AvrRpm1 Functions as an ADP-Ribosyl Transferase to Modify NOI-domain Containing Proteins, Including Arabidopsis and Soybean RPM1-interacting Protein 4

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Short title: AvrRpm1 is an ADP-ribosyl Transferase

One-sentence summary: The Pseudomonas syringae effector protein AvrRpm1 ADP-ribosylates RIN4 proteins from Arabidopsis and soybean, which promotes association of RIN4 with EXO70E2, and suppression of callose deposition.

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ABSTRACT
The *Pseudomonas syringae* effector protein AvrRpm1 activates the *Arabidopsis thaliana* intracellular innate immune receptor protein RESISTANCE TO PSEUDOMONAS MACULICOLA1 (RPM1) via modification of a second Arabidopsis protein, RPM1-INTERACTING PROTEIN4 (*At*RIN4). Prior work has shown that AvrRpm1 induces phosphorylation of *At*RIN4, but homology modeling indicated that AvrRpm1 may be an ADP-ribosyl transferase. Here we show that AvrRpm1 induces ADP-ribosylation of RIN4 proteins from both Arabidopsis and soybean within two highly conserved nitrate-induced (NOI) domains. It also ADP-ribosylates at least ten additional Arabidopsis NOI domain-containing proteins. The ADP-ribosylation activity of AvrRpm1 is required for subsequent phosphorylation on threonine 166 of *At*RIN4, an event that is necessary and sufficient for RPM1 activation. We also show that the C-terminal NOI domain of *At*RIN4 interacts with the exocyst subunits EXO70B1, EXO70E1, EXO70E2 and EXO70F1. Mutation of either EXO70B1 or EXO70E2 inhibited secretion of callose induced by the bacterial flagellin-derived peptide flg22. Substitution of RIN4 threonine 166 with aspartate enhanced the association of *At*RIN4 with EXO70E2, which we posit inhibits its callose deposition function. Collectively, these data indicate that AvrRpm1 ADP-ribosyl transferase activity contributes to virulence by promoting phosphorylation of RIN4 threonine 166, which inhibits the secretion of defense compounds by promoting the inhibitory association of RIN4 with EXO70 proteins.

INTRODUCTION
Plants are able to defend themselves from a wide range of disease causing bacteria, and do so in part by detecting the presence of Type III secretion system effector proteins delivered by these pathogens (Martin et al., 2003). Two of these effectors, AvrB and AvrRpm1, are detected in *Arabidopsis thaliana* by RPM1, a member of the nucleotide binding leucine rich repeat (NLR) family of intracellular innate immune receptors (Bisgrove et al., 1994; Grant et al., 1995; Jones et al., 2016). Although it has been known for over 20 years that RPM1 confers dual effector specificity, most research on this receptor has focused on the molecular basis of AvrB recognition, with less molecular information available on how AvrRpm1 is detected.
Recognition of AvrB by RPM1 requires a second host protein, AtRIN4 (Mackey et al., 2002). Upon delivery into the host, AvrB becomes acylated and associates with the plasma membrane (Nimchuk et al., 2000; Gao et al., 2011). There, it interacts with AtRIN4 and the protein kinase, RIPK (Liu et al., 2011). This interaction leads to the autophosphorylation of RIPK, and the subsequent phosphorylation of AtRIN4 at residues T21, S160, and T166 (Liu et al., 2011). Phosphorylation of AtRIN4 T166 is necessary and sufficient for activation of RPM1 (Chung et al., 2011).

Like the detection of AvrB, detection of AvrRpm1 also requires the presence of AtRIN4 (Mackey et al., 2002). Furthermore, expression of AvrRpm1 in rpm1 mutant Arabidopsis plants induces a modification of AtRIN4 (Mackey et al., 2002). This modification was observed as reduced band mobility when resolved by SDS-PAGE. When treated with calf intestine alkaline phosphatase, this shift in AtRIN4 size was eliminated, indicating that AvrRpm1 induces phosphorylation on AtRIN4 (Mackey et al., 2002). However, the specific AtRIN4 residues modified by AvrRpm1 have not been identified, and whether AvrRpm1 possesses kinase activity has not been determined.

The possibility that AvrRpm1 induces a distinct or additional modification of AtRIN4 arose from computational structural modeling of AvrRpm1, which indicated that AvrRpm1 may contain a fold homologous to ADP-ribosyltransferases such as diphtheria toxin. Elimination of AvrRpm1 function by mutagenesis of putative catalytic residues supported this model (Cherkis et al. 2012).

Glycine max (soybean) is also able to detect and respond to the presence of AvrB and AvrRpm1 (Ashfield et al., 1995). Unlike Arabidopsis, however, recognition of AvrB and AvrRpm1 in soybean is divided among two NLR receptors, RPG1b and RPG1r, respectively (Ashfield et al., 1995). The soybean genome contains four homologs of RIN4, designated GmRIN4a (Glyma03g19920), GmRIN4b (Glyma16g12160), GmRIN4c (Glyma18g36000), and GmRIN4d (Glyma08g46400) (Supplemental Figure 1). Thus, the role of GmRIN4 in disease resistance might be sub-functionalized. In support of this idea, virus-induced gene silencing (VIGS) of GmRIN4a or GmRIN4b, but not GmRIN4c or GmRIN4d, suppresses AvrB recognition by RPG1b (Selote and Kachroo, 2010). In addition, recognition of AvrRpm1 by RPG1r can be suppressed by AvrRpt2, a cysteine protease that cleaves all four GmRIN4 orthologs.
(Ashfield et al., 2014), consistent with findings from Arabidopsis (Ritter and Dangl, 1996; Mackey et al., 2003; Kim et al., 2005a). This result indicates that one or more of the $GmRIN4$s likely are required for RPG1r-mediated recognition of AvrRpm1.

In plants genetically unable to recognize them, type III effectors such as AvrRpm1 contribute to the ability of bacteria to proliferate and cause disease (Ritter and Dangl, 1995). This virulence activity is mediated in many cases via suppression of host defenses, including those triggered by microbe-associated molecular patterns (MAMPs). RIN4 negatively regulates the ability of Arabidopsis plants to produce cell wall fortifications, such as deposition of callose, in response to a prototypical MAMP consisting of a 22 amino acid peptide from the bacterial flagellin protein (flg22) (Kim et al., 2005b). AvrRpm1 appears to promote the activity of RIN4, as transgenic expression of AvrRpm1 suppresses flg22-induced callose deposition (Kim et al., 2005b). Thus, AvrRpm1-induced modifications of RIN4 that contribute to activation of NLR receptors in Arabidopsis, soybean and other plants may also underlie virulence activity in susceptible host plants that lack a responsive NLR.

With the working hypothesis that at least one $GmRIN4$ is required for RPG1r-mediated recognition of AvrRpm1 in soybean, we sought to elucidate the underlying molecular mechanisms. Specifically, we wanted to determine whether AvrRpm1 could induce modification of one or more of the $GmRIN4$ proteins, and if so, what these modifications might be. Here we show that AvrRpm1 induces mono-ADP ribosylation on $AtrIN4$, $GmRIN4a$, and $GmRIN4b$ within their conserved nitrate induced (NOI) domains. A D153A substitution in the C-terminal NOI domain of $AtrIN4$, which blocks ADP-ribosylation at this position, inhibits phosphorylation on T166, and thereby inhibits activation of RPM1 by AvrRpm1. We also show that RIN4 proteins from both Arabidopsis and soybean associate with Arabidopsis exocyst subunits EXO70B1, EXO70E1, EXO70E2 and EXO70F1, and that these interactions are influenced by modification of T166. Lastly, we show that mutation of $EXO70B1$ and $EXO70E2$ impairs flg22-induced callose deposition, indicating that AvrRpm1 likely inhibits callose deposition, at least in part, by enhancing the ability of RIN4 to bind to EXO70 proteins and interfere with EXO70-mediated secretion.
RESULTS

AvrRpm1 Modifies GmRIN4b in planta

Prior work has shown that the GmRIN4b gene can complement the ability of an Arabidopsis rin4 mutant to recognize either AvrB or AvrRpm1 (Selote and Kachroo, 2010). This observation indicates that GmRIN4b is likely modified by these effectors. To test this hypothesis, we generated an N-terminal Myc-tagged GmRIN4b construct under control of a dexamethasone-inducible promoter in pTA7001 (Aoyama and Chua, 1997) and transiently co-expressed it in Nicotiana benthamiana along with transiently expressed AvrB, AvrRpm1 or an empty vector control (EV). To detect possible phosphorylation of GmRIN4b, we isolated total protein from N. benthamiana leaves and separated the proteins on a polyacrylamide gel supplemented with Phos-tag reagent (Kinoshita et al., 2006), followed by immunoblot analysis. The mobility of GmRIN4b was markedly reduced when co-expressed with AvrRpm1, but not when co-expressed with AvrB or empty vector (Figure 1).

To determine if the AvrRpm1-induced modification of GmRIN4b was phosphorylation, the above samples were treated with Lambda protein phosphatase (λPP) prior to being separated by SDS-PAGE. The post-translational modification to GmRIN4b was not sensitive to λPP treatment (Figure 1A), indicating that the modification was most likely not phosphorylation.

