Molecules and Cells

Minireview

Role of RIN4 in Regulating PAMP-Triggered Immunity and Effector-Triggered Immunity: Current Status and Future Perspectives

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As sessile organisms, plants have developed sophisticated system to defend themselves against microbial attack. Since plants do not have specialized immune cells, all plant cells appear to have the innate ability to recognize pathogens and turn on an appropriate defense response. The plant innate immune system has two major branches: PAMPs (pathogen associated molecular patterns)-triggered immunity (PTI) and effector-triggered immunity (ETI). The ability to discriminate between self and non-self is a fundamental feature of living organisms, and it is a prerequisite for the activation of plant defenses specific to microbial infection. Arabidopsis cells express receptors that detect extracellular molecules or structures of the microbes, which are called collectively PAMPs and activate PTI. However, nucleotide-binding site leucine-rich repeats (NB-LRR) proteins mediated ETI is induced by direct or indirect recognition of effector molecules encoded by *avr* genes. In Arabidopsis, plasma-membrane localized multifunctional protein RIN4 (RPM1-interacting protein 4) plays important role in both PTI and ETI. Previous studies have suggested that RIN4 functions as a negative regulator of PTI. In addition, many different bacterial effector proteins modify RIN4 to destabilize plant immunity and several NB-LRR proteins, including RPM1 (resistance to *Pseudomonas syringae* pv. *maculicola* 1), RPS2 (resistance to *P. syringae* 2) guard RIN4. This review summarizes the current studies that have described signaling mechanism of RIN4 function, modification of RIN4 by bacterial effectors and different interacting partner of RIN4 in defense related pathway. In addition, the emerging role of the RIN4 in plant physiology and intercellular signaling as it presents in exosomes will be discussed.

Keywords: AvrB, AvrRpm1, AvrRpt2, effector-triggered immunity, PAMP-triggered immunity, RIN4

INTRODUCTION

Various factors affect the coevolution of pathogenic bacteria and plants, such as plant immunity, elicitors, and interactions among pathogen-derived effectors (Song and Ryu, 2018). The first defense mechanism comprises transmembrane pattern recognition receptors (PRRs), which recognize pathogen- or microbial-associated molecular patterns (PAMPs or MAMPs) (Zipfel and Felix, 2005), thereby initiating PAMP/MAMP-triggered immunity (PTI). PTI includes defense gene

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activation, oxidative burst, ion fluxes, callose deposition, hormonal action, stomatal closure, and gene silencing (Nicaise et al., 2009). Pathogens overcome PTI through the use of effectors and toxins. The second defense mechanism, ETI comprises an interplay between pathogen-employed effectors and the polymorphic nucleotide-binding site leucine-rich repeat (NB-LRR) proteins encoded by most *R* genes in plants. This interaction between effectors and NB-LRR proteins could be direct or indirect; for example, the NB-LRR protein could guard an effector target protein (guard model) (Jones and Dangl, 2006); another host protein could evolve with a similar binding site, such as an effector target protein (decoy model) (van der Hoorn and Kamoun, 2008); or NB-LRR may form a complex with an additional protein domain that helps identify pathogen effectors (Sarris et al., 2015), modification of which activates the resistance proteins. Both direct and indirect interactions activate NB-LRR proteins and initiate defense signaling. Many effectors suppress one or more components of PTI to promote nutrient leakage, pathogen dispersal, and bacterial growth (Schulze-Lefert and Panstruga, 2003). The RPM1-interacting protein 4 (RIN4) is a small protein of 211 amino acids (aa) guarded by two resistance proteins, RPM1 and RPS2. RIN4 plays an important role in regulating PTI and ETI. In this mini-review, we discuss interactions of RIN4 with various proteins and the mechanism of RIN4 modification by various effectors. We also speculate the function of RIN4 in PTI, ETI, and intercellular immune signaling.

RIN4 ACTS AS A MOLECULAR SWITCH WHEN REGULATING PTI

It was reported before that RIN4 is a negative regulator of PTI; absence of RIN4 enhances PAMP-induced response (Fig. 1A), whereas *RIN4* overexpression inhibits PTI. RIN4 controls callose deposition upon PAMP perception; no callose deposition is observed with flg22 or *Pto hrcC* infiltration of plants overexpressing RIN4. RIN4-mediated PTI signaling is independent of AvrRpt2, AvrRpm1, RPM1, and RPS2. *Pseudomonas syringae* effectors AvrRpm1 and AvrRpt2 modify RIN4 to inhibit PTI signaling (Fig. 1C) (Kim et al., 2005b). RIN4 derivatives lacking C-terminal Cys residues as well as RIN4 fragments cleaved by AvrRpt2 are hyperactive PTI suppressors (Afzal et al., 2011). The NOI (No_3-induced) domains of RIN4 play important roles in suppressing PTI. RIN4 contains two NOI domains at N-terminal (aa 6-42) and C-terminal (aa 145-187) end of the protein. N-NOI domain contains conserved motif $PxFGxW^{6-12}$ required for effector mediated RIN4 cleavage and C-NOI domain contains two conserved sequence, $Y/FTxxF^{165-169}$ for effector mediated RIN4 phosphorylation and $PxFGxW^{145-156}$ for RIN4 cleavage. RIN4 derivatives lacking both NOI domains are unable to suppress PTI; however, the presence of a single NOI domain was enough to suppress effective defense against *Pto hrcC*, but only the N-NOI domain suppressed flg22-induced callose deposition (Afzal et al., 2011). During pathogen invasion, stomata act as an active regulator of plant immune responses. Immediately

