responses to avirulent and virulent pathogens mainly differ quantitatively (in the speed and amplitude of transcriptional changes), not qualitatively (in the identity of regulated genes; Tao et al., 2003; Katagiri and Tsuda, 2010). The layered nature of the plant innate immune system, where ETI is layered on top of the pathogen-associated molecular pattern-triggered immunity (PTI) network, makes it difficult to distinguish between genuine ETI-specific signaling steps, the guarding of PTI nodes by resistance proteins, and an accelerator function of resistance proteins that speeds up and amplifies an underlying PTI response (Shen et al., 2007; Gassmann and Bhattacharjee, 2012). Here we briefly review existing evidence for and against a nuclear function of resistance proteins and other ETI-associated proteins, but mainly focus on gaps that need to be filled to understand how to connect resistance proteins to the vast transcriptional response observed during ETI.

From a pathogen's perspective, ETI is an unintended consequence of deploying effector proteins to colonize a host (Dangl and Jones, 2001; Jones and Dangl, 2006; Dodds and Rathjen, 2010). Effector proteins evolved to increase the fitness of a pathogen on its host by modulating host physiology in a variety of ways. Some examples of diverse effector functions include modifying components of the immune system to evade detection (Block and Alfano, 2011), and redirecting nutrients to the apoplast to support pathogen growth (Chen et al., 2010). Detection of these effectors by resistance proteins can occur when resistance proteins directly bind cognate effectors, or indirectly when resistance proteins detect changes to an associated host protein brought about by effectors (van der Biezen and Jones, 1998). In terms of their virulence function, one might postulate that the most potent effectors would target transcription architectures that regulate defense genes. However, in a recent comprehensive screening, transcriptional regulators are under-represented in the identified hubs targeted by multiple effectors from two different pathogens, *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* (Mukhtar et al., 2011). This deficiency may be caused by a general under-representation in the libraries screened or by elimination from consideration of auto-activating transcription factors and chromatin-associated components in yeast two-hybrid assays. In biological terms, this finding could also signify that the

transcriptional response is a late event that is not a primary barrier to an invading pathogen, or more likely that a robust transcriptional network is not an ideal target for disruption (Tsuda et al., 2009).

RESISTANCE PROTEINS AS DIRECT SIGNAL TRANSDUCERS

The activation of *Arabidopsis* resistance to *Ralstonia solanacearum*-resistant allele (RRS1-R) in the presence of the *Ralstonia solanacearum* effector *Pseudomonas* outer protein P2 (PopP2) was, until recently, considered a classic example of a system in which an activated resistance protein may directly stimulate ETI-related transcriptional changes (Deslandes et al., 2002). RRS1-R contains a WRKY transcription factor-like C-terminal domain. Native RRS1-R is unstable, and co-expression of PopP2 stabilizes nuclear RRS1-R (Deslandes et al., 2003; Tasset et al., 2010). However, subsequent findings showed that RRS1-R functions as a negative regulator of defense and that PopP2 acetyltransferase activity is required for RRS1-R activation, but not stabilization (Noutoshi et al., 2005; Tasset et al., 2010). This suggests that a yet to be identified PopP2 substrate or a protein interacting with activated RRS1-R functions in co-ordination with RRS1-R to mediate the majority of ETI gene modulations. Candidates include the resistance protein RPS4, which genetically was shown to function with *RRS1-R* in providing resistance to multiple pathogen effectors from diverse organisms (Birker et al., 2009; Narusaka et al., 2009, 2013), and ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), which was found to be in protein complexes with RPS4 and related resistance proteins (Bhattacharjee et al., 2011; Heidrich et al., 2011; see below).

A second example is the barley resistance protein Mildew locus A 10 (MLA10), which upon activation by powdery mildew effector Avr_{A10} interacts with WRKY1 and WRKY2 in the nucleus. Silencing of these WRKYs enhances resistance to both compatible and incompatible pathogens, suggesting that these WRKYs function as defense repressors (Shen et al., 2007). *Arabidopsis* WRKY18, WRKY40 and WRKY60, which have sequence homology to barley WRKY1/2, bind to promoter elements of the positive defense regulator *EDS1* and the jasmonate pathway repressor gene *JASMONATE-ZIM-DOMAIN PROTEIN8* (*JAZ8*) to repress their expression (Pandey et al., 2010). However, constitutive activation of defenses is not apparent in *wrky18 wrky40 wrky60* mutants. Instead, up-regulated basal defense genes prime these plants for enhanced resistance toward both virulent and avirulent pathogens (Shen et al., 2007; Pandey et al., 2010). WRKYs that have recently been identified as positive regulators of defenses also affect both layers of immunity (Bhattarai et al., 2010; Gao et al., 2013).

The transcriptome alterations that characterize ETI likely involve specialized transcription factors that cue from activated resistance proteins and amplify an existing PTI response. A recent advancement in understanding MLA10-mediated immunity supports this model (Chang et al., 2013). At resting state, MLA10 cannot interfere with the WRKY1 function to sequester the positive defense transcription factor MYB6. Upon activation, MLA10 not only abolishes WRKY1 repression of MYB6 but also potentiates the DNA-binding activity of MYB6. A remaining question is which transcription factor enables the reported conserved function of MLA1 in *Arabidopsis* (Maekawa et al., 2012), since *HvMYB6*-orthologous genes are likely limited to the grasses and are absent in *Arabidopsis* (Chang et al., 2013).

Padmanabhan et al. (2013) also recently reported that the tobacco resistance protein N, upon activation, acquires nuclear binding to SQUAMOSA PROMOTER BINDING PROTEIN-LIKE6 (SPL6). They demonstrated that SPL6 controls the expression of several defense genes such as *PR1* and *PAD4*, and is essential for TIR-NBS-LRR-triggered ETI. Interestingly, both MLA10 and N only interacted with MYB6 and SPL6, respectively, after activation, possibly reflecting conformational changes or oligomerization of resistance proteins as a prerequisite for these protein interactions (Chang et al., 2013; Padmanabhan et al., 2013). More recently, Panicle blast 1 (Pb1), a broad-spectrum rice resistance protein against *Magnaporthe oryzae*, was reported to interact with and stabilize nuclear-localized WRKY45 by inhibiting its ubiquitin-mediated degradation (Inoue et al., 2013). Knockdown plants in a susceptible background were unaffected in basal resistance against the blast fungus. For Pb1 it is not clear yet what the activation step is, since Pb1 possesses a degenerate NB domain that lacks a functional P-loop (Inoue et al., 2013). Nevertheless, these studies demonstrate direct induction of defense genes by resistance proteins via specific transcription factors.

ONE SIZE DOES NOT FIT ALL: MULTIPLE PATHWAYS TO RESISTANCE

Resistance proteins are deployed where they can intercept effector functions. Plasma membrane localized RPM1 is activated upon sensing host-modification of RIN4 by the action of membrane-targeted effectors AvrRpm1 and AvrB (Nimchuk et al., 2000; Mackey et al., 2002; Liu et al., 2011). Nucleocytoplasmic N is activated upon sensing and interacting with the liberated chloroplast protein NRIP1 following perturbations by the tobacco mosaic virus effector p50 (Caplan et al., 2008). While a nuclear subpool of some resistance proteins are required for their immune functions (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007; Cheng et al., 2009), RPM1 relocalization from the plasma membrane to the nucleus is not required to induce an ETI-response to AvrRpm1 (Gao et al., 2011). Therefore, nuclear signaling during ETI does not always involve activated resistance proteins as the sole carriers. This is also supported by the evolutionary evidence that, while several resistance-like proteins from other plant species like *Populus* (Tuskan et al., 2006) have domains resembling DNA-binding elements, most characterized *Arabidopsis* resistance proteins neither possess transcription factor-like domains nor have been generally identified as direct associates of transcription factors. Thus, nucleotide binding-leucine-rich repeat (NB-LRR) proteins did not evolve from transcriptional regulators. This conclusion may not be very surprising, since resistance-like proteins are increasingly being identified in defense-independent roles, not all of which directly relate to transcription (Faigón-Soverna et al., 2006; Kim et al., 2012). In addition, a small but measurable nuclear pool for many resistance proteins already exists at resting state, and the majority of these proteins remain cytoplasmic even after activation. Small changes in amounts of nuclear protein are therefore difficult to measure, and it has not been shown convincingly yet that

resistance proteins relocate to the nucleus after activation. Nevertheless, within the confines of the nucleus even small changes in the number of protein molecules relative to the bulk protein in the cytoplasm, either by import or by preventing cycling out of the nucleus, may increase the concentration of nuclear protein considerably.

An in-depth understanding of immune signaling is also being formed by studies of the activated resistance-like protein SUPPRESSOR OF npr1-1, CONSTITUTIVE1 (SNC1) that is proposed to function by repressing transcription of negative regulators of defense (Johnson et al., 2013). Even though a *bona fide* avirulence gene recognized by wild-type *SNC1* has not been identified, it was shown that SNC1 exists in comparable protein complexes as the resistance proteins RPS4 and RPS6, and contributes to AvrRps4 recognition in the absence of *RPS4* (Kim et al., 2010; Bhattacharjee et al., 2011). Genetic screens and subsequent molecular approaches on the auto-active mutant allele of *SNC1* identified *TOPLESS* (*TPL*) gene family involvement, suggesting a nuclear function for activated *snc1*. TPL members function as transcriptional co-repressors in many plant signaling pathways (Pauwels et al., 2010; Krogan et al., 2012; Wang et al., 2012). The demonstration that a TPL family member, TOPLESS RELATED1 (TPR1), forms a complex with SNC1 leads to a model in which SNC1 interacts with TPR1 to recruit HISTONE DEACETYLASE 19 (HDA19) to remodel chromatin at promoters of negative defense regulators (Zhu et al., 2010). A recent large scale search for interactors of TPL members identified transcriptional regulators belonging to diverse families, suggesting a wide role of TPL members as co-repressors (Causier et al., 2012). Intriguingly, one of the identified members, TCP14, is a target of at least two unrelated pathogen effectors (Mukhtar et al., 2011). Members of the TCP transcription factor family regulate leaf morphology and have been recently implicated in hormonal signaling (Koyama et al., 2007; Danisman et al., 2012; Steiner et al., 2012). Interestingly, a TCP-family protein was reported to be involved in the activation of several *WRKY* genes in cotton (Hao et al., 2012). It is a common observation that uncontrolled induction of immunity compromises regular growth and development of plants (Alcazar et al., 2011). Whether TCPs are direct transcriptional mediators that contribute to this fine balance needs to be determined.

VIRULENCE TARGETS AS CO-SIGNALING COMPONENTS OF ETI

A recent large protein interactome dataset identified multiple host targets that a given effector may act upon in its pursuit for virulence (Mukhtar et al., 2011). However, what is the *modus operandi* of an effector in this ever-expanding protein–protein interaction network of resistance-associated proteins? The *P. syringae* type III effectors are functionally versatile and may mediate processes as diverse as proteolytic processing, ubiquitination, or nucleotide transfer on host targets (Block and Alfano, 2011). These manipulations of host targets may play synergistic roles with activated resistance proteins toward transcriptional modulation during ETI. The *P. syringae* effectors AvrRps4 and HopA1 cause disruptions of EDS1 associations with their cognate resistance proteins RPS4 and RPS6, respectively, at a microsomal location (Bhattacharjee et al., 2011). The effector AvrRps4 is processed *in planta* (Sohn et al., 2009), and although it was deduced from transient overexpression studies in turnip that the processed C-terminal domain is sufficient for the triggering of ETI in *Arabidopsis*, two independent reports seem to suggest the potential of each of these processed AvrRps4 domains as interactors with EDS1 and an RPS4-containing complex (Bhattacharjee et al., 2011; Heidrich et al., 2011). The precise functions of these interactions require further experimentation to resolve the issue. The observation that EDS1 is enriched in the nucleus during ETI (García et al., 2010) may indicate that EDS1 liberated from tight molecular associations in the cytoplasm is a candidate transcriptional modulator. However, as seen with resistance proteins, forced nuclear enrichment of EDS1 alone does not trigger ETI. Therefore, biochemical functions of these unrelated effectors on EDS1 need to be identified.

Plasma membrane localized RPM1 and RPS2 resistance proteins guard RIN4, a common virulence target of the unrelated effectors AvrB, AvrRpm1, and AvrRpt2 (Nimchuk et al., 2000; Axtell and Staskawicz, 2003; Jin et al., 2003). The cysteine protease activity of AvrRpt2 cleaves RIN4 (Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003), whereas in the presence of AvrB or AvrRpm1 the host kinase RIPK phosphorylates RIN4 (Liu et al., 2011). These alterations of RIN4 trigger activation of the cognate resistance proteins RPS2 and RPM1, respectively. Since a nuclear pool of activated RPM1 is not necessary for function (Gao et al., 2011), other components of these systems are likely mediators for nuclear signaling. Indeed, Holt et al. (2002) identified the interaction of specific RPM1 domains with a DNA-binding protein, TIP49a. TIP49a functions as a negative regulator of plant defense, and mammalian orthologs of TIP49a are involved in transcriptional regulation (Kanemaki et al., 1997). The interaction between RPM1 and AtTIP49a is suggestive of a cytoplasmic sequestering of negative regulators by an activated resistance protein. The AvrRpt2/RPS2 system also identifies a putative component that may act in transcriptional reprogramming. Unlike the membrane-tethered native RIN4, the AvrRpt2-processed RIN4 fragments are soluble (Afzal et al., 2011). Whether these fragments translocate to the nucleus or remain cytoplasmic, and whether other host proteins that are substrates for AvrRpt2 protease function mediate gene induction regulation, requires further study. Perhaps strengthening the above notion is the observation that modified RIN4 proteins which are deficient in plasma membrane binding constitutively activate ETI-type responses (Afzal et al., 2011).

Other post-translational modifications of proteins, for example through ubiquitination or SUMOylation, are likely to play a role in ETI as well. Ubiquitination has been observed to regulate resistance protein stability (Goritschnig et al., 2007; Tasset et al., 2010), and its roles in plant immunity have been reviewed recently (Cheng and Li, 2012; Furlan et al., 2012). The covalent attachment of SMALL UBIQUITIN-LIKE MODIFIER (SUMO) to a protein also affects its function (Mazur and van den Burg, 2012; Cubenas-Potts and Matunis, 2013). SUMOylation, predominantly a nuclear event, can also modulate activities of transcription factors, co-repressors such as the TPL family, and DNA-modifying components such as histones (Gill, 2005). Interestingly, a mutation in *Arabidopsis SIZ1*, which encodes an E3 SUMO ligase, induces constitutive salicylic acid (SA)-mediated defenses and

confers enhanced resistance toward *P. syringae* DC3000 (Lee et al., 2007). Key proteins associated with innate immunity such as PAD4, EDS1, SAG101, and NPR1 contain putative SUMOylation motifs (Lee et al., 2007). Whether these proteins are real substrates for SUMO-modifications and whether the SUMOylation machinery is recruited in ETI remains to be determined. Multiple effectors from *Xanthomonas campestris pv. vesicatoria* such as XopD and AvrXv4 either possess de-sumoylation activities or cause a global decrease in the host SUMOylation profile (Hotson and Mudgett, 2004; Roden et al., 2004). This strongly suggests that SUMOylation regulates aspects of nuclear ETI signaling.

CHROMATIN CHANGES AT IMMUNE-RELATED GENES

Post-translational modifications on core histones include methylation, acetylation, and phosphorylation (Fuchs et al., 2006; Pfluger and Wagner, 2007). The chromosomal environments these modifications create for ETI-responsive genes may determine the speed and amplitude of defense responses. Indeed, several chromatin-related proteins are often identified in plant defenses (Ma et al., 2011). Typical post-translational modifications mark nucleosome assemblies of defense regulators (Alvarez et al., 2010). Immune-responsive genes such as *WRKY* genes and *PR1* are maintained in a "ready" state via the extent of methylation status (tri-, in contrast to mono- or di-methylation) on histone H3 at lysine4 position (H3K4me3; Santos-Rosa et al., 2002). Although primed, actual transcription of these genes is regulated by specialized activators and repressors. For example, *ARABIDOPSIS TRITHORAX1* (*ATX1*) encodes a histone methyltransferase that directly affects the H3K4 methylation intensity of several *WRKY* promoters and governs the expression of several *TCP* transcription factor and NBS-LRR genes, including *CSA1* and *SNC1* (Alvarez-Venegas et al., 2006, 2007). Interestingly, ATX1 is mostly cytoplasmic in un-elicited cells, suggesting that directed transcriptional reprogramming during ETI may involve coordinated recruitment of specific histone methyltransferases and nuclear transcription factors. SET (Su(var)3-9, E(z) and trithoraxconserved) DOMAIN GROUP8 (SDG8), another histone methyltransferase, was recently reported to affect the H3K4me3 status-dependent expression of an *RPS4*-like resistance gene (Palma et al., 2010).

Histone acetylation and deacetylation modulate transcriptional efficiencies through activation and repression, respectively (Wang et al., 2010; Shakespear et al., 2011). A histone deacetylase (HDAC), REDUCED POTASSIUM DEPENDENCY3/HDAC1 from maize, confers resistance to the fungus *Cochliobolus* (*Helminthosporium*) *carbonum* through an unknown mechanism (Johal and Briggs, 1992). *Arabidopsis* AtHDAC19 has been identified to interact with several WRKYs and co-repressors (Zhou et al., 2005; Kim et al., 2008; Zhu et al., 2010). More recently, the *Arabidopsis* Elongator complex subunit 2 (ELP2), an active histone acetylase, was reported to influence the expression kinetics

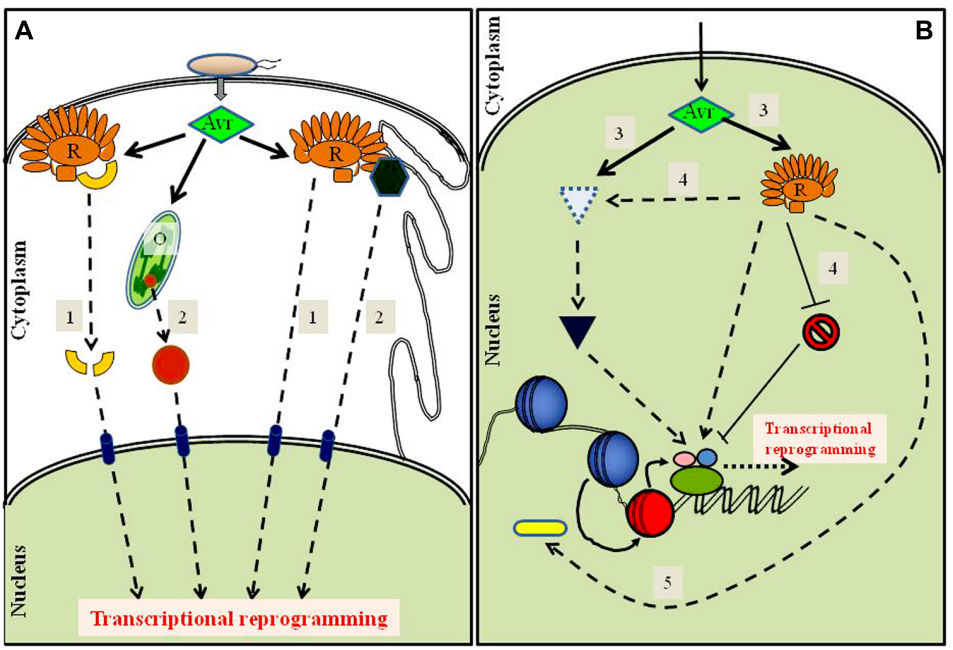

FIGURE 1 | Schematic diagram of possible cytoplasmic and nuclear routes to transcriptome reprogramming by an activated resistance protein. Detection of avirulence effector (Avr) presence or activities by a cognate resistance protein (R) may drive nuclear-directed signaling through multiple processes. **(A)** The cytoplasmic events may include, (1) direct nuclear translocation of effector-modified virulence targets or of the activated resistance protein itself, or (2) nuclear enrichment of a transcription-modulating protein sequestered in an organelle (O) or tethered to a membrane (e.g., ER). **(B)** Nuclear-targeted effector activities that trigger ETI may include, (3) promoting the stability of the sensing R protein itself or of a transcriptional activator, or (4) enabling an activated R protein either to sequester a negative regulator from or to recruit a positive regulator of defense to its target genes, or (5) altering chromatin by Avr- or R-mediated recruitment of chromatin remodeling components that further facilitate access by transcription factors. The strength and success of an effective ETI likely is determined by a tight co-ordination and possible synergism between some or all of the above processes.

of *EDS1*, *PAD4*, and *PR1*, likely through the histone acetylation/methylation status (Wang et al., 2013). Further implication of histone acetylation in immune responses can also be extrapolated from the PopP2 acetyltransferase activity in RRS1-R elicitation (Tasset et al., 2010). Although this activity of PopP2, which may include histones as substrates, would likely aim to suppress defense, stabilized RRS1 as a result of the effector presence may hijack the mechanism to induce resistance-associated genes.