Previous work with Arabidopsis RIN4 has shown that AvrB induces phosphorylation of AtRIN4 at threonine 21, serine 160, and threonine 166 (Liu et al., 2011). Furthermore, substitution of T166 with a phosphomimicking amino acid (T166E or T166D) induces an RPM1-mediated hypersensitive response in the absence of AvrB (Chung et al., 2011; Liu et al., 2011). Equivalent substitutions in GmRIN4b (T22D, T198D), induce an RPG1b-mediated HR (Selote et al., 2013). To determine if these residues are modified in the presence of AvrRpm1, GmRIN4b (T22A, T198A) or GmRIN4b (T22D, T198D) were transiently expressed in N. benthamiana in the presence of AvrRpm1 or EV. Total protein was harvested from leaves and resolved by SDS-PAGE on a gel supplemented with Phos-tag reagent. Regardless of the substitutions at these positions, GmRIN4b mobility was reduced in the presence of
AvrRpm1 (Figure 1B). Additionally, multiple distinct bands were visible when GmRIN4b was expressed with AvrRpm1 (Figure 1B). Taken together, these results indicate that GmRIN4b is modified at multiple locations in the presence of AvrRpm1, and that these modifications are at residues that differ from those involved in AvrB-induced activation of RPG1b.

**GmRIN4b is ADP-Ribosylated by AvrRpm1 in vivo**

To identify the modifications induced by AvrRpm1, and their locations on GmRIN4b, we performed immunoprecipitation of GmRIN4b followed by mass spectrometry. N-terminal sYFP-tagged GmRIN4b was co-expressed with empty vector or AvrRpm1 in *N. benthamiana* using a dexamethasone-inducible vector (Aoyama and Chua, 1997). Protein was allowed to accumulate for 3.5 hours after dexamethasone induction and the clarified total protein extract was incubated for 1 hour at 4°C with GFP-Trap beads in order to immunoprecipitate sYFP-GmRIN4b. The washed beads were then subjected to on-bead trypsin digestion and the recovered peptides analyzed by liquid chromatography coupled to mass spectrometry (see Methods).

The mass spectral analyses obtained from two independent experiments ranged in coverage from 62% to 84% depending on the experiment and sample (Figure 2; Supplemental Dataset 1). We screened these mass data for known posttranslational modifications, including phosphorylation on S, T, or Y and ADP-ribosylation on C, D, E, K, N, R, S, or T. Based on known phosphorylation sites induced by AvrB on AtRIN4, we first assessed whether GmRIN4b residues T22 and T198 were phosphorylated in the presence of AvrRpm1. These analyses revealed that neither T22 nor T198 were specifically phosphorylated in the presence of AvrRpm1. This is consistent with our observation that neither the T22A/D nor T198A/D substitutions prevented modification of GmRIN4b in the presence of AvrRpm1 (Figure 1B) and the fact that Rpg1b, which responds to GmRIN4b with phospho-mimicking residues at T22 and T198, does not respond to the presence of AvrRpm1.

Although we did not identify any phosphorylation sites that were specifically associated with AvrRpm1 treatment, we observed several residues on GmRIN4b that were phosphorylated in both empty vector and AvrRpm1-treated samples, including
S89, S130, S143, T158 and T173, indicating that GmRIN4b is phosphorylated by endogenous kinases in *N. benthamiana* (Figure 2; Supplemental Dataset 1).

The most notable of the modifications identified, however, were found on two peptides located within the N-terminal and C-terminal NOI domains of GmRIN4b: FGNWDSGENVPYTAYFDK (N-NOI) and FGDWDVNNPASADGFTHIFNK (C-NOI), respectively (Figure 2; Supplemental Dataset 1). Both of these peptides were found to be mono-ADP-ribosylated only when expressed in the presence of AvrRpm1. Based on the fragmentation data of the N-NOI domain peptide (FGNWDSGENVPYTAYFDK), it was ADP-ribosylated on the first asparagine residue (N12) (Figure 2). Fragmentation data for the C-NOI domain peptide (FGDWDVNNPASADGFTHIFNK) revealed that it was ADP-ribosylated at the first aspartate residue (D185), thus both NOI domains are modified at the same relative position, but on different amino acids (N versus D; Figure 2B). For the ADP-ribosylated versions of the peptides, we also observed characteristic marker ions resulting from fragmentation of the ADP-ribose moiety at m/z values of 136.06, 250.09, 348.07 and 428.03 (Rosenthal et al., 2015).

To confirm that AvrRpm1 induces ADP-ribosylation of GmRIN4b, we used an anti-pan-ADP-ribose binding reagent (anti-panADPR), which is a recombinant protein that selectively binds to mono- and poly-ADP-ribosylated proteins (Gibson et al., 2016). sYFP-tagged GmRIN4b was co-expressed with empty vector or AvrRpm1 in *N. benthamiana* and total protein, isolated 4 hours after induction, was immunoblotted with either anti-panADPR or anti-GFP. The anti-panADPR immunoblot showed a strong band corresponding in size to sYFP-GmRIN4b only in the samples co-expressed with AvrRpm1 (Figure 3A). In addition, we observed a second lower molecular weight band in the AvrRpm1 samples, even in the absence of GmRIN4b, indicating that AvrRpm1 can modify an endogenous *N. benthamiana* protein. These results confirm that AvrRpm1 possesses ADP-ribosylation activity.

**AvrRpm1 ADP-Ribosylates Multiple NOI-containing Proteins**

Having established that the anti-panADPR reagent was an effective tool for detecting ADP-ribosylation of GmRIN4b, we used it to assess whether AvrRpm1 could induce ADP-ribosylation of sYFP-tagged versions of the other three GmRIN4 proteins,
and the Arabidopsis AtRIN4 protein. These analyses revealed strong modification of both sYFP-AtRIN4 and sYFP-GmRIN4a, but very little modification of sYFP-GmRIN4c and sYFP-GmRIN4d (Figure 3A). The relatively weaker modification of sYFP-GmRIN4c and sYFP-GmRIN4d may be a consequence of sequence differences in the N-NOI domains of GmRIN4c and GmRIN4d relative to GmRIN4a and GmRIN4b (Figure 3B). We observed, however, that the mobility of sYFP-GmRIN4c and sYFP-GmRIN4d was retarded in the presence of AvrRpm1 (Figure 3A), indicating that these two proteins may still be modified, but for unknown reasons the modification is not detected by the anti-panADPR reagent.

The specificity of AvrRpm1 for the NOI domains of RIN4 indicated that AvrRpm1 may be able to modify other NOI domain-containing proteins. We thus co-expressed in N. benthamiana AvrRpm1 and each of 14 additional Arabidopsis proteins (AtNOIs) that, aside from containing predicted NOI domains and C-terminal acylation motifs, lack any other similarity to RIN4. AvrRpm1-dependent ADP-ribosylation was observed for 10 of the 14 AtNOI proteins (NOI1, NOI2, NOI3, NOI4, NOI5, NOI6, NOI7, NOI11, NOI12 and NOI13) and AvrRpm1 co-immunoprecipitated with the same 10 AtNOI proteins (Figure 3C). Alignment of the NOI domains from these 14 proteins revealed that NOI9 and NOI14 have non-preferred residues at the predicted ADP-ribosylation site (S and G, respectively), but it is not obvious why NOI8 and NOI10 were not modified (Supplemental Figure 2). In summary, AvrRpm1 associates with and ADP-ribosylates most AtNOI proteins (Figure 3C).

To further support our conclusion that AvrRpm1 functions as an ADP-ribosyl transferase, we assessed putative ADP ribosylation catalytic domain mutants of AvrRpm1 (Cherkis et al. 2012) for their ability to ADP-ribosylate GmRIN4b. These AvrRpm1 mutants were dramatically reduced in their ADP-ribosylation activity (Figure 3D), consistent with their reduced virulence function and ability to activate RPM1 (Cherkis et al., 2012). We also assessed whether AvrB could induce ADP-ribosylation on AtRIN4, but observed no evidence for this (Figure 3E), indicating that AvrB likely possesses a different enzymatic activity.

To confirm that AvrRpm1 can induce ADP-ribosylation of AtRIN4 when delivered by P. syringae, we inoculated Arabidopsis rpm1-3 mutant plants with P. syringae strain
DC3000 carrying empty vector or a plasmid expressing wild-type *avrRpm1* or catalytic site mutants. Native AtRIN4 protein was then immunoprecipitated and immunoblotted with α-panADPR reagent. These analyses revealed ADP-ribosylation of AtRIN4 by wild-type but not mutant forms of AvrRpm1 (Figure 3F), indicating that AvrRpm1 delivered by *P. syringae* is able to induce ADP-ribosylation of native AtRIN4 protein.

Lastly, we assessed whether the ADP-ribosylation activity of AvrRpm1 is required to induced a resistance response on soybean (Figure 3G). Inoculation of soybean cultivar Flambeau, which expresses Rpg1-r, with *P. syringae pv. glycinea* Race 4 expressing wild-type AvrRpm1 induced a strong cell death response by 25 hours post-inoculation, while neither the D185A nor the catalytic site triple mutant induced a visible response. Thus, ADP-ribosylation activity is required for recognition of AvrRpm1 by both RPM1 and Rpg1-r.