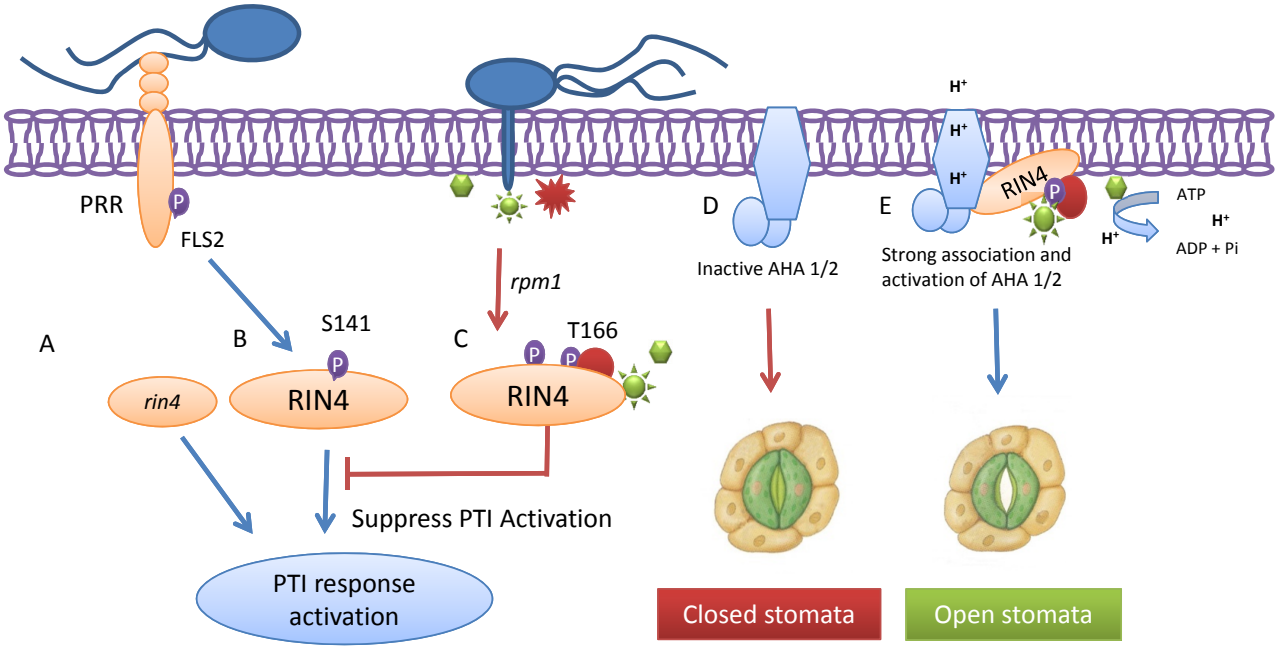

Fig. 1. Effector-mediated RIN4 modification inhibits PTI. (A) RIN4 negatively regulates PAMP-triggered immunity. (B) PAMP perception by PRR and PRPs increases FLS2-dependent RIN4 phosphorylation at the S141 residue and contributes to PTI. (C) In the absence of RPM1, two effector proteins, AvrB and AvrRpm1, induce $RIN4^{T166}$ phosphorylation, which is epistatic to $RIN4^{S141}$ phosphorylation, and repress PTI activation. (D) Activation and inactivation of AHA1 and AHA2 control stomata opening and closing. (E) RIN4 binds to the C-terminal region of H^+-ATPase and activates it. The effectors AvrRpm1 and AvrB phosphorylate $RIN4^{T166}$, resulting in increased association of RIN4 with H^+-ATPase and activation of H^+-ATPase. AvrRpt2: , AvrB: , AvrRpm1: , RIPK: .