CONCLUSION

An increasing amount of experimental evidence suggests a difference primarily in amplitude between PTI and ETI responses. To date, most identified modulators of transcription affect both branches of immunity, thereby clouding the interpretation of PTI-versus ETI-specific effects. Because in many cases effector activity and not simply the effector presence itself is the primary stimulus of ETI, an inherent deficiency of the routinely used yeast two-hybrid approach to identify resistance-associated proteins is the failure to incorporate this effector function. We have highlighted several potential areas where the function of an effector modulates the function of a host protein (**Figure 1**). Perhaps a more refined and directed approach is necessary in our search for transcriptional components. Stable lines expressing chemical-inducible effectors in susceptible and resistant hosts may provide one such PTI-independent system for proteomic approaches to identify differentially regulated nuclear proteins. In addition, genome-wide chromatin immunoprecipitation-sequencing (ChIP-seq)-based determination of transcriptional associations of activated NB-LRRs can be undertaken with this system. In parallel, precise biochemical functions of effectors need to be elucidated to understand host protein modifications. The vast interconnected ETI signaling web is clearly complex. Furthermore, any effector likely targets multiple host proteins. Whether robust and rapid ETI-associated transcriptome changes require synergistic signaling from different sectors or whether specific perturbations are direct transcriptional triggers needs to be elucidated. Transcriptional alterations require the coordinated actions of multiple DNA remodeling components, including specific transcription-associated proteins. Unraveling how nuclear signaling is achieved post-effector sensing and how this signal impinges on chromatin components is therefore necessary to understand and apply sustained resistance-developing technologies.

ACKNOWLEDGMENTS

We apologize to the many colleagues whose relevant research could not be cited because of limited space. We thank Sharon Pike for critical reading of the manuscript. Christopher M. Garner is funded by an MU Life Sciences Graduate Fellowship. Walter Gassmann acknowledges funding by NSF Integrative Organismal Systems Program grants IOS-0715926 and IOS-1121114. Saikat Bhattacharjee is supported by funds from the Regional Centre for Biotechnology, India and by a Ramalingaswami Re-Entry Fellowship 2012-2013, Ministry of Science and Technology, Department of Biotechnology, Government of India.

REFERENCES

Adams-Phillips, L., Wan, J., Tan, X., Dunning, F. M., Meyers, B. C., Michelmore, R. W., et al. (2008). Discovery of ADP-ribosylation and other plant defense pathway elements through expression profiling of four different *Arabidopsis–Pseudomonas* R-avr interactions. *Mol. Plant Microbe Interact.* 21, 646–657. doi: 10.1094/MPMI-21-5-0646

Afzal, A. J., da Cunha, L., and Mackey, D. (2011). Separable fragments and membrane tethering of *Arabidopsis* RIN4 regulate its suppression of PAMP-triggered immunity. *Plant Cell* 23, 3798–3811. doi: 10.1105/tpc.111.088708

Alcazar, R., Reymond, M., Schmitz, G., and de Meaux, J. (2011). Genetic and evolutionary perspectives on the interplay between plant immunity and development. *Curr. Opin. Plant Biol.* 14, 378–384. doi: 10.1016/j.pbi.2011.04.001

Alvarez, M. E., Nota, F., and Cambiagno, D. A. (2010). Epigenetic control of plant immunity. *Mol. Plant Pathol.* 11, 563–576. doi: 10.1111/j.1364-3703.2010.00621.x

Alvarez-Venegas, R., Abdallat, A. A., Guo, M., Alfano, J. R., and Avramova, Z. (2007). Epigenetic control of a transcription factor at the cross section of two antagonistic pathways. *Epigenetics* 2, 106–113. doi: 10.4161/epi.2.2.4404

Alvarez-Venegas, R., Sadder, M., Hlavacka, A., Baluska, F., Xia, Y., Lu, G., et al. (2006). The *Arabidopsis* homolog of trithorax, ATX1, binds phosphatidylinositol 5-phosphate, and the two regulate a common set of target genes. *Proc. Natl. Acad. Sci. U.S.A.*103, 6049–6054. doi: 10.1073/pnas.0600944103

Axtell, M. J., Chisholm, S. T., Dahlbeck, D., and Staskawicz, B. J. (2003). Genetic and molecular evidence that the *Pseudomonas syringae* type III effector protein AvrRpt2 is a cysteine protease. *Mol. Microbiol.* 49, 1537–1546. doi: 10.1046/j.1365-2958.2003.03666.x

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. doi: 10.1016/S0092-8674(03)00036-9

Bhattacharjee, S., Halane, M. K., Kim, S. H., and Gassmann, W. (2011). Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators. *Science* 334, 1405–1408. doi: 10.1126/science.1211592

Bhattarai, K. K., Atamian, H. S., Kaloshian, I., and Eulgem, T. (2010). WRKY72-type transcription factors contribute to basal immunity in tomato and *Arabidopsis* as well as gene-for-gene resistance mediated by the tomato R gene Mi-1. *Plant J.* 63, 229–240. doi: 10.1111/j.1365-313X.2010.04232.x

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. doi: 10.1111/j.1365-313X.2009.03984.x

Block, A., and Alfano, J. R. (2011). Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? *Curr. Opin. Microbiol.* 14, 39–46. doi: 10.1016/j.mib.2010.12.011

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

Caldo, R. A., Nettleton, D., and Wise, R. P. (2004). Interaction-dependent gene expression in Mla-specified response to barley powdery mildew. *Plant Cell* 16, 2514–2528. doi: 10.1105/tpc.104.023382

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. doi: 10.1016/j.cell.2007.12.031

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. doi: 10.1104/pp.111.186999

Chang, C., Yu, D., Jiao, J., Jing, S., Schulze-Lefert, P., and Shen, Q. H. (2013). Barley MLA immune receptors directly interfere with antagonistically acting transcription factors to initiate disease resistance signaling. *Plant Cell* 25, 1158–1173. doi: 10.1105/tpc.113.109942

Chen, L. Q., Hou, B. H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X. Q., et al. (2010). Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 468, 527–532. doi: 10.1038/nature09606

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. doi: 10.1105/tpc.108.064519

Cheng, Y. T., and Li, X. (2012). Ubiquitination in NB-LRR-mediated immunity. *Curr. Opin. Plant Biol.* 15, 392–399. doi: 10.1016/j.pbi.2012.03.014

Cubenas-Potts, C., and Matunis, M. J. (2013). SUMO: a multifaceted modifier of chromatin structure and function. *Dev. Cell* 24, 1–12. doi: 10.1016/j.devcel.2012.11.020

Dangl, J. L., and Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411, 826–833. doi: 10.1038/35081161

Danisman, S., van der Wal, F., Dhondt, S., Waites, R., de Folter, S., Bimbo, A., et al. (2012). *Arabidopsis* class I and class II TCP transcription factors regulate jasmonic acid metabolism and leaf development antagonistically. *Plant Physiol.* 159, 1511–1523. doi: 10.1104/pp.112.200303

Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., et al. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8024–8029. doi: 10.1073/pnas.1230660100

Deslandes, L., Olivier, J., Theulières, F., Hirsch, J., Feng, D. X., Bittner-Eddy, P., et al. (2002). Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. *Proc. Natl. Acad. Sci. U.S.A.* 99, 2404–2409. doi: 10.1073/pnas.032485099

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Faigón-Soverna, A., Harmon, F. G., Storani, L., Karayekov, E., Staneloni, R. J., Gassmann, W., et al. (2006). A constitutive shade-avoidance mutant implicates TIR-NBS-LRR proteins in *Arabidopsis* photomorphogenic development. *Plant Cell* 18, 2919–2928. doi: 10.1105/tpc.105.038810

Fuchs, J., Demidov, D., Houben, A., and Schubert, I. (2006). Chromosomal histone modification patterns – from conservation to diversity. *Trends Plant Sci.* 11, 199–208. doi: 10.1016/j.tplants.2006.02.008

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

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

Gao, Z., Chung, E. H., Eitas, T. K., and Dangl, J. L. (2011). Plant intracellular innate immune receptor resistance to *Pseudomonas syringae* pv. *maculicola* 1 (RPM1) is activated at, and functions on, the plasma membrane. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7619–7624. doi: 10.1073/pnas.1104410108

García, A. V., Blanvillain-Baufumé, S., Huibers, R. P., Wiermer, M., Li, G., Gobbato, E., et al. (2010). Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS Pathog.* 6:e1000970. doi: 10.1371/journal.ppat.1000970

Gassmann, W., and Bhattacharjee, S. (2012). Effector-triggered immunity signaling: from gene-for-gene pathways to protein–protein interaction networks. *Mol. Plant Microbe Interact.* 25, 862–868. doi: 10.1094/MPMI-01-12-0024-IA

Gill, G. (2005). Something about SUMO inhibits transcription. *Curr. Opin. Genet. Dev.* 15, 536–541. doi: 10.1016/j.gde.2005.07.004

Goritschnig, S., Zhang,Y., and Li, X. (2007). The ubiquitin pathway is required for innate immunity in *Arabidopsis*. *Plant J.* 49, 540–551. doi: 10.1111/j.1365-313X.2006.02978.x

Hao, J., Tu, L., Hu, H., Tan, J., Deng, F., Tang, W., et al. (2012). GbTCP, a cotton TCP transcription factor, confers fibre elongation and root hair development by a complex regulating system. *J. Exp. Bot.* 63, 6267–6281. doi: 10.1093/jxb/ers278

Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L., and Parker, J. E. (2011). *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* 334, 1401–1404. doi: 10.1126/science.1211641

Holt, B. F., Boyes, D. C. III, Ellerstrom, M., Siefers, N., Wiig, A., Kauffman, S., et al. (2002). An evolutionarily conserved mediator of plant disease resistance gene function is required for normal *Arabidopsis* development. *Dev. Cell* 2, 807–817. doi: 10.1016/S1534-5807(02)00174-0

Hotson, A., and Mudgett, M. B. (2004). Cysteine proteases in phytopathogenic bacteria: identification of plant targets and activation of innate immunity. *Curr. Opin. Plant Biol.* 7, 384–390. doi: 10.1016/j.pbi.2004.05.003

Inoue, H., Hayashi, N., Matsushita, A., Xinqiong, L., Nakayama, A., Sugano, S., et al. (2013). Blast resistance of CC-NB-LRR protein Pb1 is mediated by WRKY45 through protein-protein interaction. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9577–9582. doi: 10.1073/pnas.1222155110

Jin, P., Wood, M. D., Wu, Y., Xie, Z., and Katagiri, F. (2003). Cleavage of the *Pseudomonas syringae* type III effector AvrRpt2 requires a host factor(s) common among eukaryotes and is important for AvrRpt2 localization in the host cell. *Plant Physiol.* 133, 1072–1082. doi: 10.1104/pp.103.025999

Johal, G. S., and Briggs, S. P. (1992). Reductase activity encoded by the HM1 disease resistance gene in maize. *Science* 258, 985–987. doi: 10.1126/science.1359642

Johnson, K. C., Dong, O. X., Huang, Y., and Li, X. (2013). A rolling stone gathers no moss, but resistant plants must gather their mOSes. *Cold Spring Harb. Symp. Quant. Biol.* 77, 259–268. doi: 10.1101/sqb.2013.77.014738

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

Kanemaki, M., Makino, Y., Yoshida, T., Kishimoto, T., Koga, A., Yamamoto, K., et al. (1997). Molecular cloning of a rat 49-kDa TBP-interacting protein (TIP49) that is highly homologous to the bacterial RuvB. *Biochem. Biophys. Res. Commun.* 235, 64–68. doi: 10.1006/bbrc.1997.6729

Katagiri, F., and Tsuda, K. (2010). Understanding the plant immune system. *Mol. Plant Microbe Interact.* 23, 1531–1536. doi: 10.1094/MPMI-04-10-0099

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

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

Kim, T. H., Kunz, H. H., Bhattacharjee, S., Hauser, F., Park, J., Engineer, C., et al. (2012). Natural variation in small molecule-induced TIR-NB-LRR signaling induces root growth arrest via EDS1- and PAD4-complexed R protein VICTR in *Arabidopsis*. *Plant Cell* 24, 5177–5192. doi: 10.1105/tpc.112.107235

Koyama, T., Furutani, M., Tasaka, M., and Ohme-Takagi, M. (2007). TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in *Arabidopsis*. *Plant Cell* 19, 473–484. doi: 10.1105/tpc.106.044792

Krogan, N., Hogan, K., and Long, J. (2012). APETALA2 negatively regulates multiple floral organ identity genes in *Arabidopsis* by recruiting the co-repressor TOPLESS and the histone deacetylase HDA19. *Development* 139, 4180–4190. doi: 10.1242/dev.085407

Lee, J., Nam, J., Park, H. C., Na, G., Miura, K., Jin, J. B., et al. (2007). Salicylic acid-mediated innate immunity in *Arabidopsis* is regulated by SIZ1 SUMO E3 ligase. *Plant J.* 49, 79–90. doi: 10.1111/j.1365-313X.2006.02947.x

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. doi: 10.1016/j.chom.2011.01.010

Ma, K. W., Flores, C., and Ma, W. (2011). Chromatin configuration as a battlefield in plant-bacteria interactions. *Plant Physiol.* 157, 535–543. doi: 10.1104/pp.111.182295

Mackey, D., Belkhadir, Y., Alonso, J. M., Ecker, J. R., and Dangl, J. L. (2003). *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112, 379–389. doi: 10.1016/S0092-8674(03)00040-0

Mackey, D., Holt, B. F., Wiig, A., and Dangl, J. L. (2002). RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell* 108, 743–754. doi: 10.1016/S0092-8674(02)00661-X

Maekawa, T., Kracher, B., Vernaldi, S., Ver Loren van Themaat, E., and Shulze-Lefert, P. (2012). Conservation of NLR-triggered immunity across plant lineages. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20119–20123. doi: 10.1073/pnas.1218059109

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.2012.002.

Moscou, M. J., Lauter, N., Caldo, R. A., Nettleton, D., and Wise, R. P. (2011). Quantitative and temporal definition of the Mla transcriptional regulon during barley-powdery mildew interactions. *Mol. Plant Microbe Interact.*

24, 694–705. doi: 10.1094/MPMI-09-10-0211

Mukhtar, M., 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. doi: 10.1126/science.1203659

Narusaka, M., Kubo, Y., Hatakeyama, K., Imamura, J., Ezura, H., Nanasato, Y., et al. (2013). Interfamily transfer of dual NB-LRR genes confers resistance to multiple pathogens. *PLoS ONE* 8:e55954. doi: 10.1371/journal.pone.0055954

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., et al. (2009). RRS1 and RPS4 provide a dual resistance-gene system against fungal and bacterial pathogens. *Plant J.* 60, 218–226. doi: 10.1111/j.1365-313X.2009.03949.x

Nimchuk, Z., Marois, E., Kjemtrup, S., Leister, R. T., Katagiri, F., and Dangl, J. L. (2000). Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. *Cell* 101, 353–363. doi: 10.1016/S0092-8674(00)80846-6

Noutoshi, Y., Ito, T., Seki, M., Nakashita, H., Yoshida, S., Marco, Y., et al. (2005). A single amino acid insertion in the WRKY domain of the *Arabidopsis* TIR-NBS-LRR-WRKY-type disease resistance protein SLH1 (sensitive to low humidity 1) causes activation of defense responses and hypersensitive cell death. *Plant J.* 43, 873–888. doi: 10.1111/j.1365-313X.2005.02500.x

Padmanabhan, M. S., Ma, S., Burch-Smith, T. M., Czymmek, K., Huijser, P., and Dinesh-Kumar, S. P. (2013). Novel positive regulatory role for the SPL6 transcription factor in the N TIR-NB-LRR receptor-mediated plant innate immunity. *PLoS Pathog.* 9:e1003235. doi: 10.1371/journal.ppat.1003235

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

Pandey, S. P., Roccaro, M., Schon, M., Logemann, E., and Somssich, I. E. (2010). Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of *Arabidopsis*. *Plant J.* 64, 912–923. doi: 10.1111/j.1365-313X.2010.04387.x

Pauwels, L., Barbero, G., Geerinck, J., Tilleman, S., Grunewald, W., Pérez, A., et al. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* 464, 788–791. doi: 10.1038/nature08854

Pfluger, J., and Wagner, D. (2007). Histone modifications and dynamic regulation of genome accessibility in plants. *Curr. Opin. Plant Biol.* 10, 645–652. doi: 10.1016/j.pbi.2007.07.013

Roden, J., Eardley, L., Hotson, A., Cao, Y., and Mudgett, M. B. (2004). Characterization of the *Xanthomonas* AvrXv4 effector, a SUMO protease translocated into plant cells. *Mol. Plant Microbe Interact.*17, 633–643. doi: 10.1094/MPMI.2004.17.6.633

Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., et al. (2002). Active genes are tri-methylated at K4 of histone H3. *Nature* 419, 407–411. doi: 10.1038/nature01080

Shakespear, M. R., Halili, M. A., Irvine, K. M., Fairlie, D. P., and Sweet, M. J. (2011). Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol.* 32, 335–343. doi: 10.1016/j.it.2011.04.001

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. doi: 10.1126/science.1136372

Sohn, K. H., Zhang, Y., and Jones, J. D. (2009). The *Pseudomonas syringae* effector protein, AvrRPS4, requires in planta processing and the KRVY domain to function. *Plant J.* 57, 1079–1091. doi: 10.1111/j.1365-313X.2008.03751.x

Steiner, E., Efroni, I., Gopalraj, M., Saathoff, K., Tseng, T. S., Kieffer, M., et al. (2012). The *Arabidopsis* O-linked N-acetylglucosamine transferase SPINDLY interacts with class I TCPs to facilitate cytokinin responses in leaves and flowers. *Plant Cell* 24, 96–108. doi: 10.1105/tpc.111.093518

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H. S., Han, B., et al. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15, 317–330. doi: 10.1105/tpc.007591

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

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. (2009). Network properties of robust immunity in plants. *PLoS Genet.* 5:e1000772. doi: 10.1371/journal.pgen.1000772

Tuskan, G. A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., et al. (2006). The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* 313, 1596–1604. doi: 10.1126/science.1128691

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. doi: 10.1016/S0968-0004(98)01311-5

Wang, C., Gao, F., Wu, J., Dai, J., Wei, C., and Li, Y. (2010). *Arabidopsis* putative deacetylase AtSRT2 regulates basal defense by suppressing PAD4, EDS5 and SID2 expression. *Plant Cell Physiol.* 51, 1291–1299. doi: 10.1093/pcp/pcq087

Wang, L., Kim, J., and Somers, D. (2012). Transcriptional corepressor TOPLESS complexes with pseudoresponse regulator proteins and histone deacetylases to regulate circadian transcription. *Proc. Natl. Acad. Sci. U.S.A.* 110, 761–766. doi: 10.1073/pnas.1215010110

Wang, Y., An, C., Zhang, X., Yao, J., Zhang, Y., Sun, Y., et al. (2013). The *Arabidopsis* elongator complex subunit2 epigenetically regulates plant immune responses. *Plant Cell* 25, 762–776. doi: 10.1105/tpc.113.109116

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. doi: 10.1016/j.cub.2007.10.042

Zhou, C., Zhang, L., Duan, J., Miki, B., and Wu, K. (2005). HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. *Plant Cell* 17, 1196–1204. doi: 10.1105/tpc.104.028514

Zhu, Z., Xu, F., Zhang, Y., Cheng, Y. T., Wiermer, M., Li, X., et al. (2010). *Arabidopsis* resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13960–13965. doi: 10.1073/pnas.1002828107

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 May 2013; paper pending published: 01 July 2013; accepted: 27 August 2013; published online: 13 September 2013.