**ADP-Ribosylation of AtRIN4 by AvrRpm1 is Required for Full Phosphorylation of T166 and Modulates Activation of RPM1**

Because T166 of AtRIN4 contributes to activation of RPM1 by AvrRpm1 and because a phosphomimic mutation of AtRIN4 (T166D) is sufficient to activate RPM1 (Chung et al., 2011), we hypothesized that ADP-ribosylation of the AtRIN4 NOI domains is mechanistically related to phosphorylation of T166. To test this hypothesis, we extended the previously defined RPM1-activation system in *N. benthamiana* (Chung et al., 2011). We confirmed that activation of RPM1 in *N. benthamiana* required both AtRIN4 and AvrRpm1, and that only the combination of all three proteins induced cell death (Supplemental Figure 3A). We then tested the ability of AvrRpm1 ADP-ribosyl transferase catalytic-site mutants to elicit AtRIN4-dependent activation of RPM1. We observed that *avrRpm1*<sup>D185A</sup> retained partial activation of RPM1, while the more severe allele *avrRpm1*<sup>AAA</sup> was unable to activate RPM1 (Figure 4A). As a control in these experiments, we included the non-phosphorylatable AtRIN4<sup>T166A</sup> allele, which also retains partial activation of RPM1 in response to wild type AvrRpm1 (Figure 4A) (Chung et al., 2011).

AtRIN4 is predicted to be ADP-ribosylated by AvrRpm1 on D153, which is located in the C-terminal portion of AtRIN4 (AA142-211) previously demonstrated to be
sufficient for RPM1 activation (Chung et al., 2011). To test the requirement of AtRIN4 D153 in AvrRpm1-mediated RPM1 activation, we replaced this residue with alanine (D153A), which should block ADP-ribosylation at that site. This substitution reduced RPM1 activation to a level equivalent to AtRIN4<sup>T166A</sup> (Figure 4B). These results support a model in which ADP ribosylation of D153 promotes full phosphorylation on T166, a modification previously demonstrated to be sufficient for RPM1 activation (Chung et al., 2011). To further examine the relationship between D153 ribosylation and T166 phosphorylation, we constructed cis double mutants of AtRIN4<sup>D153A</sup> with either non-phosphorylatable T166, AtRIN4<sup>T166A</sup> (D153A T166A), or phosphomimic T166, AtRIN4<sup>T166D</sup> (D153A T166D). Assessing effector-independent RPM1 activation indicated that the AtRIN4 D153A T166D double mutant retained full activation of RPM1 (Figure 4C). We generated Arabidopsis transgenic plants expressing native levels of AtRIN4<sup>D153A</sup> (D153A) or AtRIN4<sup>N11AD153AT166A</sup> (N11A D153A T166A) mutants in an RPM1-<i>myc rpm1 rps2 rin4</i> background (N11 in AtRIN4 is equivalent to N12 in <i>GmRIN4b</i>). We activated RPM1 in leaves of these transgenics via delivery of AvrRpm1 from <i>P. syringae</i>. Conductivity measurements using two independent T2 transgenic lines expressing each AtRIN4 mutant demonstrated that AtRIN4<sup>D153A</sup> retained partial function in RPM1 activation, again at levels similar to that observed in plants expressing AtRIN4<sup>T166A</sup> (Figure 4D). AtRIN4<sup>N11AD153AT166A</sup>, which cannot be ribosylated and cannot be phosphorylated on T166, was a complete loss of function allele. These results indicate that ribosylation of D153 and phosphorylation of T166 act additively for complete RPM1 activation.

Arabidopsis transgenic lines expressing native levels of AtRIN4<sup>D153AT166D</sup> (D153A T166D) and AtRIN4<sup>N11AD153AT166D</sup> (N11A D153A T166D) displayed the dwarf phenotypes and diminution of RPM1 accumulation typical of RPM1 auto-immune responses previously attributed to AtRIN4<sup>T166D</sup> (gT166D) (Supplemental Figure 3B) (Chung et al., 2011). The combined results of Figures 4A-D and Supplemental Figures 3B-C indicate that AvrRpm1-mediated ribosylation of D153 contributes to activation of RPM1. Furthermore, the epistasis of the gain-of-function T166D mutation over D153A, which blocks ADP-ribosylation, is consistent with the hypothesis that ADP-ribosylation of D153 promotes phosphorylation of T166 during activation of RPM1 by AvrRpm1.
As expected, the \textit{AtRIN4} N11 residue had no effect on \textit{AvrRpm1}-mediated RPM1 activation, nor did N11A alter the RIN4 T166D RPM1 activation phenotype (Supplemental Figure 3C). We confirmed that the partial loss of \textit{AvrRpm1}-mediated RPM1 activation in RIN4 D153A was unlikely due to a structural defect, since this allele retains interaction with both \textit{AvrRpm1} and RPM1 in planta, as measured by co-immunoprecipitation (Supplemental Figure 3D). We also verified the expression of \textit{AvrRpm1}, RPM1 and RIN4 alleles used in effector-dependent or independent RPM1 activation assays in \textit{N. benthamiana}, again noting that expression of \textit{AtRIN4} T166D alleles results in less RPM1 accumulation due to RPM1 activation (Supplemental Figure 3B and 3E) (Chung et al., 2011).

We tested ADP-ribosylation and T166 phosphorylation in \textit{N. benthamiana} following co-infiltration of \textit{AtRIN4} with \textit{AvrRpm1}, \textit{AvrRpm1}^{D185A}, or \textit{AvrRpm1}^{AAA} mutants. We observed that \textit{AvrRpm1} catalytic activity is required for both ADP-ribosylation of \textit{AtRIN4} and for full phosphorylation of \textit{AtRIN4} T166 (Figure 4E).

We further tested whether the ability of \textit{AvrRpm1} to induce ADP-ribosylation is required for full phosphorylation of \textit{AtRIN4} T166 in \textit{N. benthamiana} (in the absence of RPM1) following transient expression of either \textit{AtRIN4} wild type or D153A with wild type \textit{AvrRpm1}. Following immuno-precipitation of \textit{AtRIN4} or D153A, we detected either RIN4 ADP-ribosylation or pT166 accumulation (using a phospho-specific antisera; (Chung et al., 2011). Results displayed in Figure 4F demonstrate that \textit{AvrRpm1} induces less ADP-ribosylation and T166 phosphorylation on the D153A derivative of \textit{AtRIN4} compared to wild-type \textit{AtRIN4}. Consistent with this observation, in the Arabidopsis transgenic \textit{RIN4} lines described above, we observed no ADP-ribosylation and drastically reduced T166 phosphorylation in lines complemented with \textit{AtRIN4}^{D153A} compared to lines complemented with wild-type \textit{AtRIN4} following delivery of \textit{AvrRpm1} from DC3000 (Figure 4G).

Lastly, we assessed whether \textit{AvrRpm1} ADP-ribosylation activity was required for a typical \textit{AvrRpm1}-mediated virulence activity, namely the suppression of callose deposition at sites of infection. We inoculated leaves of \textit{rpm1}-3 mutant Arabidopsis with DC3000(EV), (\textit{avrRpm1}), (\textit{avrRpm1}^{D185A}) or (\textit{avrRpm1}^{AAA}) and counted callose depositions 18 hours later. As displayed in Figure 4H, wild type \textit{AvrRpm1} suppressed
callose deposition triggered by DC3000, and this suppression required AvrRpm1
catalytic activity. These results confirm that AvrRpm1-dependent ADP-ribosylation of
RIN4, and likely other host NOI domain-containing targets, is relevant to its virulence
activity (Belkhadir et al., 2004).

Taken together, results in Figure 4E-H indicate that AvrRpm1-dependent ADP-ribosylation of
AtRIN4 D153 is required for full phosphorylation of AtRIN4 T166 to drive
RPM1 activation and for successful repression of plant defense responses in the
absence of RPM1.

\textbf{AtRIN4 Interacts with Multiple EXO70 Family Members via the C-Terminal NOI}

Domain

The Arabidopsis RIN4 protein can associate with the exocyst subunit EXO70B1
in an NOI-domain dependent manner (Sabol et al., 2017). EXO70B1 is a member of a
large family of EXO70 proteins in Arabidopsis (23 family members in total;
Supplemental Figure 4; Cvrčková et al., 2012). To assess whether AtRIN4 can interact
with other Arabidopsis EXO70 family members, we used yeast two-hybrid analysis to
test interactions with 11 members distributed across the Arabidopsis EXO70
phylogenetic tree (Supplemental Figure 4). Of these 11 pairwise tests, four showed a
robust interaction (EXO70B1, EXO70E1, EXO70E2 and EXO70F1 (Figure 5A)). To
assess whether this interaction was mediated by either NOI domain in RIN4, we
constructed two deletion derivatives of AtRIN4, one eliminating the first 141 amino
acids, including N-NOI, and one in which only C-NOI (amino acids 149-176) was
removed. The latter deletion eliminated the interaction with all four EXO70 proteins,
while the former did not, indicating that all of the RIN4-EXO70 interactions are mediated
by C-NOI (Figure 5B).