after bacteria infiltrate the plant, stomata close (within 1 h); however, virulent bacteria use the coronatine insensitive1 (COI1)-dependent pathway (Geng et al., 2016) to reopen the stomata (within 3 h) (Liu et al., 2009b). Several bacteria and fungi control stomata during pathogen infection (Allègre et al., 2007; Axelsen et al., 1999; Oerke, 2006). Activation and inactivation of plasma membrane H^+-ATPase (proton pump) control the opening and closing of stomata. The C-terminal end of H^+-ATPase inhibits proton pump activation under normal conditions (Fig. 1D). RIN4 is expressed in guard cells and mesophyll cells (Liu et al., 2009b), and positively regulates stomatal apertures by controlling H^+-ATPase activity. RIN4 interacts with the C-terminal end of $AHA1^{837\text{-}950}$ and $AHA2^{837\text{-}949}$ in yeast two-hybrid assay and bimolecular fluorescence complementation (BiFC) assay (Liu et al., 2009a). Increased phosphorylation of RIN4 accelerates AHA activity because of a strong association between RIN4 and AHA (Fig. 1E) (Lee et al., 2015; Liu et al., 2009b). RIN4 and a receptor-like cytoplasmic kinase (RLCK) RPM1-induced protein kinase (RIPK) are crucial for stomatal regulation; this is indicated by the inability of coronatine to reopen stomata in *rin4* and *ripk* mutant plants (Lee et al., 2015; Liu et al., 2009a). Under normal conditions, the basal level of phosphorylated $RIN4^{S141}$ ($pRIN4^{S141}$) is low. The level of phosphorylation increases greatly upon the sensing of flg22 by FLS2 (Fig. 1B), which initiates several immune outputs such as callose deposition, ROS burst, and defense gene expression. BIK1 and/or PBL1 are sufficient for induced level of phosphorylated $RIN4^{S141}$ (Chung et al., 2014). Plants maintain a basal level of phosphorylated $RIN4^{T166}$ ($pRIN4^{T166}$) under normal conditions, possibly for regular stomatal regulation (Lee et al., 2015). Pathogens deploy effectors to induce RIPK-mediated phosphorylation of RIN4 at T166 to suppress PTI, reopen stomata and effector triggered susceptibility (Figs. 1C and 1E). It is possible that pathogens accelerate the pre-established process of stomata opening to enter the cells using the virulence of effector proteins. All these data clearly indicate RIN4 act as a molecular switch to regulate PTI responses. It is not yet clear why AvrRpt2 cleaves a negative regulator of PTI; however, a recent study indicates that AvrRpt2 cleavage products are also hyperactive PTI suppressors (Afzal et al., 2011). HopF2 inhibits stomatal closure during *Pst* infection (Hurley et al., 2014). In the plasma membrane, both RIN4 and HopF2 interact with AHA2 and other proteins (Lee et al., 2015; Liu et al., 2009a). HopF2 has also been reported to suppress AvrRpt2-mediated RIN4 degradation (Wilton et al., 2010), suggesting that an increase in the level of RIN4 leads to greater activation of H^+-ATPase. Pathogens evolve simultaneously with the plant immune system and introduce new effectors to modulate stomatal immunity.

RIN4 IS A MULTI-EFFECTOR TARGET PROTEIN CRITICAL FOR PATHOGEN VIRULENCE AND NLR-MEDIATED ETI

Since RIN4 is an important regulator of PTI, it is expected that pathogens will try to modify RIN4 using various effectors. RIN4 is guarded by two NB-LRR proteins, which recognize RIN4 modifications (Table 1) caused by three *P. syringae* type

Table 1. List of RIN4 (AT3G25070)-associated proteins along with their gene identifiers and functions

RIN4-associated proteins (RAPs)	Gene identifier	Function	References
RPM1 (resistance to *Pseudomonas syringae* pv. *maculicola* 1)	At3G07040	Resistant protein that guards RIN4	(Mackey et al., 2002)
RPS2 (resistance to *P. syringae* 2)	At4G26090	Resistant protein that guards RIN4	(Axtell et al., 2003; Mackey et al., 2002)
NDR1 (nonrace-specific disease resistance 1)	At3G20600	RIN4-NDR1 required for RPS2 function	(Day et al., 2006)
ROC1 (rotamase CYP 1)	At4G38740	Regulates RPM1 & RPS2 activation	(Coaker et al., 2005)
RIPK (RPM1-induced protein kinase)	At2G05940	Phosphorylates RIN4	(Liu et al., 2011)
MPK4 (MAP kinase 4)	At4G01370	Phosphorylates RIN4	(Cui et al., 2010)
FLS2 (flagellin-sensitive 2)	At5G46330	Controls RIN4 phosphorylation at S141	(Chung et al., 2014)
AHA1 (H^+-ATPase 1)	At2G18960	Regulates stomata	(Liu et al., 2009a; 2009b)
AHA2 (H^+-ATPase 2)	At4G30190	Regulates stomata	(Liu et al., 2009a; 2009b)
Cys/His rich proteins	At3G46810	Involved in intracellular signaling	(Afzal et al., 2011)
ERD4 (early responsive to dehydration 4)	At1G30360	Upregulates upon abiotic stress	(Liu et al., 2009b)
Remorin	At3G61260	Immunity signaling component	(Liu et al., 2009b)
MATH domain (*Meprin And TRAF Homology*)	At3G28220	Protein interactor	(Liu et al., 2009b)
Jacalin domain	At3G16420	Upregulates upon biotic stress	(Liu et al., 2011)
Phloem filament protein	At3G01670	Required for filament formation	(Anstead et al., 2012)
EXO70E2 Exo70B1 (component of exocyst complex)	At5G61010, At5G58430	Vesicle trafficking	(Afzal et al., 2011; Sabol et al., 2017)
Pto (serine/threonine protein kinase)	*Solanum lycopersicum* P93215	Binds with AvrPto and activates defense	(Luo et al., 2009a)

III effectors, including AvrB, AvrRpm1, and AvrRpt2 (Table 2). A recent study has identified new effectors that modify RIN4 or interfere with other effector-mediated RIN4 modifications. Here, we discuss the mechanisms of RIN4 modifications mediated by different effectors, and related resistance proteins involved in the defense signaling pathway.