*Citation: Bhattacharjee S, Garner CM and Gassmann W (2013) New clues in the nucleus: transcriptional reprogramming in effector-triggered immunity. Front. Plant Sci. **4**:364. doi: 10.3389/fpls.2013.00364*

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

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 08 October 2013
doi: 10.3389/fpls.2013.00396

Partitioning, repressing and derepressing: dynamic regulations in MLA immune receptor triggered defense signaling

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Plants and animals have evolved intracellular nucleotide-binding domain and leucine-rich repeat-containing immune receptors (NLRs) to perceive non-self and trigger immune responses. Plant NLRs detect strain-specific pathogen effectors and activate immune signaling leading to extensive transcriptional reprogramming and termination of pathogen infection. Here we review the recent findings in barley MLA immune receptor mediated immune responses against the barley powdery mildew fungus. We focus on nucleocytoplasmic partitioning of immune receptor, bifurcation of immune signaling, transcriptional repression and derepression connecting receptor activation to immune responses. We also discuss similar findings from other plant NLRs where appropriate.

Keywords: plant NLRs, MLA, barley, cell death, immune signaling, transcription factors, transcription regulation

INTRODUCTION

Plants have evolved two major classes of immune receptors to detect non-self and defend themselves against pathogen infection (Jones and Dangl, 2006). The surface resident pattern recognition receptors (PRRs) mainly recognize conserved microbe-associated molecular patterns (MAMPs) while the intracellular nucleotide-binding and leucine-rich repeat receptors (NLRs) perceive strain-specific pathogen effectors that are delivered inside host cells (Zipfel, 2009; Dodds and Rathjen, 2010). Both PRR and NLR mediated cellular defense responses share an overlapping signaling network (Tsuda et al., 2009; Tsuda and Katagiri, 2010) but differ quantitatively and kinetically in nature (Tao et al., 2003; Caldo et al., 2004), nevertheless, NLR-triggered immunity is usually associated with rapid and localized host cell-death, termed hypersensitive reaction (HR), at the attempted pathogen infection sites (Shen and Schulze-Lefert, 2007; Boller and Felix, 2009; Maekawa et al., 2011b).

Plant NLRs are typically modular-structured, consisting of a central nucleotide-binding domain, C-terminal leucine-rich repeats, and a diversified N-terminal domain of either coiled-coil (CC) or TOLL/interleukin-1 receptor (TIR) subtype. The NLR receptors act as molecular switches to regulate immune responses by switching from an inactive form to an active form upon recognition of pathogen effector(s) and induced conformational changes from ADP- to ATP-bound state (Collier and Moffett, 2009; Lukasik and Takken, 2009; Takken and Goverse, 2012). The N-terminal CC or TIR domain may act as a signaling module for triggering host cell death (Swiderski et al., 2009; Krasileva et al., 2010; Bernoux et al., 2011; Collier et al., 2011; Maekawa et al., 2011a; Bai et al., 2012).

The barley MLA locus is highly polymorphic encoding a large number of allelic CC-subtype NLRs, each conferring isolate-specific disease resistance against the barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*; Seeholzer et al., 2010). The N-terminal CC domains of MLA are highly conserved in sequence (Seeholzer et al., 2010; Jordan et al., 2011), containing an EDVID motif shared with many other CC-subtype NLRs (Collier and Moffett, 2009). The more diversified C-terminal LRR region of MLA was shown to confer recognition specificity (Shen et al., 2003). Here we summarize our recent progresses towards understanding MLA-triggered immune signaling, emphasizing on receptor partitioning, signaling bifurcation, interacting transcription factors (TFs) linking receptor activation to defense response regulations. We also touch upon analogies in other plant NLR-mediated immune signaling pathways.

DYNAMIC NUCLEOCYTOPLASMIC PARTITIONING OF MLA IMMUNE RECEPTORS

The barley intracellular MLA immune receptor has been shown to distribute between the nucleus and the cytoplasm (Shen et al., 2007). Using stable transgenic barley lines expressing a single copy of MLA1-HA fusion under the control of native 5′ regulatory sequences, fractionation experiments revealed that the majority of MLA1 is located in the cytoplasm and a small fraction (∼5%) resides in the nucleus; and interestingly, its nuclear pool is increased upon inoculation of an incompatible *Bgh* isolate (Shen

et al., 2007). Transient expression of a YFP-tagged natural MLA variant, MLA10, revealed that a MLA10-YFP fusion resides in both compartments in barley leaf epidermal cells (Shen et al., 2007; Bai et al., 2012). A mutation in the P-loop motif of MLA10 resulted in apparent increase of overall YFP signal intensity of MLA10-YFP in both compartments for unknown reasons (Bai et al., 2012), excluding the possibility that the P-loop motif of MLA10 is involved in nucleocytoplasmic partitioning. Similar nucleocytoplasmic distribution of the MLA10-YFP fusion was observed in the heterologous *N. benthamiana* system upon Agrobacterium-mediated transient expression and confocal imaging (Bai et al., 2012). Interestingly, similar nucleocytoplasmic partitioning of MLA1 was observed in *Arabidopsis* using a transgenic lines expressing MLA1-HA in a triple mutant background (Maekawa et al., 2012). Whether MLA immune receptors are regulated by conserved or distinct import/export machinery in these two plant species is currently unknown.

In recent years several plant NLR immune receptors have been shown to distribute between cytoplasm and nucleus (Deslandes et al., 2002; 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; Hoser et al., 2013; Inoue et al., 2013; Ma et al., 2013). Some of them possess a canonical or predicted nuclear localization signal (NLS), for example the *Arabidopsis* RPS4/RRS1-R receptor pair and snc1, tobacco N and tomato I-2 resistance protein; while others, like MLA and potato Rx, do not harbor any discernible NLS signal. In this regard, it remains to be shown how the nucleocytoplasmic partitioning is regulated for most of these NLRs (Meier and Somers, 2011; Wirthmueller et al., 2013).

BIFURCATION OF MLA-TRIGGERED CELL DEATH AND DISEASE RESISTANCE SIGNALING

Forced localization of MLA10 to either the cytoplasm or the nucleus, by adding either nuclear export signal (NES) or NLS to its C-terminus (CT), revealed distinct receptor activities in signaling (Shen et al., 2007; Bai et al., 2012). The nuclear pool of MLA10 is essential for powdery mildew disease resistance as transient expression of the MLA10-YFP-NES fusion, that is depleted from the nucleus, fails to restrict the growth of an avirulent *Bgh* isolate (Shen et al., 2007). Further, expression and enforced nuclear localization of the MLA10-NLS fusion revealed that the MLA nuclear pool alone is sufficient to confer disease resistance against *Bgh* in barley (Bai et al., 2012). Unexpectedly, upon transient expression in the heterologous *N. benthamiana* leaves, the MLA10-NES fusion was able to trigger markedly enhanced cell death signaling, whereas MLA10-NLS was unable to induce cell death (Bai et al., 2012). Although MLA10-triggered cell death in the heterologous *N. benthamiana* system is effector-independent, combined with functional analysis in barley these data strongly suggest a model for bifurcation of MLA signaling, in which MLA triggers cell death signaling in the cytoplasm but mediates disease resistance signaling in the nucleus, and these signaling activities of MLA can be uncoupled in a cell compartment-dependent manner (**Figure 1A**).

Signaling bifurcation was also shown for a TIR-type immune receptor, the *Arabidopsis* RPS4 (Heidrich et al., 2011), which recognizes the type III effector AvrRps4 secreted by *Pseudomonas syringae* (Gassmann et al., 1999) and triggers EDS1-dependent transcriptional reprogramming and disease resistance (García et al., 2010; Heidrich et al., 2011). RPS4 was detected in association with EDS1 in complexes in *Arabidopsis* or *N. benthamiana* upon coexpression (Bhattacharjee et al., 2011; Heidrich et al., 2011). AVR effector-dependent activation of RPS4 in *Arabidopsis* nuclei restricted *P. syringae* growth without inducing cell death, however, it triggered weak cell death if the cognate AVR was forced to localize in the cytoplasm (Heidrich et al., 2011). It was proposed that nuclear or cytoplasmic RPS4-EDS1 pools specify distinct subcellular defense signaling branches, and that coordinated action of both defense signals is required for full defense responses (García et al., 2010; Heidrich et al., 2011, Heidrich et al., 2012; **Figure 1C**).

Several recent reports have shown uncoupling of host cell death from disease resistance for both TIR- and CC-subtype NLR immune receptors (Coll et al., 2010; Heidrich et al., 2011; Bai et al., 2012; Sohn et al., 2012; Gao et al., 2013), together these add unambiguous evidence to support a model that for some NLRs HR-cell death and disease resistance are distinct but interconnected subcellular functions.

MLA CC DOMAIN AS A PLATFORM FOR INTERACTING AND SIGNALING

MLA fragments harboring the N-terminal CC domain or other domains have been used for identifying MLA interactors in yeast two-hybrid screenings. The CC domain containing fragments identified the most MLA interactors, and interestingly, almost all of them interacted with the CC domain but not with the MLA full-length protein in further analysis in yeast and *in planta* upon transient coexpression (Shen et al., 2007; Chang et al., 2013; Chang and Shen, unpublished data), suggesting that the MLA CC domain alone can serve as a platform for interacting with or docking to signaling partners post MLA activation.

A crystal structure of MLA10 CC reveals that this domain can form a homodimer and this dimer configuration is shown to be critical for MLA activity (Maekawa et al., 2011a). In the heterologous *N. benthamiana* system, a role of the MLA10 CC domain in cell death signaling has been established by Agrobacterium-mediated transient expression (Maekawa et al., 2011a; Bai et al., 2012). MLA10 full-length protein triggered cell death requires an intact P-loop motif; and mutations in the MHD motif render MLA10 autoactive, triggering cell death in *N. benthamiana* and barley (Bai et al., 2012), together these findings point to a likely scenario in which MLA activation involves conformational changes driven by ATP-binding and hydrolysis cycles and releasing of the N-terminal CC domain, which adopts a homodimer conformation that could serve as a platform for signaling initiation (Maekawa et al., 2011a; Takken and Goverse, 2012). Since the MLA cytoplasmic pool alone is sufficient to trigger cell death we envisage the death signaling might first initiate from the cytoplasm and then transduced by as yet unknown signaling components.

TRANSCRIPTION FACTORS AS DIRECT DOWNSTREAM COMPONENT IN MLA-ACTIVATED SIGNALING

Earlier studies thoroughly characterized the association between MLA and two barley WRKY TFs, WRKY1 and WRKY2 (Shen et al., 2007). WRKY1 and WRKY2 interact with the MLA CC domain

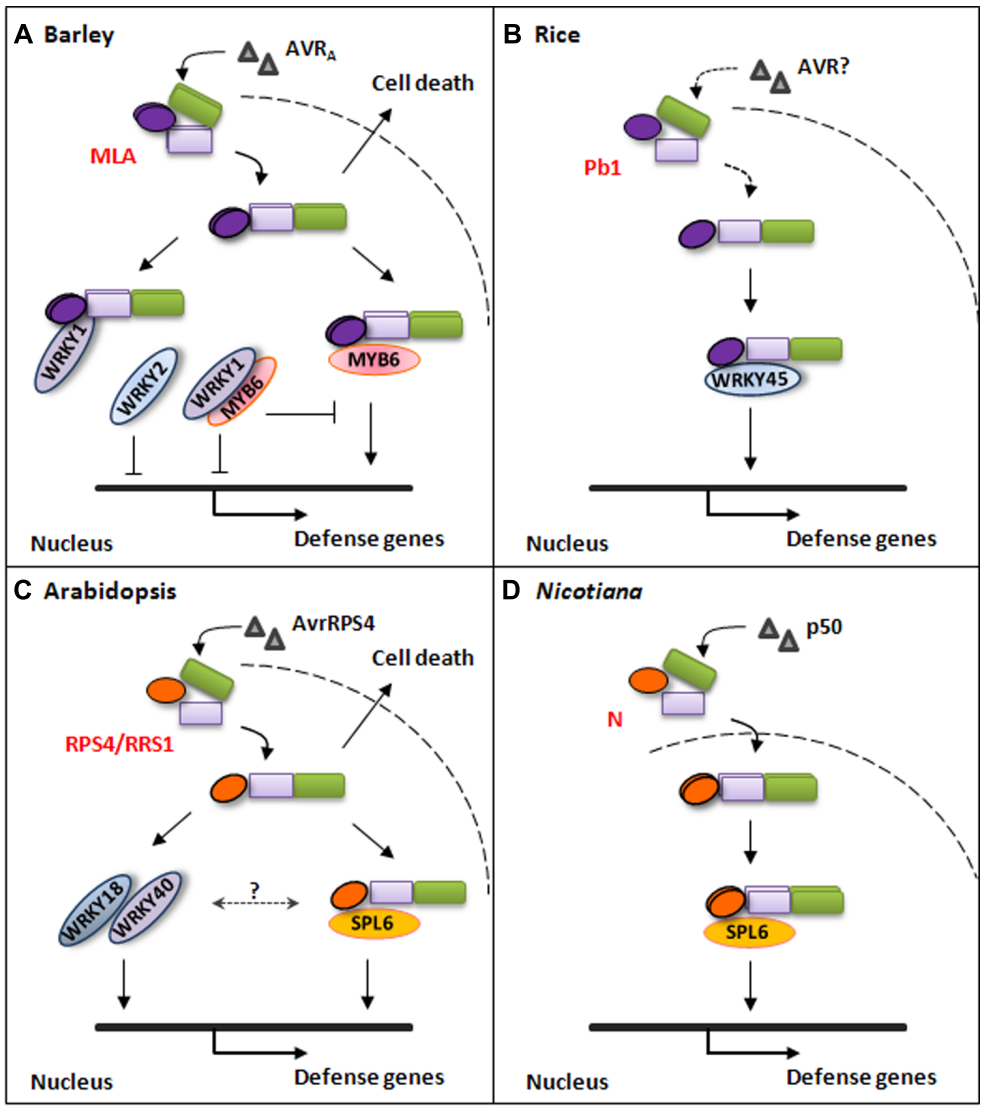

FIGURE 1 | Simplified models for plant NLR-triggered immune signaling pathways. **(A)** Barley MLA immune receptor recognizes cognate AVR$_A$ effector from *B. graminis* fungal pathogen and triggers disease resistance signaling in the nucleus or cell-death signaling in the cytoplasm. The activated MLA interacts with WRKY1 through its N-terminal CC domain to release MYB6 and by itself directly interacts with MYB6 to initiate defense gene expression. Barley WRKY1 and WRKY2 are repressors of defense responses. **(B)** Rice atypical NLR Pb1 interacts with WRKY45 to mediate immune responses against the rice blast fungal pathogen. The Pb1-WRKY45 association can prevent the TF from being degraded by the ubiquitin/proteasome system. **(C)** *Arabidopsis* NLR pair RPS4/RRS1 mediate disease resistance signaling against *Pst*DC3000(avrRPS4) through direct interaction with *At*-SPL6 or through WRKY18 and WRKY40 in the nucleus. RPS4 can also trigger cell-death signaling in the cytoplasm. **(D)** *Nicotiana* N immune receptor specifically recognizes a 50KD helicase domain (p50) from Tobacco mosaic virus (TMV) in the cytoplasm and activated N associates with SPL6 within distinct nuclear compartments to mediate immune responses against TMV.

but not with the full-length MLA protein in yeast, importantly, an AVR$_A$ effector-dependent association of full-length MLA10 with WRKY2 was detected in the nucleus of barley cells using fluorescence life time imaging-fluorescence resonance energy transfer (FLIM-FRET) analysis (Shen et al., 2007). Barley WRKY1 and WRKY2 were demonstrated to act as repressors of basal immunity against the *Bgh* fungus in barley. It was hypothesized that MLA immune receptors target these WRKY repressors to derepress PAMP-triggered immunity thus potentiating defense responses (Shen et al., 2007; Shen and Schulze-Lefert, 2007).

Recently, we reported the identification of barley MYB6 as another MLA interactor (Chang et al., 2013). MYB6 interacts with the CC domain of MLA receptors, MLA1, MLA6 and MLA10, and interestingly MYB6 appears to specifically interact with the homodimeric form of the functional CC domain (Chang et al., 2013). Since the full-length MLA protein was unable to interact with MYB6 we interpret the association of MLA CC with MYB6 as event post MLA receptor activation, somewhat analogous to the interaction between MLA and WRKY1/2. Nevertheless, contrary to the WRKY1/2 repressor, MYB6 acts as a positive regulator in

basal and MLA-triggered disease resistance against the powdery mildew fungus, demonstrated by virus-induced gene silencing (VIGS) and functional gene expression analysis in barley (Chang et al., 2013).

Since WRKY1/2 and MYB6 interact with the MLA CC domain, the potential interaction between WRKY1/2 and MYB6 was tested. Significantly, WRKY1, but not WRKY2, interacts with MYB6 and interferes with MYB6 DNA binding activity (Chang et al., 2013). It is noteworthy that barley WRKY1 and WRKY2 share the same domain structure and 72% sequence similarity, and their *Arabidopsis* homologues, *At*-WRKY18, *At*-WRKY40 and *At*-WRKY60, act redundantly as negative regulators in disease resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) and the *Arabidopsis*-infecting powdery mildew fungus *Golovinomyces orontii* (Xu et al., 2006; Shen et al., 2007; Pandey et al., 2010). However, surprisingly, it was recently reported that *At*-WRKY18 and *At*-WRKY40 are specifically required for mediating disease resistance against *Pst*DC3000 expressing effector AvrRPS4, shown by the specific susceptibility phenotype of the *wrky18 wrky40* mutant line infected with *Pst*DC3000(avrRPS4) but not with other tested *Pst*DC3000 strains (Schön et al., 2013). These findings indicate that WRKY18 and WRKY40 may function redundantly as positive regulators downstream of the RPS4/RRS1 pair, or alternatively that these WRKYs may be targeted and modified by AvrRPS4 which can be perceived by RPS4/RRS1, although the direct physical interaction between RPS4 and WRKY18 or WRKY40 was not detected in the presence or absence of the AVR effector (Schön et al., 2013; **Figure 1C**).