Because the RIN4:EXO70 interaction is mediated by C-NOI and this domain is
targeted by AvrRpm1, we tested whether AvrRpm1 could affect this interaction by
transiently expressing Arabidopsis and soybean RIN4 proteins with Arabidopsis EXO70
proteins in \textit{N. benthamiana} and performing co-immunoprecipitation analyses. We
observed that all four soybean RIN4 proteins and AtRIN4 co-immunoprecipitated with
Arabidopsis EXO70B1 in the absence of AvrRpm1 (Figure 5C). \textit{AtRIN4} also co-
immunoprecipitated with EXO70E1 (Supplemental Figure 5). Co-expression with AvrRpm1 strongly reduced these interactions for AtRIN4, GmRIN4a, GmRIN4b, and GmRIN4c, but had no effect on the interactions of EXO70 proteins with GmRIN4d. However, we also observed that the AvrRpm1D185A and AvrRpm1AAA mutants blocked RIN4-EXO70 interactions (Figure 5C and Supplemental Figure 5). This latter result indicates that AvrRpm1 and EXO70 compete for binding to the C-NOI domain, and that when AvrRpm1 is overexpressed, it can block the interaction with EXO70. This may not be a biologically relevant result, considering that AvrRpm1 is likely delivered at much lower levels during a natural infection. Never-the-less, it points to a common interaction surface on RIN4. Perhaps related to this observation, the NLR protein RPS2, which is inhibited by RIN4 and is typically only weakly activated by AvrRpm1, is strongly activated by inactive mutants of AvrRpm1, perhaps through competition for binding to RIN4 (Kim et al., 2009; Cherkis et al., 2012).

**EXO70 Proteins Contribute to flg22-Induced Callose Deposition in Cell Walls**

Prior work has shown that transgenic overexpression of either AvrRpm1 or AtRIN4 in Arabidopsis suppresses flg22-induced callose secretion (Kim et al., 2005b). The observation that RIN4 proteins interact with EXO70 proteins raised the possibility that callose secretion may depend on EXO70 function, and that RIN4 may regulate callose secretion via its interactions with EXO70. We therefore quantified the level of flg22-induced callose deposition in various single, double, and triple exo70 mutants (see Supplemental Figure 6 for details on the mutations). Figure 5D shows that knockout of either EXO70B1 or EXO70E2 significantly reduced callose deposition, while knockout of EXO70E1 or knockdown of EXO70F1 did not, indicating that a subset of EXO70 proteins contribute to callose deposition.

**Mutation of AtRIN4 T166 Stabilizes RIN4-EXO70 Interactions**

Because overexpression AvrRpm1 in Arabidopsis has the same effect on callose deposition as overexpression of AtRIN4, we hypothesized that AvrRpm1 may be suppressing callose deposition by enhancing the interaction of AtRIN4 with EXO70. As described above, however, transient overexpression of AvrRpm1 in *N. benthamiana*
had the opposite effect, possibly due to the artificially high levels of AvrRpm1 resulting in competition for binding to the C-NOI domain of RIN4. To circumvent this issue, we used a yeast two-hybrid assay to test whether a T166D phosphomimic substitution in AtRIN4 would promote the AtRIN4-EXO70 interaction, using the rationale that since AvrRpm1 promotes T166 phosphorylation, this might function to stabilize the AtRIN4:EXO70 interaction. These analyses showed that AtRIN4^{T166D} interacted with EXO70E2 more strongly than did wild-type AtRIN4 (Figure 6), supporting the model that AvrRpm1 suppresses callose deposition by enhancing the affinity of RIN4 for specific EXO70 proteins (Figure 7).

**DISCUSSION**

Our conclusion that AvrRpm1 functions as an ADP-ribosyltransferase is supported by the work of Cherkis et al. (2012), who generated computational models for AvrRpm1 using an alignment with the catalytic domains of several PARP containing proteins, including several members of the Diphtheria toxin-like family of proteins as well as the human PARP-1 protein. These analyses showed that AvrRpm1 may contain a fold homologous to other ADP-ribosyltransferases. Significantly, mutation of the predicted catalytic residues in AvrRpm1 eliminated the ADP-ribosyl transferase activity (Figure 4A), which is consistent with the observation of Cherkis et al. (2012) that these mutations strongly attenuate AvrRpm1’s ability to activate RPM1. These data indicate that AvrRpm1 is indeed acting as an ADP-ribosyltransferase. However, we were unable to confirm this with purified proteins due to instability of recombinant AvrRpm1 protein (Cherkis et al., 2012).

The *Pseudomonas* effectors HopF1 and HopF2 both share a PARP domain fold that is similar to diphtheria toxin (Singer et al., 2004; Wang et al., 2010). Although the enzymatic activity of HopF1 has not been described, HopF2 has been shown to have ADP-ribosyltransferase activity and to directly ADP-ribosylate Arabidopsis MAP Kinase Kinase 5 (M KK5) at R313 (Wang et al., 2010). This results in suppressing M KK5 kinase activity and inhibits PAMP-induced MAP Kinase activation (Wang et al., 2010). Interestingly, it has also been shown that HopF2 is capable of directly ADP-ribosylating AtRIN4 *in vitro*; however, the residues modified were not identified (Wang et al., 2010;
Wilton et al., 2010). They are presumably different from the residues modified by AvrRpm1, because HopF2 does not activate RPM1 and, unlike AvrRpm1, does interfere with AvrRpt2-induced cleavage of RIN4 and the resultant activation of RPS2 (Wilton et al. 2010; Ritter et al. 1996). Notably, however, transgenic expression of HopF2 in Arabidopsis suppresses callose deposition induced by a type III secretion system deficient mutant of *P. syringae* strain DC3000, and this ability is dependent on ADP-ribosyl transferase activity (Hurley et al., 2014). It is tempting to speculate that HopF2-mediated modification of RIN4 also serves to suppress EXO70 function.

Our mass spectrometry data indicates that *Gm*RIN4b is ADP-ribosylated twice, once within each NOI domain present in *Gm*RIN4b: FGWNDSGENVPYTAYFDK and FGWDVNNPASADGFTHIFNK (Figure 2) (Afzal et al., 2013). The C-NOI domain of *At*RIN4 has previously been shown to mediate the association of *At*RIN4 with the exocyst subunits EXO70B1 and EXO70E1 at the plasma membrane (Sabol et al., 2017). The role of RIN4-interacting EXO70 proteins in the secretion of defense compounds is supported by our finding that EXO70B1 and EXO70E2 are required for full induction of callose by the flagellin derived peptide flg22 (Figure 5). Because RIN4 is known to suppress flg22-induced callose deposition (Kim et al., 2005b), the finding that RIN4 physically associates with EXO70 indicates that this association acts to inhibit EXO70’s role in callose deposition. Thus, modification of RIN4 by AvrRpm1 should function to enhance this inhibitory activity (Figure 7). Consistent with this model, transgenic expression of AvrRpm1 also suppresses flg22-induced callose deposition (Kim et al., 2005b). We speculate that ADP-ribosylation of RIN4 functions to promote the association of RIN4 with EXO70 proteins, either by a direct effect of the ADP-ribose moiety, and/or by promoting phosphorylation on T166. In support of the latter, a T166D phosphomimic substitution in *At*RIN4 enhanced its interaction with EXO70E2 in a yeast two-hybrid assay (Figure 6). However, the T166D substitution appeared to have a slightly deleterious effect on the interaction of *At*RIN4 with EXO70B1, possibly indicating that ADP-ribosylation on D153 may be required to stabilize this interaction. Although further work is needed to solidify our model that ADP-ribosylation and/or T166 phosphorylation function to enhance *At*RIN4-EXO70 interactions, it is notable that knockout of *EXO70B2* enhances the susceptibility of Arabidopsis to infection by *P.*
syringae strain DC3000, indicating that EXO70 proteins play a role in basal defenses (Stegman et al., 2012). It thus makes sense that EXO70 proteins may be direct or indirect targets of bacterial effectors.

Consistent with EXO70 proteins being targeted by effectors, EXO70B1 is known to associate with an NLR-family member named TN2 (Zhao et al., 2015), suggesting that EXO70B1 is being “guarded”. Indeed, knock-out of EXO70B1 activates TN2-mediated cell death, at least in older plants grown under short days (Zhao et al., 2015). Under our growth conditions, we observed less exaggerated macroscopic cell death in 6 week-old exo70b1 plants and no symptoms yet in the 5 week-old plants used for measuring callose deposition.

EXO70-NOI domain interactions in the context of plant immunity have also recently been reported in rice. The rice NLR protein Pii-2 contains an NOI domain that appears to function as an integrated ‘decoy’ to sense a fungal effector protein (Fujisaki et al., 2017). In that system, the Pii-2 NOI domain interacts with a rice EXO70 protein, OsEXO70-F3, which is targeted by the AVR-Pii effector protein from the fungus Magnaporthe oryzae. Mutations in the NOI core motif of Pii-2 (PxFGxWD) eliminated its interaction with OsEXO70-F3 and detection of AVR-Pii. Notably, AvrRpm1 modifies both RIN4 NOI domains within this core motif, on the N or D residue between the G and W (Figure 2). The finding that, similar to AvrRpm1 from P. syringae, an effector protein from M. oryzae perturbs an NOI-EXO70 interaction indicates that this type of perturbation is likely a virulence strategy of diverse pathogens.