Both AvrRpm1- and AvrB-maturation is critical for RIN4-effector interaction

To inactivate PTI via RIN4 modification, pathogens inject type III effectors AvrRpm1 and AvrB into the plant cell. Both effectors have no sequence homology, except in the N-terminal region. After delivery, both effectors undergo myristoylation of glycine 2 (Gly2) residue and palmitoylation of Cys3 to ensure their proper localization to the plasma membrane. Both these sites are required for maximum virulence. Palmitoylation often requires previous myristoylation (Chung et al., 2011; Johnson et al., 1994; Nimchuk et al., 2000). In the plant cell membrane, AvrRpm1 and AvrB interact with RIN4, as shown by co-immunoprecipitation experiments (Mackey et al., 2002). AvrB has two groups of residues that interact with RIN4. The first group comprises T^{125} and H^{217} residues, and the second group comprises Q^{208}, R^{209}, and Y^{210} residues; both these groups of residues interact with Y^{165}, T^{166}, H^{167}, and F^{169} residues of RIN4 (Desveaux et al., 2007). Among these residues, F^{169} is very important for the interaction of RIN4 with AvrB; it also plays important role during RIN4–RPM1 interaction and RPM1 accumulation (Chung et al., 2011). Physical interaction between RIN4 and AvrB is required for the activation of RPM1; however, RPM1 is not required for the interaction with and phosphorylation of RIN4 induced by AvrRpm1 and AvrB (Mackey et al., 2002).

AvrB-induced RIN4 phosphorylation through host kinases is critical for immune response and phytohormone regulation

Phosphorylation of RIN4 at T^{21}, S^{160}, and T^{166} residues promotes bacterial virulence; however, S^{160} is not conserved among land plants (Afzal et al., 2011; Lee et al., 2015). On the other hand, $RIN4^{T166}$ and $RIN4^{S141}$ are evolutionarily conserved among orthologous RIN4 sequences in different plant species (Chung et al., 2014). AvrB uses RIPK to increase phosphorylated $RIN4^{T166}$ which is epistatic to all PTI outputs enhanced by PAMP-induced accumulation of $pRIN4^{S141}$ (Fig. 2A). This suggests that pathogens inject effector proteins to increase the level of $pRIN4^{T166}$ and decrease PTI. RIPK also interacts with and phosphorylates AvrB but does not interact with AvrRpt2, AvrPto, AvrPtoB, and AvrRps4, indicating that the RIPK–AvrB interaction is highly specific (Liu et al., 2011). This specific interaction between RIPK and AvrB in accordance with five other RLCK members enhances the phosphorylation activity or substrate specificity of RIPK, resulting in increased phosphorylation of $RIN4^{T166}$. The type III effector AvrPphB, a cysteine protease, cleaves RIPK and other RLCK family members (Zhang et al., 2010) and blocks AvrB recognition by RPM1 (Afzal et al., 2011). Another effector, HopF2, also targets RIN4 for its virulence (Wilton et al., 2010), and inhibits the accumulation of $pRIN4^{S141}$ (Chung et al., 2014). The cumulative effect of multiple effectors decreases the level of $pRIN4^{S141}$ and suppresses PTI.

AvrB also forms a complex with other molecular chaperones, such as HSP90–RAR1-SGT1, to induce plant susceptibility (Belkhadir et al., 2004; Cui et al., 2010; Shang et al., 2006). To enhance virulence and modulate plant hormone signaling, AvrB interacts with and phosphorylates MAP KINASE 4 (MPK4) in an RPM1-independent manner. MPK4 also interacts with and phosphorylates recombinant RIN4, suggesting that RIN4 is a substrate of MPK4. RIN4 positively modulates jasmonic acid signaling. The AvrB–MPK4 and MPK4–RIN4 interactions coupled with RAR1, SGT1, and HSP90 constitute a new pathway for regulating hormone signaling (Cui et al., 2010). AvrB targets host protein kinases, such as RIPK and MPK4, for modulating their activity and/or substrate specificity and for increasing plant susceptibility. Further investigation of the association between RIN4 and other kinases will help unravel the importance of this plant immune regulator in the modulation of PTI/ETI.