Several other TFs have recently been reported to function in NLR-mediated immune signaling (Inoue et al., 2013; Padmanabhan et al., 2013; **Figure 1**). The *Nicotiana* SPL6 TF was demonstrated to interact with the N immune receptor in subnuclear bodies once immune signaling is activated and SPL6 functions as a positive regulator in N-mediated immunity against Tobacco mosaic virus in *Nicotiana* plants (**Figure 1D**); Interestingly, like *At*-WRKY18 and *At*-WRKY40, the SPL6 paralog in *Arabidopsis* is also specifically required for RPS4-triggered disease resistance against *Pst*DC3000 (avrRPS4; Padmanabhan et al., 2013; **Figure 1C**). The rice WRKY45 was demonstrated to interact with Pb1, an CC-NB-LRR protein conferring panicle blast resistance in rice, and this interaction prevents ubiquitin/proteasome-mediated degradation of WRKY45, which is believed to be involved in Pb1-triggered blast resistance (Hayashi et al., 2010; Inoue et al., 2013; **Figure 1B**).

REPRESSING AND DEREPRESSING: TRANSCRIPTIONAL REGULATIONS IN MLA-TRIGGERED IMMUNE SIGNALING

The R2R3-type MYB TF family members have undergone expansion in different plant lineages and are involved in regulating diverse biological processes (Stracke et al., 2001; Dubos et al., 2010; Feller et al., 2011; Raffaele and Rivas, 2013). One of the best characterized MYB TF is *Arabidopsis At*-MYB30 that plays a critical role in executing hypersensitive cell death in defense response to the bacterial pathogen *Xanthomonas* (Vailleau et al., 2002; Raffaele et al., 2008). Significantly, the transcriptional activity of *At*-MYB30 and resistance function is negatively regulated not only by the host protein *At*sPLA$_2$-α through physical association in the nucleus (Froidure et al., 2010), but also by the *Xanthomonas* Type III effector XopD by relocalizing it to nuclear foci (Canonne et al., 2011).

Barley MYB6 is also a R2R3-type MYB TF that binds to the cognate *cis*-element *MBS I* and acts as a transcriptional activator to regulate gene expression (Chang et al., 2013). MYB6 activity in DNA-binding was evaluated in the presence of WRKY1 or MLA CC in electrophoretic mobility shift assay (EMSA) or *Arabidopsis* protoplast transfection assay. Interestingly, WRKY1 could suppress MYB6 DNA-binding activity, whereas the MLA10 CC domain markedly stimulated this activity, suggesting that MYB6 activity is antagonistically regulated by WRKY1 and MLA CC domain (Chang et al., 2013).

The tripartite interaction among WRKY1, MYB6 and MLA were dissected in details using yeast three-hybrid, *in planta* and *in vitro* protein interaction assays. It was demonstrated that the WRKY1-MYB6 association can be abrogated by the MLA10 CC domain in a WRKY1 CT-dependent manner, and subsequently MLA10 CC forms a complex with MYB6 in the nucleus. Importantly, MLA10 CC and an autoactive MLA10 full-length variant with a mutation in the MHD motif can antagonize WRKY1 suppression and markedly stimulates MYB6 DNA-binding activity, thus increases MYB6-dependent gene expressions in the *Arabidopsis* protoplast transfection system (Chang et al., 2013).

We propose a model in which WRKY1 repressor physically sequesters barley MYB6 from binding to the promoter of downstream target genes to prevent uncontrolled cell death and defense responses; upon perception of cognate effector activated MLA interacts with WRKY1 and releases MYB6 from suppression and stimulates its binding to cognate *cis*-acting elements to initiate disease resistance signaling (Chang et al., 2013; **Figure 1A**).

CONCLUSIONS AND PERSPECTIVES

Data from barley MLA and other plant NLRs discussed here underlines the importance of nucleocytoplasmic trafficking and transcriptional regulation in plant NLR-mediated immune responses. Emerging evidence indicates that parallel mechanistics of regulation exist in mammalian NLR-mediated immunity. NLRC5 was recently presented as a transcription regulator to cooperate with TFs to induce MHC class I gene expression (Meissner et al., 2010, 2012), while CIITA was previously identified as a master transcription coactivator in regulating MHC class II gene expression (Ting and Davis, 2005); both NLRs shuttle between the cytosol and nucleus.

Specific and fundamental questions remain to be addressed to fill the gaps in MLA-activated immune signaling: what are the target genes commonly and distinctively regulated by WRKY1/2 and/or MYB6? How are MLA, WRKY1/2 and MYB6 regulated at post-translational level? What are the components/pathways involved in MLA-triggered cell death signaling in the cytoplasm? How does MLA regulate distinct immune activities in the nucleus and cytoplasm?

So far only a limited numbers of NLRs were shown to trigger defense signaling through direct association with TFs, which

is likely downstream of AVR effector perception (**Figure 1**). Nevertheless, analogous mechanistics appears to be engaged with by both CC- and TIR-subtype of NLR receptors from either monocots or dicots to coordinate defense responses against diverse pathogens, including viral, bacterial and fungal pathogens (**Figure 1**). It is reasonable to envisage that NLRs are partially nuclear localized or translocated into the nucleus upon activation may orchestrate defense gene expression through transcriptional regulation. We are only at the beginning to unravel the dynamics of NLR-mediated signaling in the cytoplasm and the nucleus.

ACKNOWLEDGMENTS

We thank all members in Shen's lab for their contribution and Paul Schulze-Lefert at the Max Planck Institute, Cologne and colleagues for collaboration. This work was supported by the National Basic Research Program of China (2011CB100700), and National Natural Science Foundation of China (31030007).

REFERENCES

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

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. doi: 10.1016/j.chom.2011.02.009

Bhattacharjee, S., Halane, M. K., Kim, S. H., and Gassmann, W. (2011). Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators. *Science* 334, 1405–1408. doi: 10.1126/science.1211592

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

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

Caldo, R. A., Nettleton, D., and Wise, R. P. (2004). Interaction-dependent gene expression in MLA-specified response to barley powdery mildew. *Plant Cell* 16, 2514–2528. doi: 10.1105/tpc.104.023382

Canonne, J., Marino, D., Jauneau, A., Pouzet, C., Brière, C., Roby, D., et al. (2011). The *Xanthomonas* type III effector XopD targets the *Arabidopsis* transcription factor MYB30 to suppress plant defense. *Plant Cell* 23, 3498–3511. doi: 10.1105/tpc.111.088815

Chang, C., Yu, D., Jiao, J., Jing, S., Schulze-Lefert, P., and Shen, Q.-H. (2013). Barley MLA immune receptors directly interfere with antagonistically acting transcription factors to initiate disease resistance signaling. *Plant Cell* 25, 1158–1173. doi: 10.1105/tpc.113.109942

Cheng, Y. T., Germain, H., Wiermer, M., Bi, D., Xu, F., García, 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. doi: 10.1105/tpc.108.064519

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. doi: 10.1126/science.1194980

Collier, S. M., Hamel, L.-P., and Moffett, P. (2011). Cell death mediated by the N-terminal domains of a unique and highly conserved class of NB-LRR protein. *Mol. Plant Microbe Interact.* 24, 918–931. doi: 10.1094/MPMI-03-11-0050

Collier, S. M., and Moffett, P. (2009). NB-LRRs work a "bait and switch" on pathogens. *Trends Plant Sci.* 14, 521–529. doi: 10.1016/j.tplants.2009.08.001

Deslandes, L., Olivier, J., Theulières, F., Hirsch, J., Feng, D. X., Bittner-Eddy, P., et al. (2002). Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. *Proc. Natl. Acad. Sci. U.S.A.* 99, 2404–2409. doi: 10.1073/pnas.032485099

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., and Lepiniec, L. (2010). MYB transcription factors in *Arabidopsis*. *Trends Plant Sci.* 15, 573–581. doi: 10.1016/j.tplants.2010.06.005

Feller, A., Machemer, K., Braun, E. L., and Grotewold, E. (2011). Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J.* 66, 94–116. doi: 10.1111/j.1365-313X.2010.04459.x

Froidure, S., Canonne, J., Daniel, X., Jauneau, A., Brière, C., Roby, D., et al. (2010). AtsPLA2-α nuclear relocalization by the *Arabidopsis* transcription factor AtMYB30 leads to repression of the plant defense response. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15281–15286. doi: 10.1073/pnas.1009056107

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

García, A. V., Blanvillain-Baufumé, S., Huibers, R. P., Wiermer, M., Li, G., Gobbato, E., et al. (2010). Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS Pathog.* 6:e1000970. doi: 10.1371/journal.ppat.1000970

Gassmann, W., Hinsch, M. E., and Staskawicz, B. J. (1999). The *Arabidopsis* RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. *Plant J.* 20, 265–277. doi: 10.1046/j.1365-313X.1999.t01-1-00600.x

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. doi: 10.1111/j.1365-313X.2010.04348.x

Heidrich, K., Blanvillain-Baufumé, S., and Parker, J. E. (2012). Molecular and spatial constraints on NB-LRR receptor signaling. *Curr. Opin. Plant Biol.* 15, 385–391. doi: 10.1016/j.pbi.2012.03.015

Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L., and Parker, J. E. (2011). *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* 334, 1401–1404. doi: 10.1126/science.1211641

Hoser, R., Żurczak, M., Lichocka, M., Zuzga, S., Dadlez, M., Samuel, M. A., et al. (2013). Nucleocytoplasmic partitioning of tobacco N receptor is modulated by SGT1. *New Phytol.* 200, 158–171. doi: 10.1111/nph.12347

Inoue, H., Hayashi, N., Matsushita, A., Xinqiong, L., Nakayama, A., Sugano, S., et al. (2013). Blast resistance of CC-NB-LRR protein Pb1 is mediated by WRKY45 through protein–protein interaction. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9577–9582. doi: 10.1073/pnas.1222155110

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

Jordan, T., Seeholzer, S., Schwizer, S., Töller, A., Somssich, I. E., and Keller, B. (2011). The wheat MLA homologue TmMla1 exhibits an evolutionarily conserved function against powdery mildew in both wheat and barley. *Plant J.* 65, 610–621. doi: 10.1111/j.1365-313X.2010.04445.x

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. doi: 10.1105/tpc.110.075358

Lukasik, E., and Takken, F. L. W. (2009). STANDing strong, resistance proteins instigators of plant defence. *Curr. Opin. Plant Biol.* 12, 427–436. doi: 10.1016/j.pbi.2009.03.001

Ma, L., Cornelissen, B. J. C., and Takken, F. L. W. (2013). A nuclear localization for Avr2 from *Fusarium oxysporum* is required to activate the tomato resistance protein I-2. *Front. Plant Sci.* 4:94. doi: 10.3389/fpls.2013.00094

Maekawa, T., Cheng, W., Spiridon, L. N., Töller, A., Lukasik, E., Saijo, Y., et al. (2011a). 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. doi: 10.1016/j.chom.2011.02.008

Maekawa, T., Kufer, T. A., and Schulze-Lefert, P. (2011b). NLR functions in

plant and animal immune systems: so far and yet so close. *Nat. Immunol.* 12, 817–826. doi: 10.1038/ni.2083

Maekawa, T., Kracher, B., Vernaldi, S., Ver Loren van Themaat, E., and Schulze-Lefert, P. (2012). Conservation of NLR-triggered immunity across plant lineages. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20119–20123. doi: 10.1073/pnas.1218059109

Meier, I., and Somers, D. E. (2011). Regulation of nucleocytoplasmic trafficking in plants. *Curr. Opin. Plant Biol.* 14, 538–546. doi: 10.1016/j.pbi.2011.06.005

Meissner, T. B., Li, A., Biswas, A., Lee, K.-H., Liu, Y.-J., Bayir, E., et al. (2010). NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13794–13799. doi: 10.1073/pnas.1008684107

Meissner, T. B., Liu, Y.-J., Lee, K.-H., Li, A., Biswas, A., van Eggermond, M. C. J. A., et al. (2012). NLRC5 cooperates with the RFX transcription factor complex to induce MHC Class I gene expression. *J. Immunol.* 188, 4951–4958. doi: 10.4049/jimmunol.1103160

Padmanabhan, M. S., Ma, S., Burch-Smith, T. M., Czymmek, K., Huijser, P., and Dinesh-Kumar, S. P. (2013). Novel positive regulatory role for the SPL6 transcription factor in the N TIR-NB-LRR receptor-mediated plant innate immunity. *PLoS Pathog.* 9:e1003235. doi: 10.1371/journal.ppat.1003235

Pandey, S. P., Roccaro, M., Schön, M., Logemann, E., and Somssich, I. E. (2010). Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of *Arabidopsis*. *Plant J.* 64, 912–923. doi: 10.1111/j.1365-313X.2010.04387.x

Raffaele, S., and Rivas, S. (2013). Regulate and be regulated: integration of defence and other signals by the AtMYB30 transcription factor. *Front. Plant Sci.* 4:98. doi: 10.3389/fpls.2013.00098

Raffaele, S., Vailleau, F., Léger, A., Joubès, J., Miersch, O., Huard, C., et al. (2008). A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in *Arabidopsis*. *Plant Cell* 20, 752–767. doi: 10.1105/tpc.107.054858

Schön, M., Töller, A., Diezel, C., Roth, C., Westphal, L., Wiermer, M., et al. (2013). Analyses of wrky18 wrky40 plants reveal critical roles of SA/EDS1 signaling and indole-glucosinolate biosynthesis for *Golovinomyces orontii* resistance and a loss-of resistance towards *Pseudomonas syringae* pv. tomato AvrRPS4. *Mol. Plant Microbe Interact.* 26, 758–767. doi: 10.1094/MPMI-11-12-0265-R

Seeholzer, S., Tsuchimatsu, T., Jordan, T., Bieri, S., Pajonk, S., Yang, W., et al. (2010). Diversity at the Mla powdery mildew resistance locus from cultivated barley reveals sites of positive selection. *Mol. Plant Microbe Interact.* 23, 497–509. doi: 10.1094/MPMI-23-4-0497

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. doi: 10.1126/science.1136372

Shen, Q. H., and Schulze-Lefert, P. (2007). Rumble in the nuclear jungle: compartmentalization, trafficking, and nuclear action of plant immune receptors. *EMBO J.* 26, 4293–4301. doi: 10.1038/sj.emboj.7601854

Shen, Q. H., Zhou, F. S., Bieri, S., Haizel, T., Shirasu, K., and Schulze-Lefert, P. (2003). Recognition specificity and RAR1/SGT1 dependence in barley MLA disease resistance genes to the powdery mildew fungus. *Plant Cell* 15, 732–744. doi: 10.1105/tpc.009258

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. doi: 10.1105/tpc.110.077537

Sohn, K. H., Hughes, R. K., Piquerez, S. J., Jones, J. D. G., 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. doi: 10.1073/pnas.1212332109

Stracke, R., Werber, M., and Weisshaar, B. (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr. Opin. Plant Biol.* 4, 447–456. doi: 10.1016/S1369-5266(00)00199-0

Swiderski, M. R., Birker, D., and Jones, J. D. G. (2009). The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. *Mol. Plant Microbe Interact.* 22, 157–165. doi: 10.1094/MPMI-22-2-0157

Takken, F. L. W., and Goverse, A. (2012). How to build a pathogen detector: structural basis of NB-LRR function. *Curr. Opin. Plant Biol.* 15, 375–384. doi: 10.1016/j.pbi.2012.05.001

Tameling, W. I. L., 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. doi: 10.1105/tpc.110.077461

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.-S., Han, B., et al. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15, 317–330. doi: 10.1105/tpc.007591

Ting, J. P.-Y., and Davis, B. K. (2005). CATERPILLER: a novel gene family important in immunity, cell death, and diseases. *Annu. Rev. Immunol.* 23, 387–414. doi: 10.1146/annurev.immunol.23.021704.115616

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

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. (2009). Network properties of robust immunity in plants. *PLoS Genet.* 5:e1000772. doi: 10.1371/journal.pgen.1000772

Vailleau, F., Daniel, X., Tronchet, M., Montillet, J. L., Triantaphylidès, C., and Roby, D. (2002). A R2R3-MYB gene, AtMYB30, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10179–10184. doi: 10.1073/pnas.152047199

Wirthmueller, L., Roth, C., Banfield, M. J., and Wiermer, M. (2013). Hop-on hop-off: importin-a-guided tours to the nucleus in innate immune signaling. *Front. Plant Sci.* 4:149. doi: 10.3389/fpls.2013.00149

Wirthmueller, L., Zhang, Y., Jones, J. D. G., 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. doi: 10.1016/j.cub.2007.10.042

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell* 18, 1310–1326. doi: 10.1105/tpc.105.037523

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Curr. Opin. Plant Biol.* 12, 414–420. doi: 10.1016/j.pbi.2009.06.003

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: 10 June 2013; paper pending published: 01 July 2013; accepted: 16 September 2013; published online: 08 October 2013.

Citation: Chang C, Zhang L and Shen Q-H (2013) Partitioning, repressing and derepressing: dynamic regulations in MLA immune receptor triggered defense signaling. Front. Plant Sci. ***4****:396. doi: 10.3389/fpls.2013.00396*

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

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 17 October 2013
doi: 10.3389/fpls.2013.00403

Arabidopsis TNL-WRKY domain receptor RRS1 contributes to temperature-conditioned RPS4 auto-immunity

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In plant effector-triggered immunity (ETI), intracellular nucleotide binding-leucine rich repeat (NLR) receptors are activated by specific pathogen effectors. The *Arabidopsis TIR* (*Toll-Interleukin-1 receptor domain*)-*NLR* (denoted *TNL*) gene pair, *RPS4* and *RRS1*, confers resistance to *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 expressing the Type III-secreted effector, AvrRps4. Nuclear accumulation of AvrRps4, RPS4, and the TNL resistance regulator EDS1 is necessary for ETI. RRS1 possesses a C-terminal "WRKY" transcription factor DNA binding domain suggesting that important RPS4/RRS1 recognition and/or resistance signaling events occur at the nuclear chromatin. In *Arabidopsis* accession Ws-0, the $RPS4^{Ws}$/$RRS1^{Ws}$ allelic pair governs resistance to *Pst*/AvrRps4 accompanied by host programed cell death (pcd). In accession Col-0, $RPS4^{Col}$/$RRS1^{Col}$ effectively limits *Pst*/AvrRps4 growth without pcd. Constitutive expression of HA-StrepII tagged $RPS4^{Col}$ (in a *35S:RPS4-HS* line) confers temperature-conditioned *EDS1*-dependent auto-immunity. Here we show that a high (28°C, non-permissive) to moderate (19°C, permissive) temperature shift of *35S:RPS4-HS* plants can be used to follow defense-related transcriptional dynamics without a pathogen effector trigger. By comparing responses of *35S:RPS4-HS* with *35S:RPS4-HS rrs1-11* and *35S:RPS4-HS eds1-2* mutants, we establish that $RPS4^{Col}$ auto-immunity depends entirely on *EDS1* and partially on $RRS1^{Col}$. Examination of gene expression microarray data over 24 h after temperature shift reveals a mainly quantitative $RRS1^{Col}$ contribution to up- or down-regulation of a small subset of $RPS4^{Col}$-reprogramed, *EDS1*-dependent genes. We find significant over-representation of WRKY transcription factor binding W-box cis-elements within the promoters of these genes. Our data show that $RRS1^{Col}$ contributes to temperature-conditioned $RPS4^{Col}$ auto-immunity and are consistent with activated $RPS4^{Col}$ engaging $RRS1^{Col}$ for resistance signaling.