Although our data strongly support our conclusion that ADP-ribosylation of AtRIN4 on D153 promotes phosphorylation on T166, we did not observe AvrRpm1-induced phosphorylation of GmRIN4b in our mass-spectrometry analyses when these two proteins were overexpressed in N. benthamiana (Supplemental Dataset 1). We believe this may be because we did not also overexpress an appropriate kinase protein capable of this phosphorylation, such as RIPK. In support of this, we failed to detect a RIN4 size shift when we co-expressed soybean or Arabidopsis RIN4s with AvrB in N. benthamiana. Either RIPK function is not conserved in N. benthamiana, or its native expression levels are not sufficient to modify the majority of the overexpressed RIN4 protein.
RIN4 cannot be the only target of AvrRpm1, however, because AvrRpm1 enhances the virulence of *P. syringae* even in a *rin4* mutant background (Ritter and Dangl, 1995; Belkhadir et al., 2004). In addition to RIN4, there are 14 NOI-domain containing proteins found in Arabidopsis, which were initially identified from a screen searching for nitrate-induced genes. Aside from possessing an NOI domain, which includes two highly conserved PxFGxWD and F/YTxxFxK motifs, and a likely prenylation or palmitoylation sequence near their C-termini, these proteins do not share homology with one another and their functions are unknown (Afzal et al., 2013). Our data show that AvrRpm1 can ADP-ribosylate many of these proteins, which might then contribute to the enhanced virulence observed for *Pseudomonas* strains expressing *avrRpm1* on susceptible Arabidopsis plants lacking *AtRIN4* (Ritter and Dangl, 1995; Belkhadir et al., 2004).

Prior work in independent laboratories has shown that AvrRpm1 induces hyperphosphorylation of *AtRIN4* (Mackey et al., 2002; Chung et al., 2011; Liu et al., 2011; Chung et al., 2014). Our observations that AvrRpm1 induces ADP-ribosylation of RIN4 and that this event is required for full phosphorylation, as well as the additive loss of function phenotype in transgenic plants expressing native levels of an *AtRIN4* mutant that cannot be ribosylated at D153 or phosphorylated at T166, are consistent with a model in which ribosylation promotes subsequent phosphorylation. We speculate that ADP-ribosylation of *AtRIN4* on D153 induces a change in conformation and/or subcellular localization that enables or enhances phosphorylation of T166 by endogenous Arabidopsis kinases such as RIPK and related RLCKs (Chung et al., 2011; Liu et al., 2011; Xu et al., 2017; Toruño et al., 2019). Under this model, maximal phosphorylation of T166 in response to AvrRpm1 would be dependent on ADP-ribosylation of D153. Consistent with this prediction, a D153A substitution reduced AvrRpm1-induced phosphorylation of T166 (Figure 4).

Alterations in conformation and/or localization resulting from T166 phosphorylation may explain how RPM1 can detect two sequence unrelated effectors, AvrB and AvrRpm1 (Grant et al., 1995). AvrB also induces hyperphosphorylation on T166 (Chung et al., 2011; Liu et al., 2011), and T166D substitutions are sufficient for activating RPM1 (Chung et al., 2011; Liu et al., 2011). The assumption that
phosphorylation of T166 alters conformation and/or localization of RIN4 is supported by
the finding that pT166 inhibits phosphorylation on S141, an event that is required to
relieve inhibition of MAMP-triggered responses (Chung et al., 2014). Collectively, these
data indicate that RPM1 is activated by an alteration of RIN4 that results from T166
phosphorylation that is itself promoted by ADP ribosylation within the C-NOI domain. In
this context, it should be noted that AvrB also binds to C-NOI (Desveaux et al., 2007),
and we speculate that AvrB modifies C-NOI in a way that promotes T166
phosphorylation. AvrB contains a filamentation-induced by c-AMP (Fic) domain, which
is a domain known to post-translationally modify proteins through AMPylation,
UMPylation, phosphorylation, or phosphocholination (Garcia-Pino et al., 2014).
AMPylation of C-NOI would be structurally similar to the addition of ADP ribose.

In soybean, recognition of AvrB and AvrRpm1 is divided amongst two NB-LRR
proteins, RPG1b and RPG1r, respectively (Ashfield et al., 1995). RPG1b and RPG1r
are closely related paralogs, but are distantly related to RPM1 (Ashfield et al., 2014).
Phylogenetic analyses indicate that the ability to recognize AvrB and AvrRpm1 evolved
independently in soybean and Arabidopsis (Ashfield et al., 2004; Ashfield et al., 2014).
Because of this, it is plausible that RPG1b and RPG1r have evolved molecular
mechanisms to detect AvrB and AvrRpm1 that differ from those used by RPM1.
However, RPM1 and RPG1r both recognize the ADP-ribosylation activity of AvrRpm1
(Figure 3), and both require RIN4, thus the molecular mechanism underlying recognition
appear similar. Notably, AvrB does not possess ADP-ribosylation activity (Figure 3),
thus it is plausible that RPG1r directly senses ADP-ribosylation of RIN4, while RPG1b
senses the specific modification catalyzed by AvrB (possibly AMPylation or
UMPylation). This explanation makes sense in evolutionary terms, since the two RPG1
loci are the result of a recent duplication event in soybean (Ashfield et al., 2014). In this
scenario, it is possible that the initial RPG1 locus encoded an NLR protein that
responded to ADP-ribosylation of RIN4, as this appears to be the more ancestral
specificity (Ashfield et al., 2014). Following gene duplication, the second RPG1 locus
was maintained because it evolved a new specificity, enabling recognition of AvrB-
modified GmRIN4, giving rise to RPG1b.
METHODS

Plant Materials and Growth Conditions

Nicotiana benthamiana seeds were sown in a 4:1:1 ratio of ProMix-B Biofungicide (Premier Horticulture) potting mix:vermiculite:perlite supplemented with Osmocote 14-14-14 slow-release fertilizer. Plants were grown in growth rooms under a 12-h light/12-h dark cycle (24 °C day/22 °C night). Illumination was provided by GE HI-LUMEN XL Starcoat 32 watt fluorescent bulbs (a 50:50 mixture of 3,500K and 5,000K spectrum bulbs; 9 hour days, ~150 µmol/m²/s at the canopy surface). Plants were used for transient expression assays at 3-4 weeks of age.

For Arabidopsis thaliana transgenic lines, all AtrIN4 mutants were expressed from the native promoter and transformed into an RPM1-myc rpm1 rps2 rin4 background. T2 lines segregating 3:1 upon basta selection were confirmed and matched for expression of RPM1 and AtrIN4 alleles by immunoblot with anti-myc or anti-T7, respectively. Three plants with similar expression of RPM1 and AtrIN4 mutant alleles were used for conductivity measurements to monitor RPM1 activation upon P. syringae strain DC3000 (avrRpm1)-infection. Previously generated transgenic lines in the same mutant background expressing wild type AtrIN4, AtrIN4T166A or AtrIN4T166D were used to compare effector-dependent and independent RPM1-activation with Arabidopsis transgenic plants generated in this study (Chung et al., 2011). Seeds from these lines were sown in a custom potting mix prepared from 4 1/2 parts sterilized Sungrow Professional Growing Mix (Sun Gro Horticulture), 2 parts white sterilized sand (Mellotts, Carrboro, NC), 1 part Horticultural perlite (Carolina Perlite Company, Gold Hill, NC), 250 PPM Peter’s special 20-20-20 fertilizer (Everris NA Inc. Dublin, OH) and 75 mg/ft³ Marathon Pesticide (1% granular; OHP, Inc., Mainland, PA). Plants were grown in growth chambers under a 9-h light/15 h dark cycle (21 °C day/18 °C night), Illumination was provided by Westinghouse cool white fluorescent bulbs (F32T8/741/ECOMAX) at ~100 µmol/m²/s at the canopy surface.

For Arabidopsis exo70 mutants, the following T-DNA insertion lines were used: exo70b1-3 (At5g58430; Zhao et al., 2015); exo70e1 (At3g29400), SALK_084145; exo70e2-2 (At5g61010), SK26782; exo70f1-1 (At5g50380), SALK_139110. See Supplemental Figure 6 for locations of T-DNA insertions and verification of mRNA
knockdown. All lines were obtained from the Arabidopsis Biological Resource Center at The Ohio State University, except for exo70b1-3, which was provided by Dr. Dingzhong Tang (Zhao et al., 2015). Double and triple exo70 mutants were generated by crossing the relevant lines and selecting homozygous segregants in the F2 generation using PCR.

Arabidopsis exo70 mutant seed were sown in a 50/50 mix of ProMix BX (Premier Horticulture) and MetroMix 360 (Sungro Horticulture) with ~35g of Osmocote 14-14-14 slow release fertilizer per flat. Plants were grown in growth chambers under an 8-h light/16-h dark cycle (24ºC day/17-18ºC night). Illumination was provided by white T5 fluorescent bulbs with an intensity of ~300 µmol/m²/s at canopy height.