AvrRpm1 mediated RIN4 phosphorylation activates both RPM1 and RPS2

Mackey et al. (2002) have shown AvrRpm1–RIN4 interaction via co-immunoprecipitation experiments (Table 2). However, no interaction has been observed between AvrRpm1 and RIN4 in yeast two-hybrid assays (Chung et al., 2011). RIN4 is

Table 2. RIN4 (AT3G25070) modifier effectors and their mode of action

Effector	Origin	Mode of action	References
AvrB	*Pseudomonas syringae* pv. *glycinea* P13835	Induces RIN4 phosphorylation	(Axtell et al., 2003; Chung et al., 2011; Liu et al., 2011; Mackey et al., 2002)
AvrRpm1	*P. syringae* pv. *maculicola* Q7BE94	Induces RIN4 phosphorylation	(Chung et al., 2011; Liu et al., 2011; Mackey et al., 2002)
AvrRpt2	*P. syringae* pv. *tomato* Q6LAD6	Cleaves RIN4 at two sites	(Mackey et al., 2003)
AvrPto	*P. syringae* pv. *tomato* Q87Y16	Degrades RIN4 in a Pto- and Prf-dependent manner	(Luo et al., 2009a)
AvrPtoB	*P. syringae* pv. *tomato* A5AC83	Degrades RIN4	(Luo et al., 2009b)
HopF2	*P. syringae* pv. *tomato* Q88A90	Inhibits *AvrRpt2*-mediated RIN4 degradation	(Wilton et al., 2010)
$HopPtoQ1\text{-}1_{DC3000}$	*P. syringae* pv. *tomato* Q888Y7	Degrades RIN4	(Luo et al., 2009a)
$HopAM1_{DC3000}$	*P. syringae* pv. *tomato* Q877R9	Degrades RIN4	(Luo et al., 2009a)