Keywords: *resistance* gene pair, temperature shift, EDS1 signaling, biotic stress, programed cell death, transcriptional reprograming

INTRODUCTION

A critical layer of plant innate immunity is conferred by intracellular nucleotide binding-leucine rich repeat (NLR) receptors that guard against disease-promoting activities of pathogen effectors during infection (Dodds and Rathjen, 2010). Genes encoding NLR proteins represent the most diverse gene family in plants, probably as a result of pathogen selection pressure (Meyers et al., 2003; Yue et al., 2012). NLR receptors behave as ATP-driven molecular switches which become activated directly by physical association with an effector or indirectly through effector perturbations of a receptor-guarded co-factor (Maekawa et al., 2011; Bernoux et al., 2011a). Receptor activation triggers a robust anti-microbial response which is often accompanied by localized host programed cell death (pcd), although pathogen resistance can be uncoupled from pcd (Maekawa et al., 2011; Heidrich et al., 2012).

The NLR receptor family is broadly divided into two sub-classes based on different N-terminal putative signaling domains containing either Toll-Interleukin-1 receptor (TIR) homology, or a coiled-coil (CC) or other features, referred to, respectively, as TNLs and CNLs (Maekawa et al., 2011; Bernoux et al., 2011a). TNL and CNL receptor types signal in different ways for resistance (Wiermer et al., 2005; Venugopal et al., 2009). However, they all converge on the transcriptional machinery to amplify gene expression programs which operate in basal resistance against virulent (non-recognized) pathogens (Tao et al., 2003; Bartsch et al., 2006). Only a handful of TNL and CNL receptors have been characterized and many questions remain about where and how NLR are activated inside cells and the sequence of downstream signaling events leading to disease resistance. A number of functional NLR representatives from both sub-classes are nucleo-cytoplasmic and there is compelling evidence that NLR nucleo-cytoplasmic partitioning is important for full triggering of an immune response (Heidrich et al., 2012). Moreover, the *Arabidopsis* TNL protein SNC1 (Zhu et al., 2010b), tobacco TNL receptor N (Padmanabhan et al., 2013) and barley CNL receptor MLA1 (Chang et al., 2013) interact with transcription factors, suggesting a short route to the transcriptional machinery.

All functionally characterized TNL receptors depend on the nucleo-cytoplasmic immune regulator EDS1 (enhanced disease sensitivity1) for triggering resistance and pcd (Wiermer et al.,

2005) and associations between several TNLs and EDS1 have been detected in *Arabidopsis* and tobacco, suggesting that EDS1 is part of an immune receptor signaling complex (Bhattacharjee et al., 2011; Heidrich et al., 2011; Kim et al., 2012). EDS1, in direct association with its signaling partner PAD4 (phytoalexin deficient4), is essential for basal resistance against virulent pathogens, measured as a slowing of pathogen growth without obvious TNL recognition or pcd (Jirage et al., 1999; Feys et al., 2001; Rietz et al., 2011). Based on interactions detected between EDS1 and *Pseudomonas syringae* Type III-secreted effectors AvrRps4 and HopA1, it was proposed that TNL receptors might guard the EDS1–PAD4 basal resistance machinery against interference by pathogen effectors as well as co-opting EDS1 as an early signaling component for execution of effector-triggered immunity (ETI; Bhattacharjee et al., 2011; Heidrich et al., 2011).

We are studying ETI in *Arabidopsis* mediated by the TNL receptor gene pair, *RPS4* and *RRS1*, in recognition of AvrRps4 derived from leaf-infecting *P. syringae* pv *pisi* (Hinsch and Staskawicz, 1996; Gassmann et al., 1999; Birker et al., 2009; Narusaka et al., 2009). Particular allelic forms of the same *RPS4 RRS1* pair also recognize an unrelated YopJ family effector, PopP2, secreted by root-infecting *Ralstonia solanacearum* bacteria (Deslandes et al., 2003; Narusaka et al., 2009). RPS4 accumulates as a nucleo-cytoplasmic protein associating with endo-membranes (Wirthmueller et al., 2007; Bhattacharjee et al., 2011). Notably, RPS4 nuclear accumulation conferred by a C-terminal NLS is essential for resistance to *P. syringae* pv *tomato* (*Pst*) expressing AvrRps4 (*Pst*/AvrRps4), although *RPS4* nucleo-cytoplasmic partitioning does not rely on the presence of either AvrRps4 or EDS1 (Wirthmueller et al., 2007; Heidrich et al., 2011). RRS1 is an atypical TNL in that it also possesses a C-terminal "WRKY" transcription factor DNA binding domain (Deslandes et al., 2002) known to recognize W-box consensus sequences within the promoters of defense-related genes (Rushton et al., 2010; Chen et al., 2013; Logemann et al., 2013). Analysis of the auto-immune phenotype of an *rrs1* (*slh1*) single amino acid insertion mutation in the WRKY domain abolishing DNA binding *in vitro*, led to the idea that *RRS1* exists as an auto-inhibited form at the chromatin in healthy tissues (Noutoshi et al., 2005). An effector trigger might then cause an RRS1 conformational switch to initiate resistance signaling. Other studies established that RRS1 interacts with *R. solanacearum* effector PopP2 (Deslandes et al., 2003; Tasset et al., 2010). PopP2 has an auto-acetyltransferase activity and this enzymatic function, coupled with recognition by a resistant RRS1-R allelic form, appear to be necessary for triggering resistance (Tasset et al., 2010). By contrast, AvrRps4 has no known enzyme activity but is proteolytically cleaved inside plant cells to produce an 11 kDa α-helical CC C-terminal fragment which is essential for *RPS4*/*RRS1* recognition (Sohn et al., 2009, 2012). While association between AvrRPS4 and EDS1 was reported based on fluorescence resonance energy transfer–fluorescence life-time imaging (FRET–FLIM) and co-immunoprecipitation assays in tobacco and *Arabidopsis* (Bhattacharjee et al., 2011; Heidrich et al., 2011), another study argued against AvrRps4–EDS1 association based on negative interaction data (Sohn et al., 2012). Clearly, much needs to be resolved about the configurations of receptor pre-activation and signaling complexes and their precise relationship with the transcriptional machinery.

Resistance conditioned by TNL receptors is acutely sensitive to temperature with higher temperatures suppressing activated immune responses (Yang and Hua, 2004; Wang et al., 2009; Kim et al., 2010; Zhu et al., 2010a; Alcazar and Parker, 2011). Previously, we described an HA-StrepII epitope tagged RPS4 over-expression line (*35S:RPS4-HS*) in *Arabidopsis* accession Columbia (Col-0) which displays *EDS1*-dependent auto-immunity and stunting at 22°C, consistent with EDS1 being recruited coincidently or immediately downstream of activated RPS4 (Wirthmueller et al., 2007; Heidrich et al., 2011). Here we establish that auto-immunity in the *35S:RPS4-HS* plants grown at 22°C or shifted from a suppressive (28°C) to permissive (19°C) temperature depends fully on *EDS1* and partially on $RRS1^{Col}$. We have used the 28–19°C temperature shift to induce $RPS4^{Col}$ immunity and examine transcriptional reprograming in leaf tissues. This reveals a mainly quantitative contribution of $RRS1^{Col}$ to up- and down-regulation of a discrete set of EDS1-dependent genes. The data suggest that RRS1 acts positively and at an early stage of RPS4 auto-immunity.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

All mutant and transgenic lines used were in *Arabidopsis* accessions Columbia (Col-0) or Wassilewskija (Ws-0). Col *eds1-2* (Bartsch et al., 2006), *rps4-2* (Wirthmueller et al., 2007), *rrs1-11* (Birker et al., 2009), Ws *eds1-1* (Parker et al., 1996), *rps4-21*, *rrs1-1*, and *rps4-21/rrs1-1* (Narusaka et al., 2009) mutant lines, *35S:RPS4-HS* and *35S:RPS4-HS eds1-2* (Wirthmueller et al., 2007) have been described. The *35S:RPS4-HS rrs1-11* line was generated by crossing *35S:RPS4-HS* with *rrs1-11*. Plants were grown in soil in chambers under a 10/14 h day/night cycle (150–200 $\mu E/m^2s$) and ∼65% relative humidity at 19, 22, or 28°C.

BACTERIAL STRAINS

Bacterial strains *Pst* strain DC3000 and *Pst* DC3000 expressing AvrRps4 (*Pst*/AvrRps4) were obtained from R. Innes (Indiana University, Bloomington, USA) and grown as described (Hinsch and Staskawicz, 1996). *Pst* strain DC3000 expressing AvrRps4-HA or the AvrRps4-HA-NLS and AvrRps4-HA-NES variants from a pEDV6 vector, or a non-pathogenic *Pseudomonas fluorescens* (*Pfo*) strain for delivery of Type III-secreted effectors (Thomas et al., 2009) expressing AvrRps4-HA in pEDV6, have been described (Heidrich et al., 2011).

BACTERIAL GROWTH ASSAYS

For *Pst* spray infections, bacteria were adjusted to 1×10^8 cfu/ml in 10 mM $MgCl_2$ containing 0.04 % (v/v) Silwet L-77 (Lehle seeds, USA). *In planta* bacterial titers were determined 3 h after spray-infection (day 0) and 3 days post-infection (dpi) by shaking leaf disks in 10 mM $MgCl_2$ with 0.01% Silwet L-77 at 28°C for 1 h, as described (Tornero and Dangl, 2001; Garcia et al., 2010). Infected plants were kept in a growth cabinet with a 10/14 h day/night cycle at 23°C. Mean values and standard errors (SEs) were calculated from at least three biological replicates per experiment. In the bacterial growth assays shown in **Figure 1A**, raw data was

$\log_{10}$ transformed and all replicate values from three independent experiments analyzed using a linear model.

ION LEAKAGE ASSAYS

For conductivity measurements after *Pfo* infiltration, leaves of 4-week-old-plants were infiltrated with 1.5×10^8 cfu/ml bacteria in 10 mM $MgCl_2$. Leaf disks were collected using a cork borer (6 mm diameter), floated in water for 30 min, and three leaf disks per measurement were subsequently transferred to a microtiter plate containing 3 ml distilled water. Conductivity of the solution was determined with a Horiba Twin B-173 conductivity meter at the indicated time points. Mean values and SE were calculated from four replicate measurements per genotype or bacterial strain. Experiments were repeated at least three times.

PROTEIN IMMUNOBLOTTING

Total protein extracts from *Arabidopsis* leaves were prepared as previously described (Garcia et al., 2010). Protein concentrations were quantified and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electro-blotted onto nitrocellulose membranes. Equal protein transfer was monitored by staining membranes with Ponceau S (Sigma-Aldrich). Membranes were blocked in a 5%-milk Tris buffer saline-Tween (TBST 20) solution before incubation in a 2% milk-TBST solution containing primary α-HA antibody (3F10; Roche) overnight. The appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) was applied and proteins were detected using enhanced chemiluminiscence reagent (ECL; Pierce Thermo Scientific).

RT-PCR ANALYSIS OF DEFENSE GENE EXPRESSION

Total RNA was extracted from leaf material of 3-week-old plants using TriReagent (Sigma-Aldrich) according to the manufacturer's protocol. A 1.5 μg of total RNA was incubated with 10 units of RNAse-free DNAse I (Roche) at 37°C for 30 min followed by heat-inactivation of the enzyme at 75°C for 10 min. Reverse transcription was performed with Super-Script II enzyme (Invitrogen) according to the manufacturer's protocol. The following primer combinations were used for semi-quantitative real-time polymerase chain reaction (RT-PCR). Actin: fw GGCGATGAAGCTCAATCCAAACG, Actin: rev GGTCAC-GACCAGCAAGATCAAGACG; EDS1: fw TCATACGCAATC-CAAATGTTTAC, EDS1: rev AAAAACCTCTCTTGCTCGATCAC; PBS3: fw CAACTTGTTAGAGGAGATCATCACACCC, PBS3: rev CCAGAAGGAGTCATGGATTCTTGTTTA; At5g26920: fw CGGAACAGCCCTAGTTTTCATGGG, At5g26920: rev GAGAA-GACGAGAACGGTCCCGTACT; At5g27420: fw CTACTATTATC-CGTGTCGGC, At5g27420: rev CGCGTCTAACCCACG.

GENE EXPRESSION MICROARRAY ANALYSIS

Total RNA was prepared from 3.5-week-old plants grown at 28°C and shifted to 19°C for 0, 2, 8, and 24 h, using a QIAGEN Plant RNeasy kit. RNA quality was assessed on a Bioanalyzer (Agilent). Biotinylated cRNA was prepared and hybridized on Affymetrix ATH1-121501 "GeneChip" arrays, as described (Hajheidari et al., 2012). Briefly, biotinylated cRNA was made from 1 μg total RNA using the MessageAmp II-Biotin Enhanced Kit (Ambion). After amplification and fragmentation, 12.5 μg of cRNA were hybridized for 16 h at 45°C. Arrays were subsequently washed and stained in the Affymetrix Fluidics Station 450 using Fluidics Script FS450-004, and scanned with a GeneChip Scanner 3000 7G. For each condition, three Affymetrix ATH1 microarrays were hybridized with independent biological samples. Raw data for gene expression signals was extracted using the Affymetrix GeneChip Operating Software (version 1.4). For further data collection and assessment, R language version 2.15 (bioconductor project) was used. Probe signal values were subjected to GeneChip-robust multiarray average algorithm (GC-RMA; Wu and Irizarry, 2004). Probes which were below the background signal in all samples were not considered for further analysis. The results were analyzed by the following linear model using the lmFit function in the limma package in the *R* environment: $S_{gyr} = GY_{gyt} + R_r + \varepsilon_{gyr}$, where S is $\log_2$ expression value, GY, genotype:time interaction, and random factors; R is biological replicate; ε, residual. The eBayes function in the limma package was used for variance shrinkage in calculating the p-values and the Storey's q-values were calculated using the q-value function in the q-value package from the p-values (Storey and Tibshirani, 2003). Genes whose expression changes were *RRS1*-dependent upon temperature shift at any time point (q-values < 0.01 and $>$2-fold change) were selected (250 genes) for the clustering analysis. Heatmaps were generated by CLUSTER using uncentered Pearson correlation and complete linkage and were visualized by TREEVIEW (Eisen et al., 1998). Promoter sequences of the 250 *RRS1*-dependent genes were retrieved from the TAIR website[1] with fixed 1000 bp sequences upstream of the translational start site. Over representation of the core W-box (TTGACY) was assessed using the promoter bootstrapping (POBO) application[2] (Kankainen and Holm, 2004). One thousand pseudo-clusters of 250 genes were generated from the *RRS1*-dependent genes (Cluster2), all induced/suppressed genes upon temperature shift in Col (q-values < 0.01 and $>$2-fold change; Cluster 3), and the *Arabidopsis* genomic background (background). Statistical significance of the t-values generated by POBO was calculated using the linked Graphpad application for a two-tailed comparison: *Comparison of Cluster 2 and background ($p < 0.0001$); *Comparison of Cluster 2 and Cluster 3 ($p < 0.0001$); *Comparison of Cluster 3 and background ($p < 0.0001$). Analysis of gene ontology (GO) terms for the 250 *RRS1*-dependent genes was performed using Agrico[3]. Microarray data have been submitted to the Gene Expression Omnibus database (GEO accession no. GSE50019).

RESULTS

ANALYSIS OF *RPS4^{Ws}* AND *RRS1^{Ws}* COOPERATIVITY IN AvrRps4-TRIGGERED RESISTANCE AND HR

In *Arabidopsis* accession Ws-0, resistance to *Pst* strain DC3000 expressing AvrRps4 (*Pst*/AvrRps4) after bacterial infiltration of leaves relies on genetic cooperation between *RPS4^{Ws}* and *RRS1^{Ws}* (Narusaka et al., 2009). We tested whether the *RPS4^{Ws}*

[1]http://www.Arabidopsis.org/

[2]http://ekhidna.biocenter.helsinki.fi/poxo/pobo/pobo

[3]http://bioinfo.cau.edu.cn/agriGO/

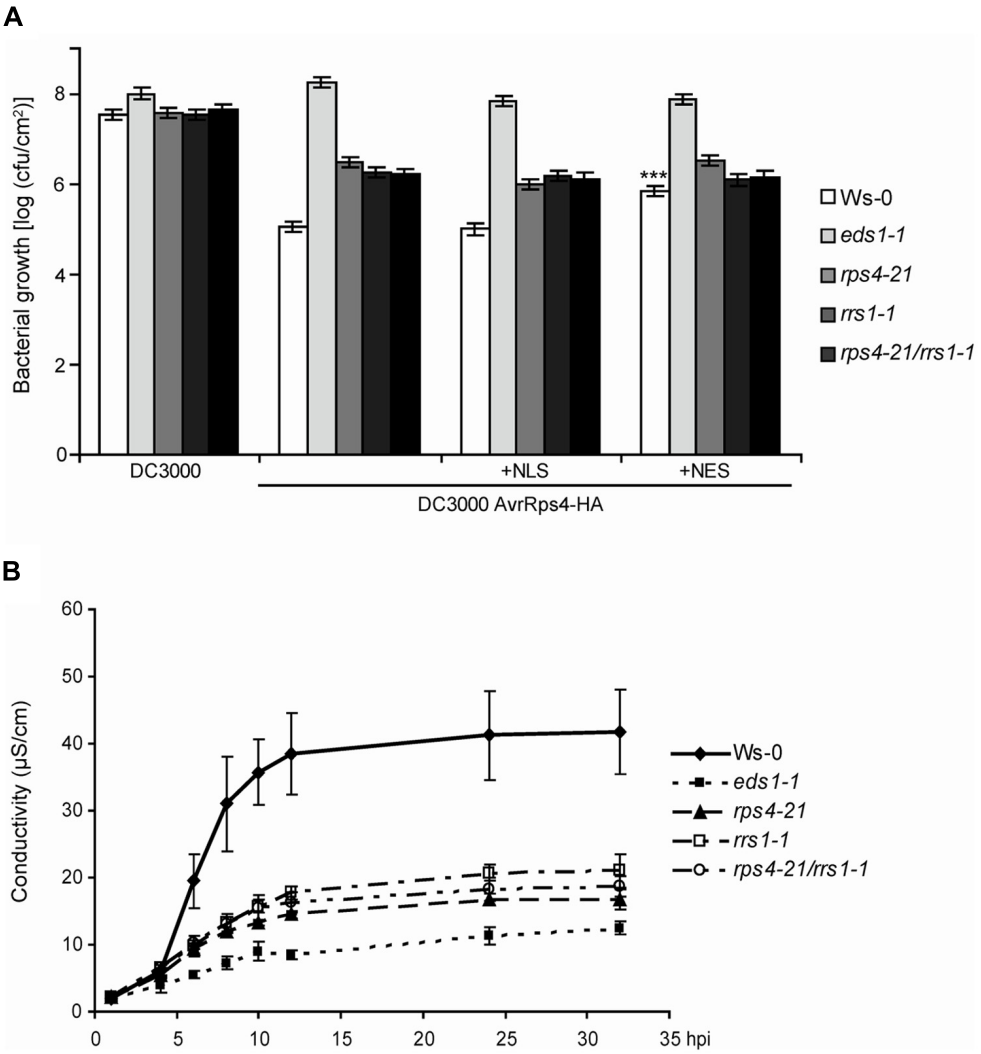


FIGURE 1 | *RPS4*^{Ws} and *RRS1*^{Ws} act cooperatively in AvrRps4-triggered bacterial resistance and pcd. (A) Four-week-old plants were spray-inoculated with virulent *Pst* DC3000 or *Pst* expressing AvrRps4-HA, AvrRps4-HA-NLS, or AvrRps4-HA-NES variants. Bacterial titers at 3 dpi are shown. All bacterial strains had similar entry rates measured at 3 hpi (data not shown). Replicate values were combined from three independent experiments with similar results and SEs calculated using a linear model. ***Significant difference ($p < 0.001$). **(B)** Ion leakage measurements were recorded at the indicated time points in leaf disks of 4-week-old Ws-0, *eds1-1*, *rps4-21*, *rrs1-1*, and *rps4-21 rrs1-1* plants after infiltration with *Pfo*-expressing AvrRps4-HA. Error bars represent standard errors of four samples per genotype. The experiment was performed three times with similar results.