**Construction of Plasmid Clones**

To generate cDNA clones of soybean RIN4 genes, total RNA was isolated from partially expanded trifoliate soybean Williams-82 leaves using the Total RNA Spectrum Kit (Sigma) and cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer’s instructions. Specific GmRIN4 sequences were then amplified from this cDNA using the primers listed in Supplemental Table 1 using Phusion High-Fidelity DNA Polymerase (ThermoFisher). PCR products were cloned into the Gateway entry vector pBSDONR P4rP2 (Qi et al., 2012) using the BP Clonase II kit (ThermoFisher). Entry clones were then recombined with the dexamethasone inducible vector pTA7001-Dest (Vinatzer et al., 2006) and N-terminal epitope tags 3xHA, 5xMyc, or sYFP in pBSDONR P1P4 (Qi et al., 2012) using the LR Clonase II kit (ThermoFisher).

AtRIN4 and AtNOI genes were also cloned using the Gateway system (Chung et al., 2011). All AtRIN4 mutants were generated via site-directed mutagenesis by using genomic AtRIN4 as a template and cloned into the entry vector pDONR207 (ThermoFisher)(Chung et al., 2011). To introduce multiple mutation sites, single mutant entry clones of AtRIN4 (T166A or T166D) were used as a template. For AtNOI genes, all intron and exons were amplified and cloned into the entry vector pENTR-D-TOPO, followed by LR recombination into a destination vector containing a T7 epitope-tag at the N-terminus under control of a 35S promoter.
To generate the AvrRpm1 triple mutant, site-directed mutagenesis was performed by using an AvrRpm1 H63A single mutant clone to amplify an N-terminal fragment with the Y122A mutation, and an AvrRpm1 D185A single mutant clone to amplify a C-terminal fragment with the Y122A mutation, followed by cloning the full-length triple mutant fragment into pENTR-D-TOPO vector (Invitrogen, Carsbad, CA; Cherkis et al., 2012). All entry clones were then cloned into the pBAR-GW destination vector for AtRIN4 mutants and the pMDC7 destination vector for \textit{avrRpm1} mutants. All primers used for site-directed mutagenesis are listed in Supplemental Table 1.

To generate EXO70 clones for yeast two-hybrid analyses, Entry clones for EXO70A1(G20002), EXO70B1(G21983), EXO70B2(G21656), EXO70D2(G24035), EXO70E2(G13181), EXO70G1(G22207), and EXO70H1(G19572) were obtained from the Arabidopsis Biological Resource Center at Ohio State. Entry clones for EXO70C1, EXO70C2, EXO70E1, and EXO70F1 were generated by PCR with Pfu DNA polymerase (Stratagene), using Arabidopsis seedling cDNA as template and cloned into pCR-CCD-F. The primer pairs used for plasmid constructs are listed in Supplemental Table 1. These entry clones were then recombined into the destination vector pDEST22 using LR Clonase (ThermoFisher).

To generate EXO70 clones for transient expression in \textit{N. benthamiana}, the above entry clones were recombined into the destination vector pCsVMV;HA-C-1300, which contains a CsVMV promoter and 3xHA C-terminal epitope tag (Kim et al., 2013).

\textbf{Agrobacterium-Mediated Transient Expression in \textit{N. benthamiana} or \textit{N. glutinosa}}

\textit{Agrobacterium tumefaciens} transformed with the pTA7001 constructs carrying the wild-type \textit{GmRIN4s}, \textit{AvrB}, \textit{AvrRpm1}, or empty vector were in strain GV3101(pMP90). Expression of the pSITE constructs carrying the phosphomimetic or phosphodeficient \textit{GmRIN4b} alleles were in \textit{A. tumefaciens} strain LBA440 (Selote et al., 2013). Liquid cultures of all Agrobacterium strains were initially grown overnight at 30°C with agitation in LB media supplemented with the appropriate antibiotics (50 μg/ml kanamycin and 50 μg/ml gentamicin for all pTA7001 constructs, or 100 μg/ml rifampicin, 100 μg/ml spectinomycin, and 50 μg/ml streptomycin for pSITE constructs).
For transient expression in *N. benthamiana*, the overnight cultures were subcultured the following morning at a dilution of 1:10 in fresh LB media (without antibiotics) and grown for an additional 5 hours at 30°C. The bacterial cells were pelleted by centrifugation at 4,000 rpm for 5 minutes. The pellet was washed once in 10 mM MgCl₂ before resuspending the Agrobacterium strains to an optical density at 600 nm (OD₆₀₀) of 0.8 in 10 mM MgCl₂. The strains harboring a *GmRIN4* construct were mixed in a 1:1 ratio with strains carrying an effector construct (or empty vector) directly before fully injecting the plant leaves. The infiltrated plants were then returned to the growth room. Transgene expression was induced within 36 hours after infiltration by spraying plants with 50 μM dexamethasone. Tissue was collected within 3.5 hours after induction and either directly prepared for analysis by immunoblot or mass spectrometry or immediately frozen in liquid nitrogen and stored at -80°C.

The *N. benthamiana* transient assay for RPM1 activation by AvrRpm1 and/or *AtRIN4* alleles was performed by infiltrating the Agrobacterium at OD₆₀₀ of 0.1, 0.3 and 0.3 for strains carrying the AvrRpm1 alleles, *AtRIN4* alleles or RPM1, and 0.1 for a strain carrying the viral p19 suppressor of genes silencing, respectively. To induce the expression of AvrRpm1 alleles, 20 μM of β-estradiol (Est) was applied by spray onto the infiltrated leaves. For the immunoblots with anti-HA for AvrRpm1, anti-panADPR for *AtRIN4* ribosylation and anti-pT166 for *AtRIN4* T166 phosphorylation, the same infiltration condition was applied for AvrRpm1 alleles and *AtRIN4* alleles without RPM1.

For transient expression in *N. glutinosa*, the overnight cultures were pelleted by centrifugation and washed once in 10 mM MgCl₂ before being resuspended in 10 mM MgCl₂ supplemented with 100 μM acetosyringone. The bacterial suspension was incubated for 3-4 hours at room temperature before it was diluted to an OD₆₀₀ of 0.3. The strains with a *GmRIN4* construct were mixed in a 1:1 ratio with strains carrying an effector construct (or empty vector) before infiltration. Transgene expression was induced 40 hours after infiltration by spraying plants with 50 μM dexamethasone with 0.02% (v/v) Silwet L-77 (OSi Specialties) surfactant added. Tissue was collected 4 hours after induction and prepared for immunoblot analysis.

**Immunoblot Analyses**
Total protein was extracted from whole *N. benthamiana* or *N. glutinosa* leaves in 4x (w/v) lysis buffer (150mM NaCl, 50 mM Tris [pH 7.5], 0.1% Nonidet P-40, 1% Plant Protase Inhibitor Cocktail (Sigma), 1% 2,2'-Dithiodipyridine) and separated on 8% polyacrylamide gels supplemented with 50 μM Phos-tag reagent (Wako Laboratory Chemical), or on 4-20% Tris-glycine gradient gels (Bio-Rad). Proteins were transferred to a nitrocellulose membrane (GE Healthcare Life Sciences) and then detected with a 1:4,000 diluted peroxidase conjugated anti-HA antibody (rat monoclonal, Roche catalog no. 12013819001), a 1:4,000 diluted peroxidase conjugated anti-c-Myc antibody (mouse monoclonal, ThermoFisher catalog no. MA1-81357), or a 1:2,000 diluted unconjugated anti-GFP antibody (mouse monoclonal, Novus Biologicals catalog no. NB600597), which was detected using 1:5,000 diluted goat anti-mouse peroxidase conjugated secondary antibody (ThermoFisher catalog no. 31460). To detect ADP-ribosylated proteins, we used a 1:5,000 dilution of an anti-pan-ADP-ribose binding reagent (anti-panADPR; EMD Millipore catalog no. MABE1016). Immunoblots with anti-T7-HRP (Milipore Sigma catalog no. 69048) or anti-HA-HRP (Santa Cruz catalog no. sc-7392 HRP) to detect *AtRIN4* or *AvrRpm1* alleles were performed as described in Chung et al., (2011). The immunoblots were visualized using the Clarity Western ECL Substrate (BioRad) or the ECL prime (Amersham).

**Immunoprecipitation of GmRIN4b, AtRIN4, and NOI proteins**

N-terminally tagged sYFP-GmRIN4b was transiently expressed with AvrRpm1 in *N. benthamiana* as described above. Total protein was extracted from whole *N. benthamiana* leaves in 4x (w/v) lysis buffer (150mM NaCl, 50 mM Tris [pH 7.5], 0.1% Nonidet P-40; Sigma, 1% Plant Protease Inhibitor Cocktail; Sigma, 1% 2,2'-Dithiodipyridine; Sigma, 0.5 mM EDTA) using a ceramic mortar and pestle. The crude lysate was filtered through a double layer of Miracloth and then centrifuged for 5 minutes at 10,000 x g (at 4°C) twice to remove plant debris. The clarified extract was then incubated with 25 μl of GFP-Trap A (Chromotek) anti-GFP bead slurry for 1 hour at 4°C with constant end-over-end tumbling. The beads were pelleted by centrifugation at 2,500 x g for 2 minutes at 4°C and washed at least three times with 20x bed-volumes of...
wash buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 0.1% Nonidet P-40, 1% 2,2'-Dithiodipyridine; Sigma, 0.5 mM EDTA).