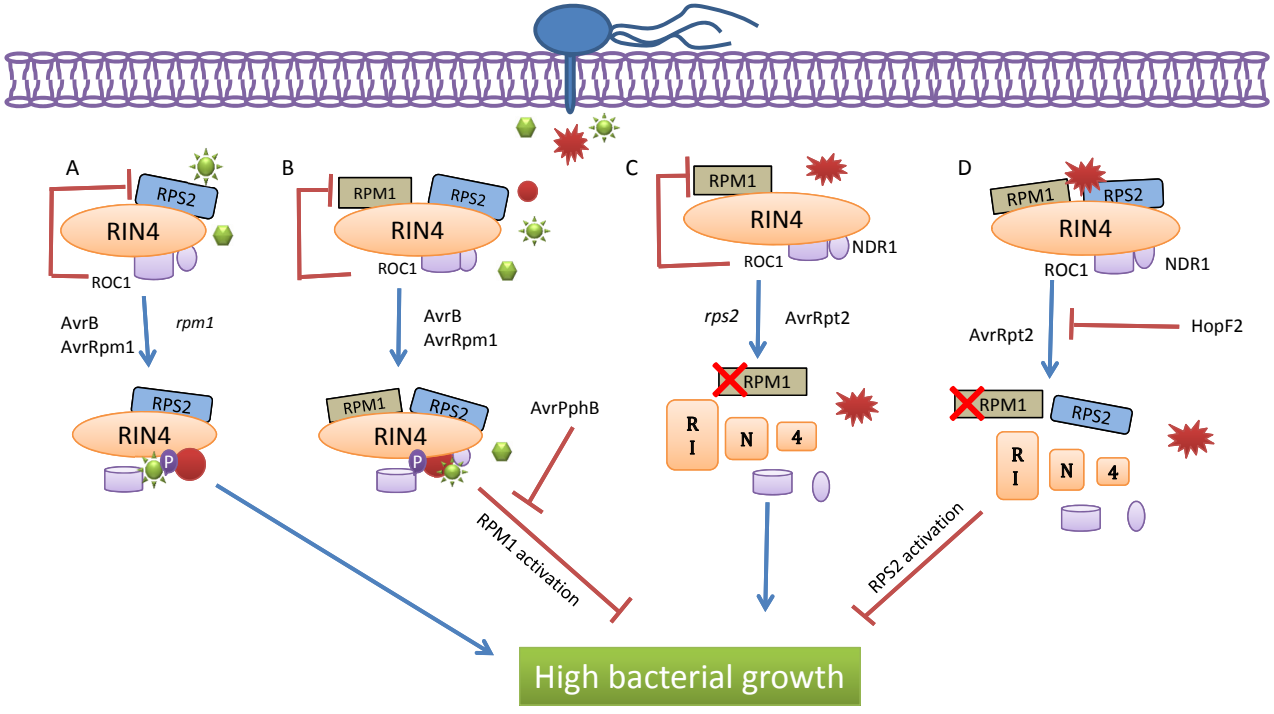

Fig. 2. Type III effectors modify RIN4 to promote bacterial growth and activate resistance proteins. (A) In *rpm1* mutant plants, the type III effectors, AvrRpm1 and AvrB, phosphorylate RIN4 to promote bacterial growth. ROC1 isomerizes RIN4^{P149} and inhibits RPS2. (B) In resistant plants, ROC1 suppresses RPM1 and RPS2. RIN4 phosphorylation at the T166 residue removes ROC1 suppression and activates RPM1. RPM1 activation initiates a hypersensitive response and decreases bacterial growth. AvrPphB inhibits AvrB-induced RPM1 activation. (C) The type III effector AvrRpt2 cleaves RIN4 upon delivery and processing. In *rps2* mutant plants, AvrRpt2 cleaves RIN4 to promote bacterial growth. RIN4 cleavage subsequently decreases the accumulation of RPM1. (D) In resistant plants, RIN4 degradation activates RPS2, which initiates hypersensitive response and decreases bacterial growth. The RIN4-NDR1 association is important for RPS2 activation. RIN4 degradation activates the resistance protein RPS2, and HopF2 inhibits AvrRpt2-mediated RIN4 cleavage. AvrRpt2: , AvrB: , AvrRpm1: , RIPK: , ROC1: , NDR1: .

not a target of AvrRpm1 alone, as it shows higher virulence in all *rpm1* and *rpm1 rps2 rin4* mutant plants (Belkhadir et al., 2004). AvrRpm1 induces phosphorylation of RIN4; however, RIN4 phosphorylation does not show a consistent decrease in *ripk* mutant background, suggesting that other kinases from the RLCK family are involved in RIN4 phosphorylation (Liu et al., 2011). AvrPphB-mediated RIPK cleavage fails to inhibit AvrRpm1-mediated RPM1 activation, thus supporting previous data (Russell et al., 2015). Increased RIN4^{T166} phosphorylation is observed in the presence of AvrRpm1 both in Arabidopsis and tobacco, but this phosphorylation partially activates AvrRpm1-triggered RPM1 defense signaling. Both AvrRpm1 and AvrB co-immunoprecipitate with RIN4 and AvrB induces phosphorylation of conserved threonine residue at position 166 of RIN4 (Chung et al., 2011). Despite the classical gene-for-gene activation of RPM1, AvrRpm1 also induces RPS2-mediated defense signaling (Kim et al., 2005a). A recent study describes that AvrRpm1 induces virulence through COI1-mediated suppression of salicylic acid (SA)-dependent or independent defense signaling pathways (Geng et al., 2016). PARP-1s are proteins that contain extra domains beyond the canonical catalytic domain. They typically facilitate the ribosylation reaction using the catalytic triad. AvrRpm1 has a fold homologous to the active catalytic triad (H^{63}-Y^{122}-D^{185}) of poly-ADP-ribosyl-polymerase-1 (PARP-1) and key structural components of PARP-1. This catalytic activity of AvrRmp1 is required for virulence in a susceptible host, or to activate RPM1-mediated defense signaling. Catalytically inactive AvrRpm1 inhibits the growth of *P. syringae* pv. *maculicola* 1 on susceptible plants through RPS2-mediated defense signaling. These data suggest that AvrRpm1 mimics ADP ribosyl transferase (Cherkis et al., 2012); however, further investigation is required to establish the ribosyl-transferase activity of AvrRpm1.

AvrB- or AvrRpm1-mediated RIN4^{T166} phosphorylation activates RPM1

Among the 211 aa of RIN4, aa 141-176 are necessary for phosphorylation, with the threonine (Thr) residue at position 166 being critical for RPM1 activation. Both Avr proteins, AvrB and AvrRpm1, phosphorylate RIN4^{T166} *in vivo* and ultimately activate RPM1 (Fig. 2B), as it recognizes effector-mediated RIN4 phosphorylation in planta (Chung et al., 2011; Liu et al., 2011). The level of activation of RPM1 is regulated by ROC1, which exists in multiple cellular compartments (Ferreira et al., 1996). ROC1 interacts with RIN4$^{142-211}$ and exhibits pro-

lyl-peptidyl isomerase (PPIase) activity, which modifies RIN4 configuration by catalyzing cis/trans isomerization of RIN4^{P149} (144-KVTVVPKFGDWD-155) and suppresses RPM1- as well as RPS2-dependent ETI. Mutation of ROC1 at R62 residue completely disrupts its PPIase activity whereas mutation of ROC1 at S58 increases the PPIase activity of ROC1. This RIN4–ROC1 interaction inhibits AvrB- or AvrRpm1-induced phosphorylation of RIN4 and the dissociation of ROC1 from RIN4 or deletion of RIN4^{P149} activates RPM1. Isomerization status of RIN4^{P149} residue can modulate proper conformational change upon induced RIN4^{T166} phosphorylation by effector proteins. In other word, RPM1 can monitor conformational change of RIN4 mediated by T166 phosphorylation and ROC1 plays a role in this conformational change on RIN4 along with T166 phosphorylation (Chung et al., 2011; Li et al., 2014).