RRS1^{Ws} dual resistance system also operates against spray-inoculated *Pst*/AvrRps4 which enter leaves through stomata. Suspensions of *Pst*/AvrRps4 were sprayed onto wild-type Ws-0, Ws *eds1-1*, the single Ws *rps4-21* and *rrs1-1* T-DNA insertion mutants or the *rps4-21 rrs1-1* double-mutant (Narusaka et al., 2009), and bacterial growth measured in leaves. At 3 h post-inoculation, titers of all bacterial strains were similar ($\sim 5 \times 10^3$ cfu/cm^2). At 3 days post-inoculation (dpi), the *rps4-21 rrs1-1* double-mutant line displayed the same level of intermediate resistance as each *rps4-21* and *rrs1-1* single mutant, lying between fully resistant Ws-0 and fully susceptible *eds1-1* plants (**Figure 1A**). Therefore, *RPS4*^{Ws} and *RRS1*^{Ws} dual resistance to *Pst*/AvrRps4 also operates after bacterial infection through leaf stomata. Residual *EDS1*-dependent resistance in *rps4-21 rrs1-1* to *Pst*/AvrRps4 infection (**Figure 1A**) is conferred by an *RPS4*- and *RRS1*-independent mechanism operating in Ws-0 and likely also in accession Col-0 expressing the respective *RPS4*^{Col} and *RRS1*^{Col} allelic variants (Birker et al., 2009; Sohn et al., 2012). We showed previously that resistance in Ws-0 and Col-0 to *Pst*/AvrRps4 could be effectively triggered by an AvrRps4-HA-NLS form targeted to nuclei and that this also required RPS4^{Col} nuclear accumulation (Heidrich et al., 2011). By contrast, enhanced nuclear export of AvrRps4-HA fused to a nuclear export sequence (AvrRps4-HA-NES) triggered low resistance but was able to trigger some pcd. Spray-inoculation of *Pst*-delivered AvrRps4-HA-NLS or AvrRps4-HA-NES variants (Heidrich et al., 2011) did not alter the partial resistance phenotype of the *rps4-21* and *rrs1-1* single or *rps4-21 rrs1-1*

double mutant lines (**Figure 1A**). Therefore, forced AvrRps4 localization to the nucleus or the cytoplasm does not alleviate the requirement for $RPS4^{Ws}$ or $RRS1^{Ws}$ in limiting bacterial infection or the extent of residual *RPS4* and *RRS1*-independent resistance.

Delivery of AvrRps4 from a non-infectious *Pfo* strain infiltrated into Ws-0 leaves triggers a strong macroscopic hypersensitive response (HR) which is abolished in Ws *eds1-1* mutant plants and reduced in *rps4-21* or *rrs1-1* mutants (Heidrich et al., 2011; Sohn et al., 2012). Resistance to *Pst*/AvrRps4 growth in *Arabidopsis* accession Col-0 is somewhat higher than in Ws-0 and depends on both the $RPS4^{Col}$ and $RRS1^{Col}$ allelic forms (Birker et al., 2009) but is accompanied by an extremely weak HR to *Pfo*/AvrRps4 (Heidrich et al., 2011; Sohn et al., 2012). Sohn et al. (2012) further showed that Col-0 transformed with a FLAG-tagged $RRS1^{Ws}$ transgene reconstituted a strong HR to infiltrated *Pfo*/AvrRps4, suggesting that $RRS1^{Ws}$ is a major determinant of AvrRps4-triggered pcd in Ws-0 or is able to boost the existing $RPS4^{Col}$/$RRS1^{Col}$ low-level pcd response. We performed a quantitative ion leakage assay over 36 h in leaves of Ws-0, the *rps4-21* and *rrs1-1* single mutants, and *rps4-21 rrs1-1* double mutants after leaf infiltration of *Pfo*/AvrRps4. Ws *eds1-1* mutant leaves were infiltrated alongside as a non-responding control. As shown previously (Heidrich et al., 2011), Ws-0 leaves produced a rapid HR reaching a peak at 12–16 h after infiltration, whereas *eds1-1* leaves produced base line conductivity of ∼10 μS/cm over the ion leakage time course (**Figure 1B**). Responses of the single and double *rps4-21 rrs1-1* mutants were all intermediate between Ws-0 and *eds1-1* (**Figure 1B**). Therefore, there is genetic cooperativity between $RPS4^{Ws}$ and $RRS1^{Ws}$ in eliciting host pcd and in partially restricting to *Pst*/AvrRps4 bacterial growth.

$RRS1^{Col}$ CONTRIBUTES TO AUTO-ACTIVATED $RPS4^{Col}$ PLANT STUNTING AND IMMUNITY

We reported that a Col-0 line constitutively expressing functional HA-StrepII-tagged genomic $RPS4^{Col}$ under control of the CaMV *35S* promoter (referred to here as *35S:RPS4-HS*) exhibits *EDS1*-dependent auto-immunity and stunting at 22°C (Wirthmueller et al., 2007; Heidrich et al., 2011). Given the tight functional relationship between the $RPS4^{Ws}$ and $RRS1^{Ws}$ allelic pairs in accession Ws-0, and presumably between $RPS4^{Col}$ and $RRS1^{Col}$ in Col-0 for resistance to *Pst*/AvrRps4, we investigated whether $RRS1^{Col}$ also has a role in *35S:RPS4-HS*-triggered auto-immunity. A Col *rrs1* null mutant allele (*rrs1-11*; Birker et al., 2009) was crossed into the *35S:RPS4-HS* background and a line selected that was homozygous for the *35S:RPS4-HS* transgene and *rrs1-11*. The same *35S:RPS4-HS* line crossed into a Col *eds1-2* null mutant was used as a control with suppressed *RPS4* auto-immunity. As anticipated, *35S:RPS4-HS* plants were severely stunted after 3–4 weeks growth and *35S:RPS4-HS eds1-2* plants exhibited no growth inhibition at 22°C (**Figures 2A,B**). Steady-state RPS4-HS protein accumulation in *35S:RPS4-HS eds1-2* was slightly reduced compared to the *35S:RPS4-HS* line (**Figure 2C**). Mutation of $RRS1^{Col}$ caused intermediate *35S:RPS4-HS* stunting at 22°C (**Figures 2A,B**) but did not affect RPS4-HS accumulation (**Figure 2C**). Therefore, $RRS1^{Col}$ contributes positively to $RPS4^{Col}$ auto-immunity at the level of plant growth inhibition. We concluded that the $RRS1^{Col}$ protein likely plays a role in resistance signaling triggered by an auto-activated $RPS4^{Col}$ receptor, besides its presumed role in AvrRps4 recognition (Birker et al., 2009; Narusaka et al., 2009).

We then tested whether *35S:RPS4-HS* plants grown at 22°C display enhanced basal resistance to virulent *Pst* strain DC3000 and the influence of *rrs1-11* compared to *eds1-2* on the *35S:RPS4-HS* basal resistance phenotype. Col-0 wild-type, *eds1-2*, and *rrs1-11* plants were grown alongside *35S:RPS4-HS*, *35S:RPS4-HS eds1-2*, and *35S:RPS4-HS rrs1-11* plants for 3.5 weeks at 22°C and then spray-inoculated with *Pst* DC3000 for bacterial growth assays. The *rrs1-11* mutant supported similar *Pst* DC3000 growth as Col-0 wild type (**Figure 3A**) and therefore did not exhibit an enhanced disease susceptibility phenotype (which would be indicative of a loss of basal resistance), in contrast to *eds1-2* (**Figure 3A**). The *35S:RPS4-HS* plants exhibited strongly enhanced basal resistance to *Pst* DC3000 which was abolished by *eds1-2* and partially suppressed by *rrs1-11* (**Figure 3A**). We concluded that auto-immunity exhibited by *35S:RPS4-HS* at 22°C involves $RRS1^{Col}$ for enhancing *EDS1*-dependent basal resistance responses.

We spray-inoculated the same set of plants with *Pst*/AvrRps4 and found that the high basal resistance in *35S:RPS4-HS* (see **Figure 3A**) was slightly increased by AvrRps4 and was also fully *EDS1*-dependent (**Figure 3B**). The *35S:RPS4-HS rrs1-11* plants displayed intermediate loss of resistance to *Pst*/AvrRps4 (**Figure 3B**), suggesting that an $RPS4^{Col}$ $RRS1^{Col}$-independent mechanism also plays a role in *35S:RPS4-HS* immunity to *Pst*/AvrRps4. The results show that $RRS1^{Col}$ contributes to $RPS4^{Col}$ auto-immunity. In genetically recruiting *EDS1* and $RRS1^{Col}$, while retaining an $RRS1^{Col}$-independent resistance component (**Figure 3B**), we reasoned that the *35S:RPS4-HS* auto-activated immune system might be useful for measuring *RPS4*/*RRS1*-triggered defense pathway transcription dynamics without needing to infect with the pathogen.

A HIGH TO LOW TEMPERATURE SHIFT INDUCES *35S:RPS4-HS* AUTO-IMMUNITY

In *Arabidopsis*, suppression of basal and effector-triggered TNL immunity at high temperature (>25°C) is associated with lowered expression of defense pathway genes, including *EDS1*, and reduced feed-forward defense amplification (Yang and Hua, 2004; Wang et al., 2009). We therefore investigated whether shifting plants from high temperature (28°C, non-permissive for *Arabidopsis* TNL resistance) to a lower temperature (19–22°C, permissive for TNL resistance) could be used to turn on *RPS4* auto-immunity synchronously in leaf tissues.

The *35S:RPS4-HS* plants grew similarly to wild type Col-0 at 28°C (**Figure 4A**) and showed no constitutive defense gene expression (**Figure 4B**). Moving *35S:RPS4-HS* plants from 28 to 19°C induced expression of *EDS1* itself and several known *Pst*/AvrRps4-responsive, *EDS1*-dependent defense-related genes (Bartsch et al., 2006) at 4 and 6 h post-temperature shift (hps; **Figure 4B**). Col-0 wild type and *35S:RPS4-HS eds1-2* plants subjected to the same temperature change did not show induction of these genes at 4 and 6 hps (**Figure 4B**). In multiple repeats, the 28 to 19°C temperature shift proved to be an easy and highly reproducible *EDS1*-requiring defense gene inductive switch for *35S:RPS4-HS* plants.

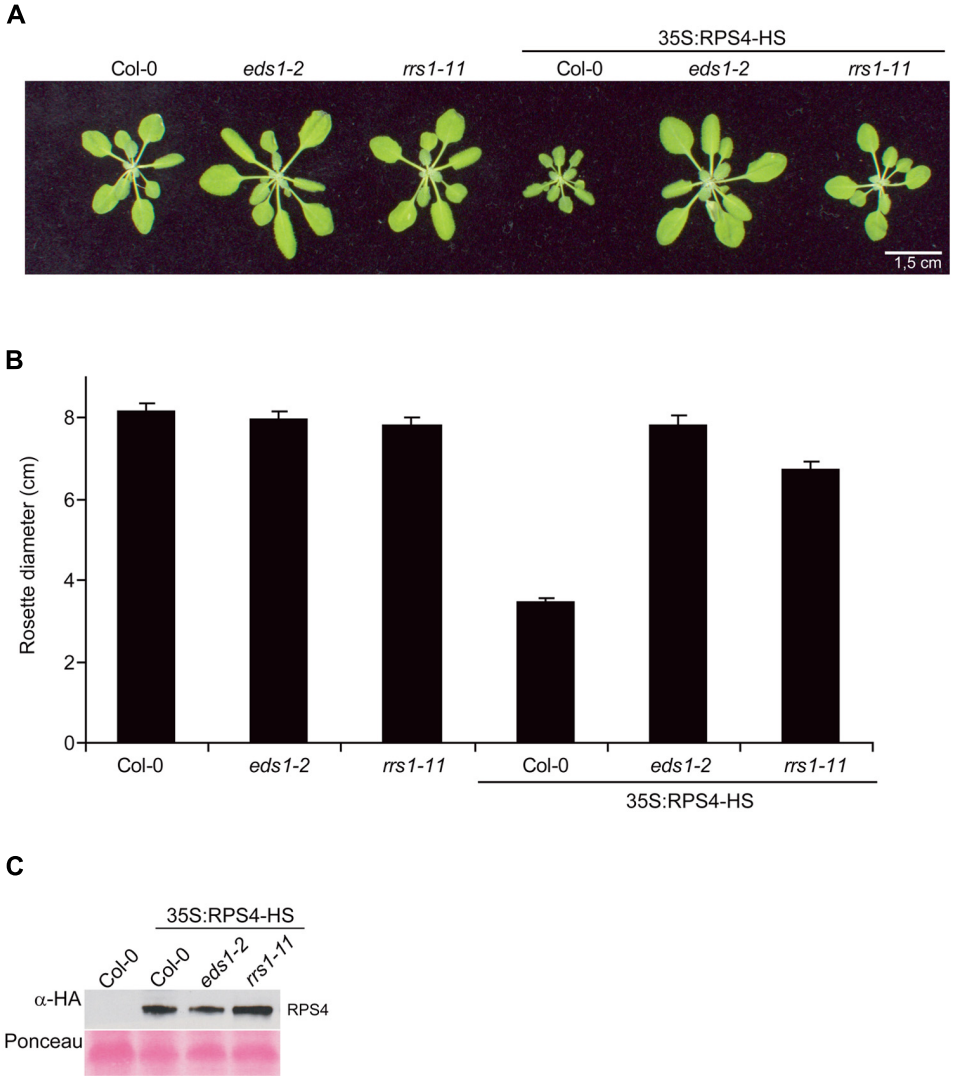


FIGURE 2 | Mutation of *RRS1^{Col}* partially suppresses *35S:RPS4-HS* stunting. (A) Growth at 22°C of representative 3.5-week-old Col-0, *eds1-2*, and *rrs1-11* and the same backgrounds containing the *35S:RPS4-HS* transgene. Scale bar, 1.5 cm. **(B)** Quantification of rosette diameters at 3.5 weeks of lines shown in **(A)**. **(C)** Immunoblot analysis of total leaf protein extracts separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) from the 3.5-week-old *35S:RPS4-HS* transgenic leaf tissues in Col-0, *eds1-2*, and *rrs1-11* backgrounds, probed with α-HA antibody. Ponceau S staining shows equal transfer of protein samples to the membrane. Two independent experiments gave similar results.

Macroscopic symptoms of auto-immunity were first seen as leaf chlorosis in *35S:RPS4-HS* plants, starting at 3–4 days after the 28 to 19°C temperature shift and showing complete *EDS1*-dependence (**Figure 4A**). In conductivity assays for cell death, ion leakage from *35S:RPS4-HS* leaf disks started to rise significantly between 4 and 6 days post-shift (dps) but did not increase in *35S:RPS4-HS eds1-2* or wild-type Col-0 (**Figure 4C**). We tested the *35S:RPS4-HS rrs1-11* line under the same conditions and found that progression of leaf chlorosis (**Figure 4A**) and ion leakage (**Figure 4C**) was intermediate between *35S:RPS4-HS* and *35S:RPS4-HS eds1-2* plants. Steady-state RPS4-HS protein accumulation was not strongly affected by temperature or the *rrs1-11* mutation, but was slightly lower in *eds1-2* at 8 h after temperature shift (**Figure 4D**). Collectively, these data show that *RRS1^{Col}* contributes to temperature-conditioned *35S:RPS4-HS* auto-immunity at the level of leaf chlorosis and pcd.

RRS1^{Col} SUPPORTS TRANSCRIPTIONAL REPROGRAMING OF A DISCRETE SET OF *EDS1*-DEPENDENT GENES IN TEMPERATURE-SHIFTED *35:RPS4-HS* PLANTS

In the above assays, we established that *35S:RPS4-HS* 28/19°C-shifted leaf tissues resemble *Pst*/AvrRps4-infected plants at 22°C with respect to complete *EDS1*- and partial *RRS1^{Col}*-dependence in chlorotic and pcd phenotypes. However, the temperature shift will have physiological effects unrelated to immunity (Penfield, 2008; McClung and Davis, 2010). We therefore performed gene expression microarray analysis of *35S:RPS4-HS*, *35S:RPS4-HS rrs1-11*, and *35S:RPS4-HS eds1-2* leaf mRNAs at 0 h

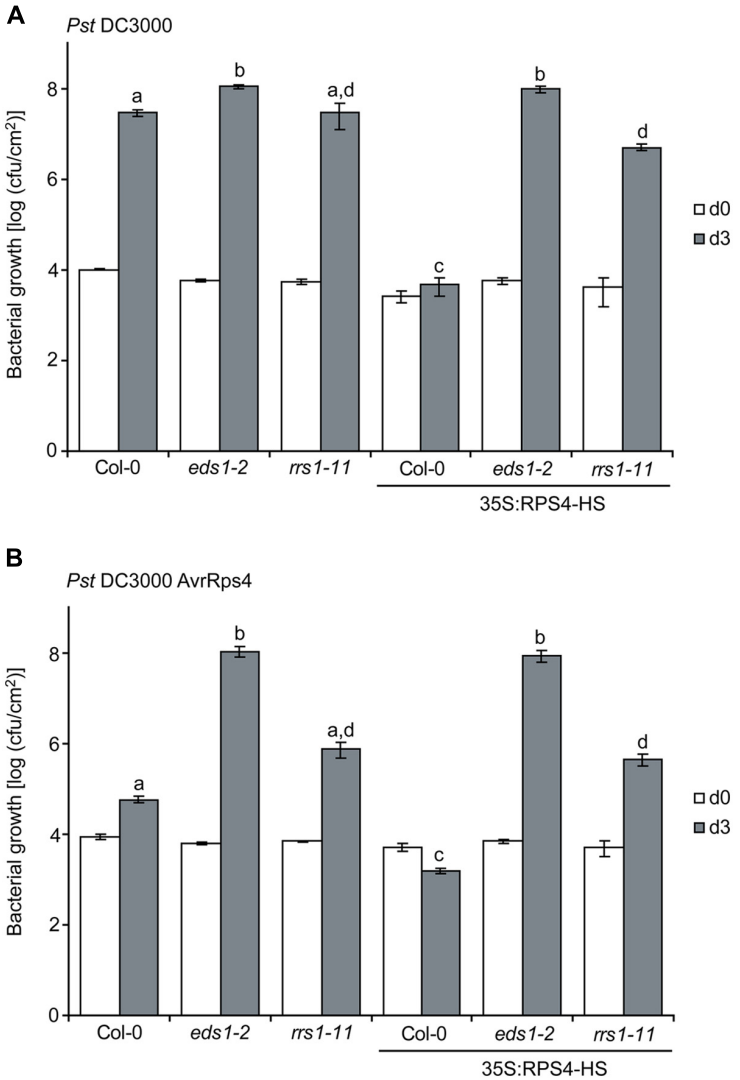


FIGURE 3 | *RRS1^{Col}* contributes to enhanced basal and AvrRps4-triggered resistance of *35S:RPS4-HS* at 22°C. 3.5-week-old plants of the indicated lines grown at 22°C were spray-inoculated with virulent *Pst* DC3000 **(A)** or avirulent *Pst*/AvrRps4 **(B)** bacteria in the same experiment. Bacterial titers were measured at 3 hpi (d0) indicating bacterial entry rates and at 3 dpi (d3). Standard errors were calculated from three biological samples per genotype. Letters (a,b,c,d) indicate significant differences ($p < 0.05$) calculated by a Student's *t*-test. Experiments were performed independently three times with similar results.