To prepare GmRIN4b samples for mass spectrometry, an on-bead digestion method was used. Briefly, GmRIN4b-bound anti-GFP beads were washed with 50 mM ammonium bicarbonate (AMBIC; pH 8.0) at least three times and then incubated with 2x bed-volumes of 50 mM AMBIC with 50 mM dithiothreitol (DTT) for 1 hour at 60°C. The AMBIC plus DTT solution was removed and then the beads were incubated in the dark with 2x bed-volumes of 50 mM AMBIC with 50 mM iodoacetamide for 1 hour at room temperature. To digest the proteins, 250 ng of Trypsin (Promega) was added to the beads and they were incubated overnight at 37°C. To collect the digested peptides, the samples were centrifuged for 3 minutes at 3,000 x g and the supernatant was transferred to a fresh Eppendorf tube. The beads were incubated with 2x bed-volumes of 60% acetonitrile with 0.1% trifluoroacetic acid for an additional 10 minutes at room temperature. The samples were centrifuged for 3 minutes at 3,000 x g and the supernatant was added to the new Eppendorf tube.

Immunoprecipitation or co-immunoprecipitation with anti-T7 antibody for AtRIN4 and AtNOI proteins was performed as described (Chung et al., 2011). Agrobacteria expressing AvrRpm1, RPM1, AtRIN4 alleles and NOI proteins were infiltrated into N. benthamiana leaves at OD_{600} of 0.1, 0.3, 0.3 and 0.3, respectively. Leaf samples for immunoprecipitation or co-immunoprecipitation were collected 12 hours post estradiol-induction for the expression of AvrRpm1 alleles. For immunoprecipitation by anti-RIN4 with Arabidopsis tissue samples was done as described previously (Mackey et al., 2002).

Mass Spectrometry and Data Analysis

Tryptic peptides were injected into an Easy-nLC 100 HPLC system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific, Bremen, Germany). More specifically, peptide samples were loaded onto an Acclaim PepMapTM 100 C18 trap column (75 μm x 20 mm, 3 μm, 100 Å) in 0.1% formic acid. The peptides were separated using an Acclaim PepMapTM RSLC C18 analytical column (75 μm x 150 mm, 2 μm, 100 Å) using an acetonitrile-based gradient (Solvent A: 0% acetonitrile, 0.1%
formic acid; Solvent B: 80% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min. A 30 min gradient was performed as follows: 0-0.5 min, 2-8% B; 0.5-24 min, 8-40% B; 24-26 min, 40-100% B; 26-30 min, 100% B, followed by re-equilibration to 2% B. Electrospray ionization was then carried out with a nanoESI source at a 275 °C capillary temperature and 1.9 kV spray voltage. The mass spectrometer was operated in data-dependent acquisition mode with mass range 400 to 2000 m/z. Precursor ions were selected for tandem mass (MS/MS) analysis in the Orbitrap with 3 sec cycle time using higher-energy collisional dissociation (HCD) at 28% collision energy as described by (Rosenthal et al., 2015). The intensity threshold was set at 5 x 10^4. The dynamic exclusion was set with a repeat count of 1 and exclusion duration of 30 s. The resulting data were searched in Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm) against the Glycine max Rin4B sequence. Carbamidomethylation of cysteine residues was set as a fixed modification. Protein N-terminal acetylation, oxidation of methionine, protein N-terminal methionine loss, pyroglutamine formation, phosphorylation on STY and ADP-ribosylation on CDEKNRST were set as variable modifications. A total of three variable modifications were allowed. Trypsin digestion specificity with one missed cleavage was allowed. The mass tolerance for precursor and fragment ions was set to 10 ppm for both. Peptide and protein identification cut-off scores were set to 15 and 22, respectively. Marker ions (ADP-ribose fragments) at m/z 136.0632, 250.0940, 348.0709, 428.0372 were used to confirm the existence of ADP-ribose on a given peptide (Rosenthal et al., 2015).

Yeast Two-Hybrid Analyses

The ProQuest yeast two-hybrid system (Invitrogen) was used. The full-length AtRIN4 coding sequence and the derivatives of AtRIN4 (RIN4 1Δ141 and RIN4 149Δ176) were cloned into the bait vector (pDEST32). EXO70 family genes from Arabidopsis (EXO70A1, EXO70B1, EXO70B2, EXO70C1, EXO70C2, EXO70E1, EXO70E2, EXO70F1, EXO70D2, EXO70G1 and EXO70H1) were cloned into the prey vector (pDEST22). The interaction between AtRIN4 and AvrB was used as positive control. The bait and prey vectors were co-transformed into yeast strain MaV203 in different combinations, and the transformants were selected on synthetic dextrose
medium without Leu and Trp (SD-Leu-Trp). The positive clones were plated on SD-Leu-Trp-His medium including 25 mM 3-amino-1,2,4-triazde (3AT, Sigma-Aldrich), or SD-Leu-Trp-Ura medium. Additionally, clones were subjected to X-gal assays following manufacturer’s instructions. For immunoblot detection of full length and derivatives of AtRIN4 proteins, total protein was extracted from yeast, as previously described (Horvath and Riezman, 1994). EXO70-GAL4 fusions were detected using anti-GAL4 monoclonal antibody (Takara catalog no. 630402), while RIN4 fusions were detected using anti-RIN4 antibody (Mackey et al., 2002).

**Co-immunoprecipitation Analyses**

Protein co-immunoprecipitation analyses were performed as previously reported using GFP-Trap A (Chromotek) anti-GFP beads (Carter et al., 2018), except that Myc-Trap A (Chromotek) anti-Myc beads were used and incubation with beads was performed for only one hour rather than overnight. Following incubation, beads were pelleted by centrifugation at 3,000 x g for 2 minutes at 4°C. Bead pellets were washed five times with 500 µl of IP wash buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% Glycerol, 1 mM DTT, 1 mM EDTA, 1% NP40, 0.1% Triton X-100). After the last wash, the immune-complexes were re-suspended in 40 µl of IP wash and 10 µl of 5X SDS loading buffer. Protein samples were denatured at 95°C for 10 minutes and were resolved on a 4-20% gradient Precise™ Protein Gels (Thermo Scientific, Waltham, MA). Gels were run for 1 hour at 150 V in 1X Tris/Glycine/SDS running buffer. Total protein was transferred to a nitrocellulose membrane (GE Water and Process Technologies, Trevose, PA) for 45 minutes at constant amperage 200 mA. Membranes were blocked with 5% Difco™ Skim Milk (BD, Franklin Lakes, N) for one hour. Proteins were detected with 1:5,000 anti-pan-ADP-ribose binding reagent (anti-pan ADPR; EMD Millipore catalog number MABE1016) or 1:5,000 horseradish peroxidase conjugated anti-HA antibody (rat monoclonal, Roche, catalog number 12013819001) or 1:5,000 diluted peroxidase-conjugated anti-c-Myc antibody (mouse monoclonal, Thermo Fisher Scientific, catalog number MA1-81357) for 1 hour. After one hour membranes were washed in 1X Tris-buffered saline (pH 7.5) solution containing 0.1% Tween 20 (TBST) three
times for 10 minutes. Membranes were incubated with 1:5,000 horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody depending on primary antibodies for one hour and then washed three times for 10 minutes in TBST solution. Protein bands were imaged using a Immuni-Star™ Reagents (Bio-Rad, Hercules, CA) or Supersignal® West Femto Maximum Sensitivity Substrates (Thermo Scientific, Waltham, MA) and X-ray film.

Electrolyte Leakage Assays

Ion leakage measurements for hypersensitive response assays in N. benthamiana or Arabidopsis were performed as described previously (Chung et al., 2011). Leaf disks were collected 2 hours post estradiol-induction. Four leaf discs were sampled and submerged into 6 mL of double distilled water with three replicates per treatment (n=12 leaf disks). Conductivity was measured using a conductivity meter (Orion, model 130) at the indicated time points. Error bars represent 2 x SE. One-way ANOVA analyses were performed to calculate significant differences (Supplemental Dataset 2).

Callose Deposition Analyses

Leaves from ~5-week-old plants were syringe-infiltrated with 100 μM flg22 or distilled water and collected after 16 hr. To visualize callose deposition, leaves were stained with aniline blue (Sigma-Aldrich) as described previously (Jin and Mackey, 2017). In short, leaves were cleared with lactophenol (mixture of 1 volume of 1:1:1:1 glycerol, saturated phenol, lactic acid, and deionized water with 2 volumes of 95 % ethanol) and washed with 50% ethanol and then water. Callose was stained with 0.01 % aniline blue in 150 mM K2HPO4 (pH 9.5). Stained leaves were mounted in 50% glycerol and visualized with a Nikon eclipse 80i microscope. The number of callose deposits was counted using ImageJ software (http://rsbweb.nih.gov/ij/).