AvrRpt2-mediated cleavage and degradation of RIN4 activates RPS2

AvrRpt2 is a cysteine protease that cleaves the membrane localized Arabidopsis protein RIN4. After delivery inside the cell AvrRpt2 cleaves itself at N terminal region between G^{71}-G^{72} in presence of host cyclophilin ROC1 (Jin et al., 2003) and activated AvrRpt*2* associates with PM through myristoylation (Toruno et al., 2018). ROC1 associates with RIN4 and enforces T166 phosphorylation to enhance RPM1-mediated response (Li et al., 2014). RPS2 and RIN4 are also localized to the plasma membrane (Axtell et al., 2003; Jin et al., 2003; Mackey et al., 2002). Among these proteins, RPS2 acts as an integral membrane protein because high salt, urea, or pH is unable to remove RPS2 from the membrane (Axtell et al., 2003; Jin et al., 2003; Mackey et al., 2002). In the absence of RPS2, AvrRpt2 promotes bacterial growth through COI1-mediated suppression of both SA-dependent and -independent defense signaling (Geng et al., 2016). The AvrRpt2-dependent RIN4 degradation is independent of the RPS2 but involves NDR1 and RAR1 (Fig. 2C) (Muskett et al., 2002; Tornero and Dangl, 2001). RPS2 is unable to recognize AvrRpt2 directly; instead, it identifies RIN4 degradation and then initiates defense signaling (Axtell et al., 2003; Mackey et al., 2002). AvrRpt2 cleaves itself and other proteins within a conserved sequence (V/I/L) PxFGxW motif (Chisholm et al., 2005). RIN4 has two cleavage sites related to AvrRpt2 cleavage sites: RIN4 cleavage site 1 (RCS1; 3-14 aa) and RCS2 (145-156 aa). Cleavage at RCS1 results in 22.2 kDa and 1.2 kDa fragments; cleavage at RCS2 results in 17 kDa and 6.4 kDa fragments; and cleavage at both RCS1 and RCS2 results in 15.9 kDa, 6.4 kDa, and 1.2 kDa fragments. After AvrRpt2 cleaves at RCS2, the 6.4 kDa product remains in the plasma membrane, indicating its membrane localization. After cleavage at RCS2, RIN4 is released from the membrane and the cleavage products undergo proteasome-dependent degradation, as indicated by the inhibition of degradation by the proteasome inhibitor (Chisholm et al., 2005; Kim et al., 2005a).

RIN4 degradation inappropriately activates RPS2-mediated defense signaling

RPS2 is an integral membrane protein, which shows direct or indirect interaction with RIN4 in the plant cell. RPS2 recognizes RIN4 degradation and shows a hypersensitive response; thus the *rin4* knockout mutation is viable only in the *rps2* mutant background (Mackey et al., 2002). Only the C-terminal half of RIN4 is required for the negative regulation of and association with RPS2. Cleavage of RIN4 removes the negative regulation of RPS2, thereby activating RPS2 (Fig. 2D). Deletion of 6-9 aa from the C-terminal end of RIN4 results in the complete loss of RPS2 regulation by RIN4 (Day et al., 2005). A recent study has shown that RIN4^{P149} acts as a conformational switch, which regulates RPS2 activation (Li et al., 2014). ROC1 suppresses RPS2 activation through its PPIase activity to isomerize RIN4^{P149} by interacting with

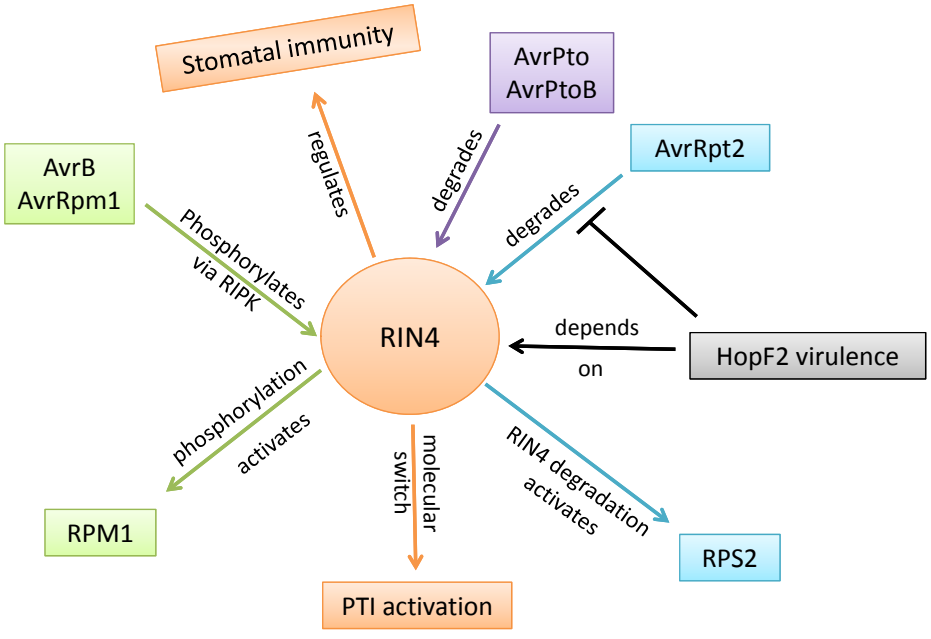

Fig. 3. A model depicting the role of RIN4 in PTI and ETI. RIN4 negatively regulates PTI response upon PAMP recognition (orange). RIN4 strongly binds to the C-terminal inhibitory region of H^+-ATPase and activates H^+-ATPase to control stomatal immunity (orange). The effector proteins AvrB and AvrRpm1 induce RIN4 phosphorylation and activate RPM1, if present (green). Another effector, AvrRpt2, degrades RIN4 and activates RPS2, if present (blue). RIN4 degradation also interfere with RPM1 accumulation and function. Expression of the effector protein HopF2 halts AvrRpt2-mediated RIN4 degradation (black). AvrPto and AvrPtoB also degrade RIN4 (purple).