(28°C), 2, 8, and 24 hps to 19°C in order to determine the relative contributions of RRS1^{Col} and EDS1 to temperature-conditioned *35S:RPS4-HS* transcriptional reprograming. Profiling of polyA$^+$ RNAs was performed using Affymetrix ATH1 GeneChips (see Materials and Methods). We first selected genes whose expression was significantly up- or down-regulated (*q*-values < 0.01 and >2-fold change) in *35S:RPS4-HS* over all time points compared to non-shifted *35S:RPS4-HS* plants at 28°C (t0; 10277 genes in total). Hence, there is extensive reprograming of transcription in *35S:RPS4-HS* leaves over 24 hps. We then compared the global gene expression profiles of *35S:RPS4-HS*, *35S:RPS4-HS rrs1-11*, and *35S:RPS4-HS eds1-2* at 0, 2, 8, and 24 hps by plotting changed transcripts in *35S:RPS4-HS rrs1-11* or *35S:RPS4-HS eds1-2* on a linear regression curve (red) against the regression curve set by *35S:RPS4-HS* transcript changes (black; **Figure 5A**). This analysis shows that expression changes in *35S:RPS4-HS rrs1-11* broadly resemble those of *35S:RPS4-HS* over the 24 h time course (**Figure 5A**). Therefore, loss of RRS1^{Col} function has little effect on RPS4-HS transcriptional reprograming overall. Many gene expression changes in *35S:RPS4-HS* at 2 hps (80%) were also similar in *35S:RPS4-HS eds1-2*, as seen by the near congruence of the red and black regression curves (**Figure 5A**). A measurable impact of *eds1-2* on expression changes in *35S:RPS4-HS* was observed at 8 and 24 hps, with most differences between the two lines established already at 8 hps (**Figure 5A**). These data show that EDS1 contributes substantially to RPS4-HS-triggered transcriptional reprograming following an early EDS1-independent phase that is likely due to the temperature shift *per se* and not

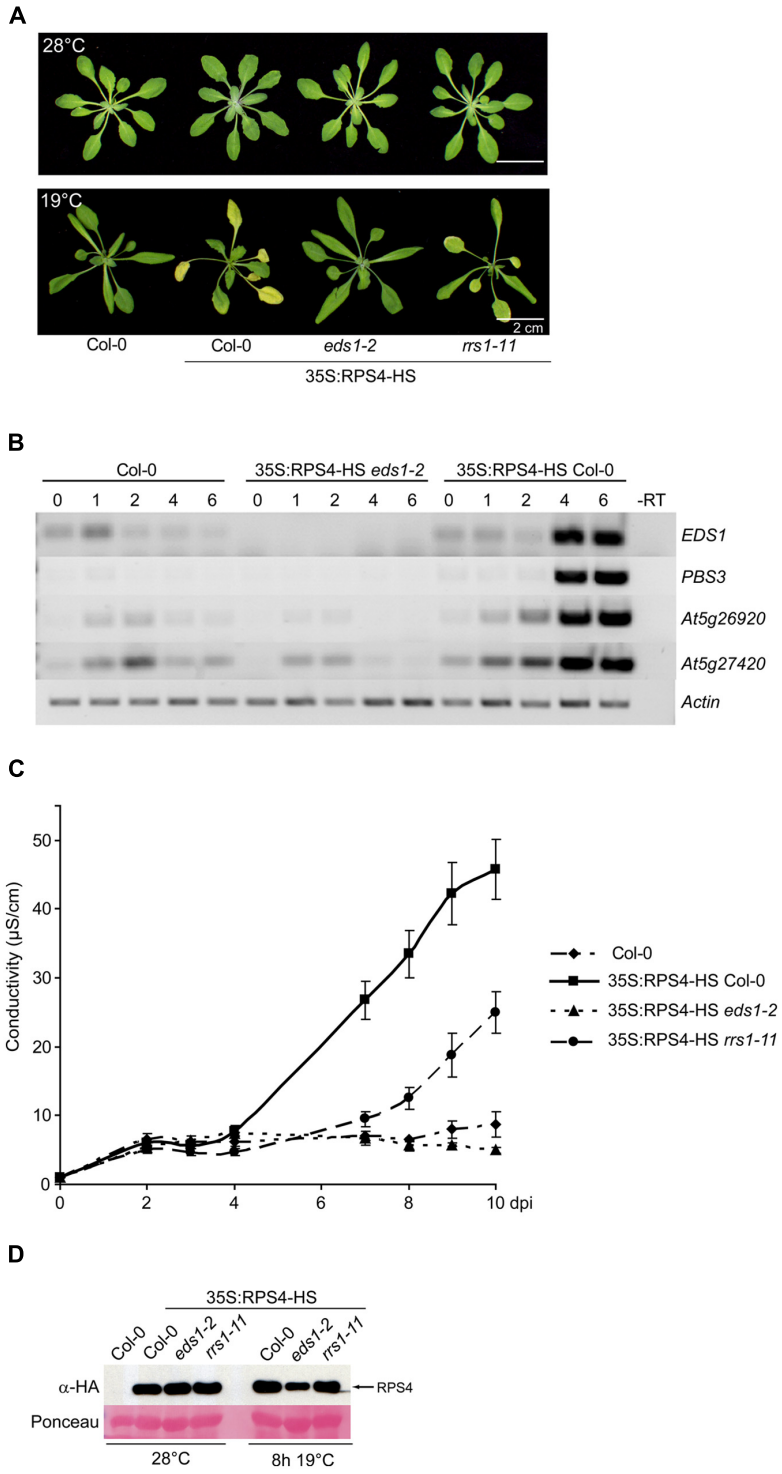
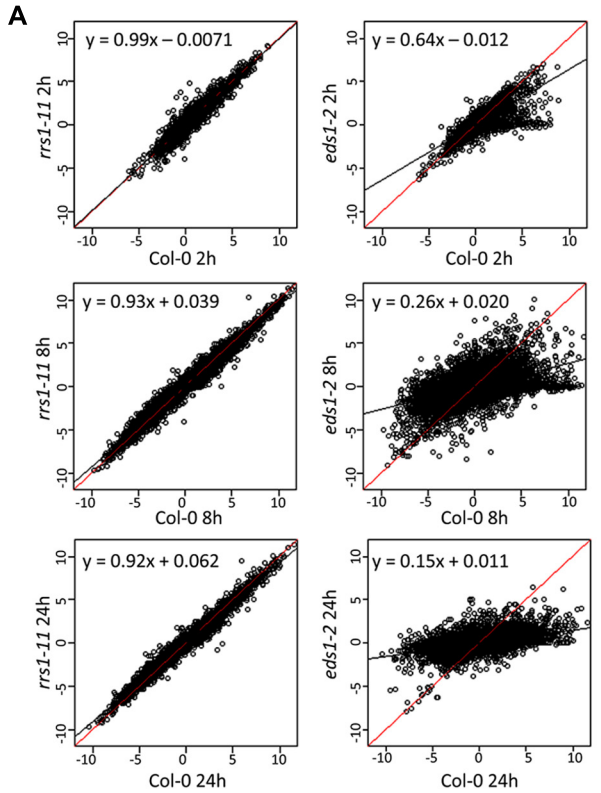
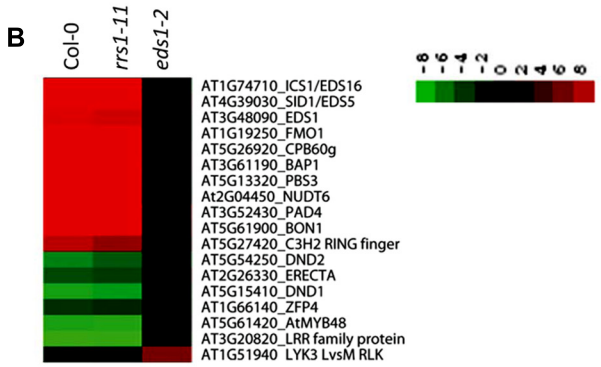
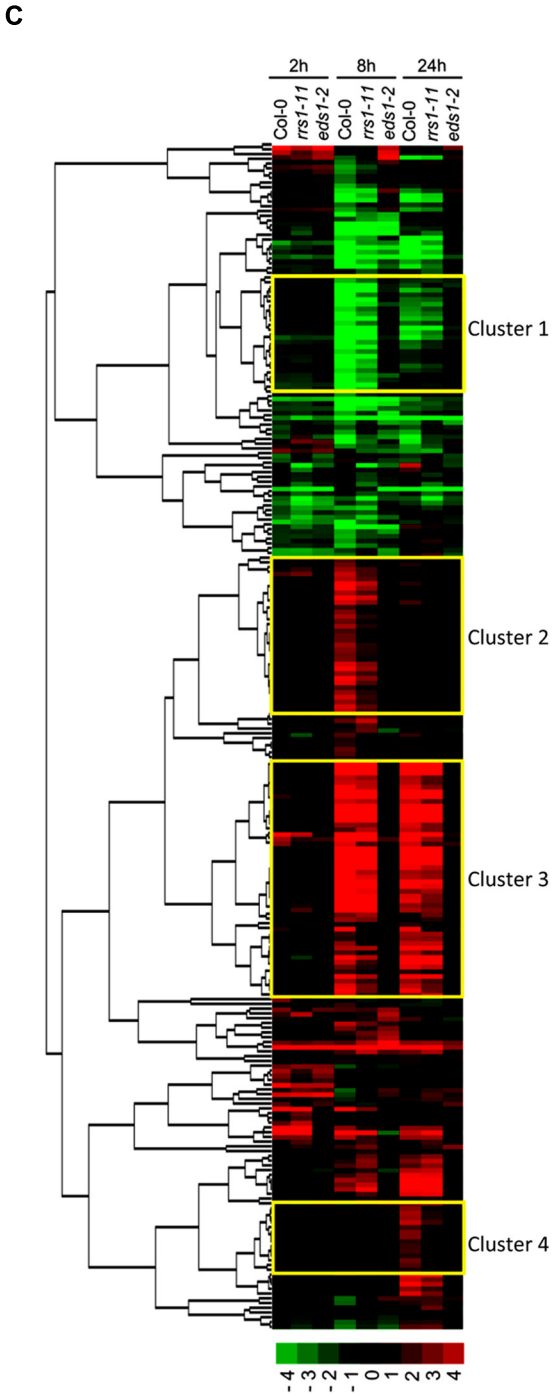


FIGURE 4 | A 28 to 19°C temperature shift induces *RPS4-HS* auto-immunity. (A) Growth of 3.5-week-old *35S:RPS4-HS* plants at 28°C (upper panel) and 6 days after moving to 19°C (lower panel). Scale bars, 2 cm. **(B)** Semi-quantitative RT-PCR of known *Pst*/AvrRps4-responsive, *EDS1*-dependent genes over 0–6 h after temperature shift of Col-0, *35S:RPS4-HS eds1-2*, and *35S:RPS4-HS* Col-0 plants, as indicated. **(C)** Ion leakage measurements made over a 10-day period after shift from high to low temperature (dps) in leaf disks of the different 3.5-week-old *35S:RPS4-HS* lines and Col-0 wild-type, as indicated. Error bars represent standard errors of four samples per genotype. Three independent experiments gave similar results. **(D)** Immunoblot analysis of total leaf protein extracts from 3.5-week-old *35S:RPS4-HS* lines grown at 28°C and shifted to 19°C for 8 h, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and probed with α-HA antibody. Ponceau S staining shows equal transfer of protein samples to the membrane.







D

GO term	p value	FDR
response to chemical stimulus	8.2 e-06	0.0022
response to organic substance	2.5 e-05	0.0034
response to stimulus	9.7 e-05	0.0089
response to hormone stimulus	0.00037	0.026
response to endogenous stimulus	0.0007	0.034

FIGURE 5 | *RRS1^{Col}* contributes to *EDS1*-dependent gene expression changes in *RPS4^{Col}* auto-immunity. Gene expression microarray analysis was performed on leaf RNAs of 3.5-week-old *35S:RPS4-HS* transgenic plants in the Col-0, *rrs1-11*, or *eds1-2* backgrounds at 0, 2, 8, and 24 h after temperature shift. **(A)** Induced or repressed genes (q-values < 0.01 and >2-fold changes) in *35S:RPS4-HS* Col-0 upon temperature shift at any time point and the $\log_2$ ratios compared to 0 h are plotted. Linear regression lines indicate $\log_2$ ratios in *35S:RPS4-HS* Col-0 (red) and the *35S:RPS4-HS rrs1-11* or *35S:RPS4-HS eds1-2* mutant lines (black). **(B)** $\log_2$ gene expression ratios at 8 h after temperature shift compared to 0 h in *35S:RPS4-HS* Col-0 plants for previously identified *Pst*/AvrRps4-triggered *EDS1*-dependent genes, shown by Heatmap clustering analysis. **(C)** Heatmap clustering of 250 genes whose expression changes are *RRS1*-dependent in *35S:RPS4-HS* after temperature shift at any time point (q-values < 0.01 and >2-fold change). Expression patterns for the 250 genes in *35S:RPS4-HS* Col-0, *35S:RPS4-HS rrs1-11*, and *35S:RPS4-HS eds1-2* lines are shown at 2, 8, and 24 h. Highlighted clusters 1–4 are described in the text. **(D)** GO term analysis of the 250 *RRS1*-dependent genes.

directly related to *RPS4* auto-immunity. We then selected a sample of defense-related genes whose up- or down-regulation was established in a previous gene expression microarray study as *EDS1*- and *PAD4*-dependent at 6 h after leaf infiltration with *Pst*/AvrRps4 bacteria at 22°C (Bartsch et al., 2006; Zhu et al., 2010b). The pattern of AvrRps4-triggered induction or repression of the genes was recapitulated at 8 h post-temperature shift in *35S:RPS4-HS* and *35S:RPS4-HS rrs1-11* and was strongly *EDS1*-dependent, as shown in a heatmap (**Figure 5B**). This suggests that major defense-related transcriptional changes requiring *EDS1* in *Pst*/AvrRps4-infected tissues are qualitatively similar at 8 hps in the temperature-conditioned *RPS4* auto-immune response.

We investigated whether a subset of the total 10227 genes exhibiting changed expression over the *35S:RPS4-HS* temperature shift experiment was affected by *rrs1-11* by selecting genes whose up- or down-regulation showed dependence on $RRS1^{Col}$ for at least one time point (q-values < 0.01 and >2-fold change). Altogether, 250 genes fitted this pattern with most showing reduced up-regulation in *35S:RPS4-HS rrs1-11* tissues compared to *35S:RPS4-HS*. The 250 genes displayed partial $RRS1^{Col}$- and strong *EDS1*-dependence for expression changes, as shown in the heatmap (**Figure 5C**). Hence, the effect of the *rrs1-11* mutation is mainly quantitative in the *35S:RPS4-HS* temperature-conditioned system. Analysis of GO terms enriched among the 250 genes shows a high representation of genes responsive to chemical, hormone, and other endogenous stimuli (**Figure 5D**). In a clustering analysis of the 250 "$RRS1^{Col}$-dependent" genes (see Materials and Methods), four gene clusters were of interest (**Figure 5C**). In Cluster 1, genes are grouped that show $RRS1^{Col}$-dependent repression at 8 and 24 h. Cluster 2 contains genes that are up-regulated at 8 hps and show an $RRS1^{Col}$ contribution to induction. Cluster 3 has genes up-regulated at 8 and 24 hps and showing $RRS1^{Col}$-dependence at both time points. In Cluster 4, a discrete set of genes displaying $RRS1^{Col}$-dependence in up-regulation at 24 hps is displayed. Interestingly, distinct sub-clusters of genes with strong $RRS1^{Col}$-dependence are observed within Clusters 3 and 4 (**Figure 5C**). We concluded that $RRS1^{Col}$ has a measurable positive effect on expression of a subset of *EDS1*-dependent genes in *35S:RPS4-HS* auto-immunity.

Because $RRS1^{Col}$ encodes a functional TNL receptor with a C-terminal "WRKY" transcription factor DNA-binding domain recognizing W-box elements, we investigated if W-box cis-elements are enriched in the promoters of the 250 $RRS1^{Col}$-dependent genes. As shown in **Figure 6**, analysis of the core W-box motif (TTGACY) in promoters of these genes by POBO (Materials and Methods) shows that enrichment of this motif is highly significant (p-value < 0.0001) compared to randomly selected promoters from all *Arabidopsis* genes. Since the W-box is known to be enriched in promoters of genes that are responsive to biotic stresses (Rushton et al., 2010), we also compared W-box enrichment between promoters of the 250 $RRS1^{Col}$-dependent genes and promoters from randomly selected *35S:RPS4-HS*-regulated genes. The POBO analysis showed that W-boxes remain significantly enriched (p-value < 0.0001) in the promoters of the $RRS1^{Col}$-dependent genes (**Figure 6**). These results suggest that $RRS1^{Col}$ acts on a subset of *35S:RPS4-HS* reprogramed genes directly or indirectly through the presence of W-box elements in their gene promoters.

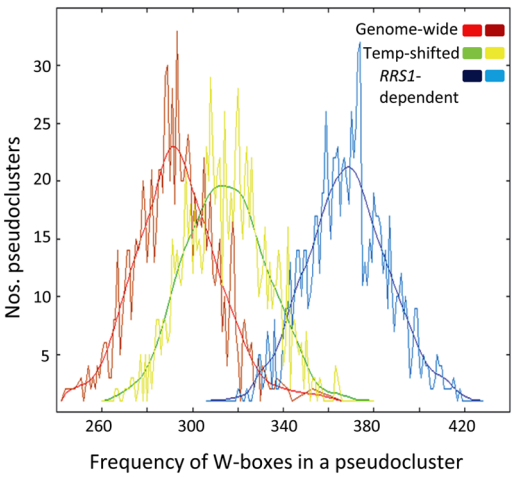


FIGURE 6 | W-boxes are highly enriched in promoters of *RRS1*-dependent genes. POBO analysis of the motif distribution in 1000 bp promoters of *RRS1*-dependent genes. One thousand pseudo-clusters of the 250 *RRS1*-dependent genes, genes regulated by the temperature shift (Temp-shifted) and randomly selected genes from *35S:RPS4-HS* (Genome-wide) are shown. Jagged lines indicate motif frequencies from which a fitted curve was derived. The W-box (TTGACY) is significantly over-represented in promoters of the *RRS1*-dependent genes compared to temperature-responsive genes or genes from the genome background with p-values < 0.0001.

DISCUSSION

NLR receptors are usually activated upon specific pathogen effector recognition to trigger a timely and balanced innate immune response. In the absence of a corresponding effector, tight regulation of NLR receptors is enforced by restricting *NLR* gene expression and ensuring NLR associations with inhibitory co-factors (Heidrich et al., 2012; Staiger et al., 2013). Auto-immunity producing stunting and constitutive activation of resistance and cell death pathways can occur when NLRs are released from inhibition either by NLR over expression or loss-of-function mutations in negative factors (Heidrich et al., 2012; Staiger et al., 2013). An outstanding question is to what extent auto-activated NLR processes mirror those triggered by authentic effector recognition. For TNLs there is compelling evidence that auto-activated receptors connect immediately to a *bona fide* TNL resistance signaling pathway involving the basal resistance regulator EDS1 (Zhang et al., 2003; Yang and Hua, 2004; Wirthmueller et al., 2007; Huang et al., 2010). Detection of EDS1 in complexes with several NLRs (Bhattacharjee et al., 2011; Heidrich et al., 2011; Kim et al., 2012) is also consistent with EDS1 being an integral and early component of TNL resistance. Thus, effector- and auto-activated TNL signaling steps are likely to be related, although constitutive resistance clearly has deleterious pleiotropic effects on growth and development.

Here we provide evidence that *EDS1*-dependent auto-immunity in an *Arabidopsis* $RPS4^{Col}$ over-expression line (*35S:RPS4-HS*) has a partial requirement for $RRS1^{Col}$, the genetic

partner of *RPS4*^{Col} in ETI (Birker et al., 2009; Narusaka et al., 2009). This partial dependence on *RRS1*^{Col} is seen in plants grown at 22°C that exhibit constitutive basal resistance (**Figure 3**) and after shifting plants from high (28°C) to moderate (19°C) temperature to induce defense-related transcriptional reprograming, chlorosis, and pcd (**Figures 4** and **5**). Hence, RPS4 auto-immunity does not fully override a requirement for RRS1. Therefore, we reasoned that the dual RPS4–RRS1 resistance system might involve RPS4–RRS1 cooperation beyond initial effector recognition steps to include aspects of downstream resistance signaling. Alternatively, part of the RPS4 auto-activation mechanism involves processes that also occur during effector activation, such as particular NLR conformational transitions (Collier and Moffett, 2009; Lukasik and Takken, 2009). Reduced *RPS4*^{Col} auto-immunity in *rrs1-11* mirrors the intermediate loss of resistance in *rrs1-11* mutants to *Pst*/AvRps4 bacteria (**Figures 1** and **3**). Therefore, it is possible that in both backgrounds an *RPS4*/*RRS1*-independent pathway contributes to the residual resistance (Birker et al., 2009; Sohn et al., 2012). Although the precise nature of effector- and auto-triggered RPS4–RRS1 activation events needs to be resolved, the fact that temperature-induced RPS4 immunity mirrors ETI in displaying complete dependence on EDS1 and partial dependence on RRS1 is significant. The temperature-conditioned RPS4 auto-immune system presents a potentially powerful tool to examine dynamic TNL signaling and transcriptional events in leaf tissues.

Pairing of *RPS4* and *RRS1* genes and their homologs in a head-to-head tandem arrangement is evolutionarily conserved, underscoring functional significance of the inverted TNL organization (Gassmann et al., 1999; Narusaka et al., 2009). *RRS1*, a representative of the TNL-A clade, exhibits higher sequence diversity among *Arabidopsis* accessions than *RPS4*, as a member of the TNL-B clade (Meyers et al., 2003; Narusaka et al., 2009). This, together with finding that the RRS1 interacts directly with the *R. solanacearum* effector PopP2 inside nuclei points to RRS1 as a direct effector recognition component, although interaction alone is not sufficient for triggering RRS1 resistance (Deslandes et al., 2003; Tasset et al., 2010). Noutoshi et al. (2005) proposed an attractive model for RRS1 "restraint" and activation based on analysis of an auto-activated *slh1* WRKY domain mutation. In the model, RRS1 in non-elicited cells resides at sites on the chromatin as an auto-inhibited NLR. Subsequent studies revealing *RRS1–RPS4* genetic cooperativity in resistance to AvrRps4 and PopP2, and an unknown *Colletotrichum higginsianum* effector (Birker et al., 2009; Narusaka et al., 2009), raised the prospect that effector recognition might be conferred by an auto-inhibited RPS4–RRS1 complex which becomes activated via RPS4–RRS1 conformational changes at the chromatin. Because our data indicate that RRS1 contributes to RPS4 auto-immunity, we propose that signaling events also involve RRS1 with RPS4, as well as EDS1, in what might be a "reconfigured" receptor complex, possibly mediated through TIR–TIR interactions (Mestre and Baulcombe, 2006; Bernoux et al., 2011b). The fact that neither *rrs1* nor *rps4* null mutant displays constitutive resistance also argues against resistance pathway activation simply being due to release of one or other component from an auto-inhibited complex. An interesting but complicating issue is that EDS1 was found to interact with the AvrRps4 effector in FRET–FLIM and co-immunoprecipitation studies, implying that EDS1 contributes to effector recognition as well as being an integral component of the TNL resistance pathways (Bhattacharjee et al., 2011; Heidrich et al., 2011). Notably, EDS1 interacts with two effectors, AvrRps4 and HopA1, recognized, respectively, by TNLs RPS4/RRS1 and RPS6 (Bhattacharjee et al., 2011; Heidrich et al., 2011). Thus, TNL pre- and post-activation events in these recognition systems might be closely intertwined.

Temperature-induced *RPS4* auto-immunity produces an exaggerated transcriptional response compared to ETI probably through an *EDS1*-dependent transcriptional feed-forward loop (Wang et al., 2009; Zhu et al., 2010a). At 2 h post-temperature shift, analysis of the gene expression microarray data revealed mainly *EDS1*-independent transcriptional reprograming of *35S:RPS4-HS* plants which we attribute to a "temperature" effect (**Figure 5A**). The small sector (20%) of *EDS1*-dependent changes at 2 h will be examined in a more detailed expression time series over 1–4 h to identify earliest *EDS1* and, potentially, *RRS1* effects. At 8 h after temperature shift, transcriptional reprograming was largely *EDS1*-dependent (**Figure 5**) and qualitatively similar to ETI for a panel of AvrRps4-triggered *EDS1*-dependent induced and repressed genes (**Figure 5B**). A quantitative contribution of *RRS1* was detected also at 8 and 24 h after temperature shift in 250 of the *EDS1*-dependent down and up-regulated genes (**Figure 5**). An auxiliary role of RRS1 in EDS1-mediated gene expression is reminiscent of the contribution of WRKY18 to NPR1-dependent basal defense responses (Wang et al., 2006) and might reflect a common feature of WRKY-containing transcriptional immune regulators. Notably, several sub-clusters within the *RRS1*-dependent genes display strong *RRS1*-dependence in expression at 8 or 24 h (**Figure 5C**; Clusters 3 and 4). Whether any of these genes are direct targets of RRS1 (or RPS4) is not known but the high representation of W-boxes in their promoter elements (**Figure 6**) suggests that WRKY-domain protein recruitment might be an important modulator of expression. Current evidence indicates that the dynamics of WRKY transcription factor binding of promoters are complex and likely to involve reconfigurations from repressive to inductive transcription complexes at the chromatin, as well as functional redundancy between WRKY transcription factors (Rushton et al., 2010; Chen et al., 2013; Logemann et al., 2013; Schon et al., 2013).

CONCLUSION

Our data show that RRS1^{Col} positively contributes to RPS4^{Col} auto-immunity induced by a high to moderate temperature shift. The temperature-activated RPS4 over-expression system can help to illuminate the molecular role of RRS1 in this TNL resistance partnership and the hierarchy of defense-related transcriptional reprograming events.

ACKNOWLEDGMENTS

We thank our colleagues for helpful discussions. This work was supported by The Max-Planck Society, a Deutsche Forschungsgemeinschaft grant in SFB 670 (Jane E. Parker, Katharina Heidrich, Jaqueline Bautor) and a BMBF-funded Plant-KBBE Trilateral "BALANCE" project grant (Jane E. Parker, Servane Blanvillain-Baufumé). Katharina Heidrich was recipient of an IMPRS PhD fellowship.

REFERENCES

Alcazar, R., and Parker, J. E. (2011). The impact of temperature on balancing immune responsiveness and growth in *Arabidopsis*. *Trends Plant Sci.* 16, 666–675. doi: 10.1016/j.tplants.2011.09.001

Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J. L., Bautor, J., et al. (2006). Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in *Arabidopsis* immunity and cell death is regulated by the monooxygenase FMO1 and the Nudix hydrolase NUDT7. *Plant Cell* 18, 1038–1051. doi: 10.1105/tpc.105.039982

Bernoux, M., Ellis, J. G., and Dodds, P. N. (2011a). New insights in plant immunity signaling activation. *Curr. Opin. Plant Biol.* 14, 512–518. doi: 10.1016/j.pbi.2011.05.005

Bernoux, M., Ve, T., Williams, S., Warren, C., Hatters, D., Valkov, E., et al. (2011b). 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. doi: 10.1016/j.chom.2011.02.009

Bhattacharjee, S., Halane, M. K., Kim, S. H., and Gassmann, W. (2011). Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators. *Science* 334, 1405–1408. doi: 10.1126/science.1211592

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. doi: 10.1111/j.1365-313X.2009.03984.x

Chang, C., Yu, D., Jiao, J., Jing, S., Schulze-Lefert, P., and Shen, Q. H. (2013). Barley MLA immune receptors directly interfere with antagonistically acting transcription factors to initiate disease resistance signaling. *Plant Cell* 25, 1158–1173. doi: 10.1105/tpc.113.109942

Chen, L., Zhang, L., Li, D., Wang, F., and Yu, D. (2013). WRKY8 transcription factor functions in the TMV-cg defense response by mediating both abscisic acid and ethylene signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1963–E1971. doi: 10.1073/pnas.1221347110

Collier, S. M., and Moffett, P. (2009). NB-LRRs work a "bait and switch" on pathogens. *Trends Plant Sci.* 14, 521–529. doi: 10.1016/j.tplants.2009.08.001

Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., et al. (2003). Physical interaction between *RRS1*-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8024–8029. doi: 10.1073/pnas.1230660100

Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D. X., Bittner-Eddy, P., et al. (2002). Resistance to *Ralstonia solanacearum* in *Arabidopsis* thaliana is conferred by the recessive *RRS1*-R gene, a member of a novel family of resistance genes. *Proc. Natl. Acad. Sci. U.S.A.* 99, 2404–2409. doi: 10.1073/pnas.032485099

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14863–14868. doi: 10.1073/pnas.95.25.14863

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

Garcia, A. V., Blanvillain-Baufume, S., Huibers, R. P., Wiermer, M., Li, G., Gobbato, E., et al. (2010). Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS Pathog.* 6:e1000970. doi: 10.1371/journal.ppat.1000970

Gassmann, W., Hinsch, M. E., and Staskawicz, B. J. (1999). The *Arabidopsis RPS4* bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. *Plant J.* 20, 265–277. doi: 10.1046/j.1365-313X.1999.t01-1-00600.x

Hajheidari, M., Farrona, S., Huettel, B., Koncz, Z., and Koncz, C. (2012). CDKF-1 and CDKD protein kinases regulate phosphorylation of serine residues in the C-terminal domain of *Arabidopsis* RNA Polymerase II. *Plant Cell* 24, 1626–1642. doi: 10.1105/tpc.112.096834

Heidrich, K., Blanvillain-Baufume, S., and Parker, J. E. (2012). Molecular and spatial constraints on NB-LRR receptor signaling. *Curr. Opin. Plant Biol.* 15, 385–391. doi: 10.1016/j.pbi.2012.03.015

Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L., and Parker, J. E. (2011). *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* 334, 1401–1404. doi: 10.1126/science.1211641

Hinsch, M., and Staskawicz, B. (1996). Identification of a new *Arabidopsis* disease resistance locus, *RPS4*, and cloning of the corresponding avirulence gene, avrRps4, from *Pseudomonas syringae* pv. *pisi*. *Mol. Plant Microbe Interact.* 9, 55–61. doi: 10.1094/MPMI-9-0055

Huang, X., Li, J., Bao, F., Zhang, X., and Yang, S. (2010). A gain-of-function mutation in the *Arabidopsis* disease resistance gene RPP4 confers sensitivity to low temperature. *Plant Physiol.* 154, 796–809. doi: 10.1104/pp.110.157610

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

Kankainen, M., and Holm, L. (2004). POBO, transcription factor binding site verification with bootstrapping. *Nucleic Acids Res.* 32, W222–W229. doi: 10.1093/nar/gkh463

Kim, S. H., Gao, F., Bhattacharjee, S., Adiasor, J. A., Nam, J. C., and Gassmann, W. (2010). The *Arabidopsis* resistance-like gene SNC1 is activated by mutations in SRFR1 and contributes to resistance to the bacterial effector avrRps4. *PLoS Pathog.* 6:e1001172. doi: 10.1371/journal.ppat.1001172

Kim, T. H., Kunz, H. H., Bhattacharjee, S., Hauser, F., Park, J., Engineer, C., et al. (2012). Natural variation in small molecule-induced TIR-NB-LRR signaling induces root growth arrest via EDS1- and PAD4-complexed R protein VICTR in *Arabidopsis*. *Plant Cell* 24, 5177–5192. doi: 10.1105/tpc.112.107235

Logemann, E., Birkenbihl, R. P., Rawat, V., Schneeberger, K., Schmelzer, E., and Somssich, I. E. (2013). Functional dissection of the PROPEP2 and PROPEP3 promoters reveals the importance of WRKY factors in mediating microbe-associated molecular pattern-induced expression. *New Phytol.* 198, 1165–1177. doi: 10.1111/nph.12233

Lukasik, E., and Takken, F. L. W. (2009). STANDing strong, resistance protein instigators of plant defence. *Curr. Opin. Plant Biol.* 12, 427–436. doi: 10.1016/j.pbi.2009.03.001

Maekawa, T., Kufer, T. A., and Schulze-Lefert, P. (2011). NLR functions in plant and animal immune systems: so far and yet so close. *Nat. Immunol.* 12, 817–826. doi: 10.1038/ni.2083

McClung, C. R., and Davis, S. J. (2010). Ambient thermometers in plants: from physiological outputs towards mechanisms of thermal sensing. *Curr. Biol.* 20, R1086–R1092. doi: 10.1016/j.cub.2010.10.035

Mestre, P., and Baulcombe, D. C. (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. *Plant Cell* 18, 491–501. doi: 10.1105/tpc.105.037234

Meyers, B. C., Kozik, A., Griego, A., Kuang, H. H., and Michelmore, R. W. (2003). Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. *Plant Cell* 15, 809–834. doi: 10.1105/tpc.009308

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., et al. (2009). *RRS1* and *RPS4* provide a dual resistance-gene system against fungal and bacterial pathogens. *Plant J.* 60, 218–226. doi: 10.1111/j.1365-313X.2009.03949.x

Noutoshi, Y., Ito, T., Seki, M., Nakashita, H., Yoshida, S., Marco, Y., et al. (2005). A single amino acid insertion in the WRKY domain of the *Arabidopsis* TIR-NBS-LRR-WRKY-type disease resistance protein SLH1 (sensitive to low humidity 1) causes activation of defense responses and hypersensitive cell death. *Plant J.* 43, 873–888. doi: 10.1111/j.1365-313X.2005.02500.x

Padmanabhan, M. S., Ma, S., Burch-Smith, T. M., Czymmek, K., Huijser, P., and Dinesh-Kumar, S. P. (2013). Novel positive regulatory role for the SPL6 transcription factor in N TIR-NB-LRR receptor-mediated plant innate immunity. *PLoS Pathog.* 9:e1003235. doi: 10.1371/journal.ppat.1003235

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

Penfield, S. (2008). Temperature perception and signal transduction in plants. *New Phytol.* 179, 615–628. doi: 10.1111/j.1469-8137.2008.02478.x

Rietz, S., Stamm, A., Malonek, S., Wagner, S., Becker, D., Medina-Escobar, N., et al. (2011). Different roles of enhanced disease susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in *Arabidopsis* immunity. *New Phytol.*

191, 107–119. doi: 10.1111/j.1469-8137.2011.03675.x

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

Schon, M., Toller, A., Diezel, C., Roth, C., Westphal, L., Wiermer, M., et al. (2013). Analyses of wrky18 wrky40 plants reveal critical roles of SA/EDS1 signaling and indole-glucosinolate biosynthesis for *Golovinomyces orontii* resistance and a loss-of resistance towards *Pseudomonas syringae* pv. *tomato avrRPS*4. *Mol. Plant Microbe Interact.* 26, 758–767. doi: 10.1094/MPMI-11-12-0265-R

Sohn, K. H., Hughes, R. K., Piquerez, S. J., Jones, J. D. G., 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. doi: 10.1073/pnas.1212332109

Sohn, K. H., Zhang, Y., and Jones, J. D. G. (2009). The *Pseudomonas syringae* effector protein, avrRPS4, requires in planta processing and the KRVY domain to function. *Plant J.* 57, 1079–1091. doi: 10.1111/j.1365-313X.2008.03751.x

Staiger, D., Korneli, C., Lummer, M., and Navarro, L. (2013). Emerging role for RNA-based regulation in plant immunity. *New Phytol.* 197, 394–404. doi: 10.1111/nph.12022

Storey, J., and Tibshirani, R. (2003). Statistical methods for identifying differentially expressed genes in DNA microarrays. *Methods Mol. Biol.* 224, 149–157.

Tao, Y., Xie, Z. Y., Chen, W. Q., Glazebrook, J., Chang, H. S., Han, B., et al. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15, 317–330. doi: 10.1105/tpc.007591

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

Thomas, W. J., Thireault, C. A., Kimbrel, J. A., and Chang, J. H. (2009). Recombineering and stable integration of the *Pseudomonas syringae* pv. *syringae* 61 hrp/hrc cluster into the genome of the soil bacterium *Pseudomonas fluorescens* Pf0-1. *Plant J.* 60, 919–928. doi: 10.1111/j.1365-313X.2009.03998.x

Tornero, P., and Dangl, J. L. (2001). A high-throughput method for quantifying growth of phytopathogenic bacteria in *Arabidopsis thaliana*. *Plant J.* 28, 475–481. doi: 10.1046/j.1365-313X.2001.01136.x

Venugopal, S. C., Jeong, R.-D., Mandal, M. K., Zhu, S., Chandra-Shekara, A. C., Xia, Y., et al. (2009). Enhanced disease susceptibility 1 and salicylic acid act redundantly to regulate resistance gene-mediated signaling. *PloS Genet.* 5:e1000545. doi: 10.1371/journal.pgen.1000545

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

Wang, Y., Bao, Z., Zhu, Y., and Hua, J. (2009). Analysis of temperature modulation of plant defense against biotrophic microbes. *Mol. Plant Microbe Interact.* 22, 498–506. doi: 10.1094/MPMI-22-5-0498

Wiermer, M., Feys, B. J., and Parker, J. E. (2005). Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* 8, 383–389. doi: 10.1016/j.pbi.2005.05.010

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. doi: 10.1016/j.cub.2007.10.042

Wu, Z. J., and Irizarry, R. A. (2004). Preprocessing of oligonucleotide array data. *Nat. Biotechnol.* 22, 656–658. doi: 10.1038/nbt0604-656b

Yang, S. H., and Hua, J. (2004). A haplotype-specific resistance gene regulated by BONZAI1 mediates temperature-dependent growth control in *Arabidopsis*. *Plant Cell* 16, 1060–1071. doi: 10.1105/tpc.020479

Yue, J.-X., Meyers, B. C., Chen, J.-Q., Tian, D., and Yang, S. (2012). Tracing the origin and evolutionary history of plant nucleotide-binding site-leucine-rich repeat (NBS-LRR) genes. *New Phytol.* 193, 1049–1063. doi: 10.1111/j.1469-8137.2011.04006.x

Zhang, Y. L., Goritschnig, S., Dong, X. N., and Li, X. (2003). A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. *Plant Cell* 15, 2636–2646. doi: 10.1105/tpc.015842

Zhu, Y., Qian, W., and Hua, J. (2010a). Temperature modulates plant defense responses through NB-LRR proteins. *PLoS Pathog.* 6:e1000844. doi: 10.1371/journal.ppat.1000844

Zhu, Z., Xu, F., Zhang, Y., Cheng, Y. T., Wiermer, M., and Li, X. (2010b). *Arabidopsis* resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13960–13965. doi: 10.1073/pnas.1002828107

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: 01 July 2013; accepted: 23 September 2013; published online: 17 October 2013.

Citation: Heidrich K, Tsuda K, Blanvillain-Baufumé S, Wirthmueller L, Bautor J and Parker JE (2013) Arabidopsis TNL-WRKY domain receptor RRS1 contributes to temperature-conditioned RPS4 auto-immunity. Front. Plant Sci. ***4****:403. doi: 10.3389/fpls.2013.00403*

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