the C-terminal end of RIN4$^{142\text{-}210}$. The RIN4–ROC1 complex dissociates following AvrB- or AvrRpm1-induced RIN4^{T166} phosphorylation. The dissociation of ROC1 from RIN4 or deletion of RIN4^{P149} activates RPS2-mediated defense signaling (Fig. 2D) (Li et al., 2014). AvrRpt2 cleavage products of RIN4 neither interact with RPS2 nor inhibit RPS2-mediated defense signaling (Day et al., 2006). The activation of RPS2-mediated defense signaling is also regulated by RIN4–NDR1 complex (Coppinger et al., 2004). The absence of NDR1-RIN4 interaction blocks hypersensitive response after AvrRpt2 inoculation. The ectopic activation of RPS2 is independent of NDR1 and the contribution of NDR1 in AvrRpt2-induced activation of RPS2 requires its interaction with RIN4 (Day et al., 2006).

Additional effector proteins that modify RIN4

P. syringae pv. *tomato* DC3000 type III effector HopF2 contains a myristoylation sequence, which is required for its localization to the plasma membrane (Robert-Seilaniantz et al., 2006). HopF2 binds with RIN4 or RIN4-associated complex in the membrane, and suppresses AvrRpt2-induced RIN4 degradation and hypersensitive response, without interfering with AvrRpt2 maturation by ROC1 (Fig. 2D). RPM1 and RPS2 have no effect on the virulence function of HopF2 (Wilton et al., 2010). Although the HopF2-mediated RIN4 modification pathway has not yet been established, a recent study shows that HopF2 mediates stomatal regulation through its interaction with AHA2 (Hurley et al., 2014). It would be interesting to determine the mechanism of HopF2-mediated RIN4 modification and its effect on plant immunity.

Arabidopsis RIN4 and its homolog in tomato interact with the N-terminal end of unrelated bacterial effectors, AvrPto and AvrPtoB. AvrPto degrades RIN4 in a Pto- (a protein kinase) and Prf- (NB-LRR protein) dependent manner using the same site as used by AvrRpt2. Two additional effectors with unrelated sequences, which also induce RIN4 degradation, include HopQ1-1 DC3000 (HopPtoQ DC3000) and HopAM1 DC3000 (AvrPpiB DC3000). The mode of action of these remain unknown (Fig. 3) (Luo et al., 2009a).

CONCLUSION AND PERSPECTIVES

Protein complex formation and protein function are very important aspects of plant immunity. RIN4 is a crucial regulator of plant immunity, the absence or modification of which activates various immune responses (Fig. 3). However, several aspects of RIN4-interacting proteins and their functions in defense signaling need further investigation. Exosomes transport immune system components within the cell and in the apoplast (Luo et al., 2009a). It would be interesting to investigate the involvement of RIN4 in intercellular signaling, as it is present in exosomes and interacts with exocyst components EXO70E2 and EXO70B1. RIN4 can recruit one of the exocyst subunit Exo70B1 to the plasma membrane wherein upon AvrRpt2 delivery, both RIN4 and Exo70B1 can be released from plasma membrane to cytoplasm (Sabol et al., 2017). Other RIN4-interacting proteins, such as Remorins and Jacalin domain-containing proteins, are involved in response biotic stress; therefore, their involvement in RIN4-mediated plant immunity should be investigated (Esch and Schaffrath, 2017; Raffaele et al., 2007). AvrRpm1 induces RIN4 phosphorylation, in part via RIPK or other RLCK kinases and in part by itself, as it has PARP1-like catalytic activity. AvrRpm1 family of type III effectors shares the PARP catalytic fold including key catalytic and structural components of PARP such as the catalytic triad H862-Y907-E998, which facilitates ribosylation reaction (Cherkis et al., 2012). It would be interesting to identify other kinases induced by AvrRpm1. The RLCK family members BIK1 or PBL1 are ideal candidates for further investigation. RIN4 phosphorylation induced by AvrB and AvrRpm1 increases its activation and association with AHA. However, this phosphorylation is monitored by resistance proteins; therefore, another effector (HopF2) interacts with AHA2 and inhibits stomatal closure. Determining the role of AvrB- and AvrRpm1-mediated RIN4 phosphorylation, in combination with HopF2, would provide further insight into the regulation of stomatal immunity.

Disclosure

The authors have no potential conflicts of interest to disclose.

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