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number(s): AvrRpm1 (NP_114197), GmRIN4a (Glyma03g19920; NP_001235221), GmRIN4b (Glyma16g12160; NP_001239973), GmRIN4c (Glyma18g36000; NP_001235235), GmRIN4d (Glyma08g46400; NP_001235252),
AtRIN4 (AT3G25070; NP_001325873), AtEXO70A1 (AT5G03540; NM_120434),
EXO70B1 (AT5G58430; Q9FGH9); EXO70B2 (AT1G07000; NM_100573), EXO70C1
(AT5G13150; NM_121318), EXO70C2 (AT5G13990; NM_121402.2), EXO70D2
(AT1G54090; NM_104286.3), EXO70E1 (AT3G29400; AEE77581), EXO70E2
(AT5G61010; Q9FNR3), EXO70F1 (AT5G50380; AED95937), EXO70G1 (AT4G31540;
NM_119303.3), EXO70H1 (AT3G55150; NM_115373.4), NOI1 (AT5G63270;
AED97726), NOI2 (AT5G40645; AED94577), NOI3 (AT2G17660; AEC06662), NOI4
(AT5G55850; O22633), NOI5 (AT3G48450; AEE78418), NOI6 (AT5G64850; ABI49424
), NOI7 (AT5G09960; ABD38888), NOI8 (AT5G18310; BAH19857), NOI9 (AT5G48500;
AED95678), NOI10 (AT5G48657; AAO44036), NOI11 (AT3G07195; AAS76281), NOI12
(AT2G04410; AEC0583), NOI13 (AT4G35655; AEE86545), and NOI14 (AT5G19473;
AED92713).

Supplemental Data

Supplemental Figure 1. RIN4 is Highly Conserved Between Species.

Supplemental Figure 2. NOI Domains From Arabidopsis NOI Proteins.

Supplemental Figure 3. ADP-ribosylation of AtRIN4 on D153 Promotes
Phosphorylation on T166.

Supplemental Figure 4. Phylogenetic Tree of Arabidopsis EXO70 Family
Members.

Supplemental Figure 5. Overexpression of AvrRpm1 Blocks Association of AtRIN4
with Arabidopsis EXO70 Proteins Independent of Catalytic Activity.

Supplemental Figure 6. Supplemental Figure 5. Characterization of exo70
Mutants Described in This Work.

Supplemental Dataset 1. GmRIN4b Mass Spectrometry Summary Data.

Supplemental Dataset 2. One-way ANOVA Tables in Support of Figure 4.

Supplemental Table 1. Primers Used for This Study

Supplemental File 1. Alignment used to generate phylogenetic tree in
Supplemental Figure 4.
ACKNOWLEDGEMENTS

This work was supported by grants from the United States National Science Foundation to R.W.I. (IOS-1551452) and J.L.D. (IOS-1758400) and by the HHMI. J.L.D. is an Investigator of the Howard Hughes Medical Institute. Support for D.M. was provided by the Rural Development Administration, Republic of Korea, Systems & Synthetic Agrobiotech Center (PJ01326904) and NIH (R01GM092772). In addition, J.H.K. was supported by a grant from the Human Frontier Science Program (LT000607/2010-L) and Q.Z. was supported by a fellowship from the China Scholarship Council (201308430497). Thanks to Laura Giese for technical support in the Mackey lab.

AUTHOR CONTRIBUTIONS


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syringae pv. syringae B728a and its role in survival and disease on host and non-host plants. Mol Microbiol 62, 26-44.


**FIGURE LEGENDS**

**Figure 1.** AvrRpm1 Induces a Size Shift in GmRIN4b that Is Insensitive to Protein Phosphatase Treatment.

(A) 5xMyc-GmRIN4b was transiently expressed in *N. benthamiana* in the presence of AvrB, AvrRpm1, or empty vector (EV). Total protein lysate was incubated with (+) or without (-) Lambda Protein Phosphatase (λPP) and separated on an 8% poly-acrylamide gel supplemented with 50 μM Phos-tag reagent. Proteins were detected by immunoblot using anti-Myc antibodies. Similar results were obtained from three independent experiments.

(B) AvrRpm1-dependent modification of GmRIN4b occurs at a novel location. Wild-type (WT), phospho-deficient (T22A T198A), or phospho-mimic (T22D, T198D) alleles of GmRIN4b were transiently expressed in *N. benthamiana* in the presence of AvrRpm1. Total protein lysate was separated for 16 h on a 10 cm 8% poly-acrylamide gel at low voltage (top), or separated on a 8% poly-acrylamide gel supplemented with 50 μM Phos-tag reagent (bottom). Proteins were detected by immunoblot using anti-Myc antibodies. Similar results were obtained from three independent experiments.

See also Supplemental Figure 1.

**Figure 2.** GmRIN4b is ADP-Ribosylated in vivo in the Presence of AvrRpm1.

sYFP-GmRIN4b was transiently expressed with AvrRpm1 or an empty vector control in *N. benthamiana* and purified YFP-GmRIN4b protein was subjected to trypsin digestion prior to analysis by LC-MS/MS.

(A) GmRIN4b coverage map. Peptides detected in the negative control empty vector (EV) samples are underlined in blue. Peptides detected in the AvrRpm1 treated samples are underlined in red with red stars indicating potentially ADP-ribosylated residues (only found in the AvrRpm1 samples; Supplemental Dataset 1). Blue stars indicate phosphorylated residues found in both EV and AvrRpm1 treated samples. The highly conserved PxFGxWD and F/YTxxFxK motifs of the NOI domains are highlighted in green.
(B) An alignment of the N- (top) and C-terminal (bottom) NOI-domain peptides found to be modified by AvrRpm1.

(C) Mass spectra resulting from fragmentation of the \( \text{GmRIN4b} \) N-terminal NOI-domain peptide, FGNWDSGENVPYTAYFDK, which was expressed in the absence of AvrRpm1.

(D) Mass spectra resulting from fragmentation of the \( \text{GmRIN4b} \) N-terminal NOI-domain peptide, FGNWDSGENVPYTAYFDK, which was expressed in the presence of AvrRpm1.

(E) Mass spectra resulting from fragmentation of the \( \text{GmRIN4b} \) C-terminal NOI-domain peptide, FGDWDVNNPASADGFTIFNK, which was expressed in the absence of AvrRpm1.

(F) Mass spectra resulting from fragmentation of the \( \text{GmRIN4b} \) C-terminal NOI-domain peptide, FGDWDVNNPASADGFTIFNK, which was expressed in the presence of AvrRpm1.

See also Supplemental Dataset 1

**Figure 3.** AvrRpm1 ADP-ribosylates \( \text{GmRIN4a} \), \( \text{AtRIN4} \) and at Least 10 Other Arabidopsis NOI Domain-containing Proteins.

(A) AvrRpm1 ADP-ribosylates \( \text{GmRIN4a} \), \( \text{GmRIN4b} \), and \( \text{AtRIN4} \). The indicated RIN4 proteins were fused to sYFP on their N-termini and transiently expressed in \( N. benthamiana \) in the presence and absence of AvrRpm1. Total protein lysates were then analyzed by immunoblot using anti-GFP antibody or an anti-pan ADP-ribose reagent. Similar results were obtained from three independent experiments.

(B) The sequences of the N-NOI domains of \( \text{GmRIN4c} \) and \( \text{GmRIN4d} \) are poorly conserved. The asterisk indicates the position of ADP-ribosylation in \( \text{GmRIN4b} \). Note, in particular, the lack of a tryptophan adjacent to the predicted ribosylation site. The C-NOI domains, in contrast, are well-conserved across all five RIN4 proteins.
AvrRpm1 ADP-ribosylates the majority of Arabidopsis NOI-domain containing proteins. AtRIN4 and fourteen Arabidopsis NOI proteins, epitope tagged with the T7 peptide, were transiently expressed in *N. benthamiana* in the presence or absence of AvrRpm1. Immunoprecipitation with anti-T7 was performed followed by immunoblotting with anti-T7, anti-panADPR, or anti-HA. The expression of AvrRpm1 was confirmed in the input samples by immunoblot with anti-HA. All images in a single row are extracted from a single blot. The molecular weight of NOI-domain containing proteins ranges from 10 kDa to 30 kDa. Similar results were obtained from two independent experiments.

Mutation of putative catalytic site residues in AvrRpm1 inhibit ADP-ribosylation activity. HA-tagged *Gm*RIN4b was transiently co-expressed with the indicated Myc-tagged AvrRpm1 mutant proteins in *N. benthamiana*. Total protein lysates were then analyzed by immunoblot to detect AvrRpm1, *Gm*RIN4b, and ADP-ribose. Similar results were obtained from three independent experiments.

AvrB does not possess ADP-ribosylase activity. AtRIN4 was transiently expressed in *N. benthamiana* with the indicated proteins. Total protein lysates were then analyzed by immunoblot using anti-Myc antibody or an anti-pan ADP-ribose reagent. Similar results were obtained from two independent experiments.

Delivery of AvrRpm1 from *P. syringae* strain DC3000 induces ADP-ribosylation of AtRIN4 and phosphorylation of AtRIN4 T166. Arabidopsis *rpm1-3* mutant plants were inoculated with 5 x 10^7 cfu/mL of *P. syringae* strains DC3000(EV), DC3000(*avrRpm1*), DC3000(*avrRpm1*D185A) or DC3000(*avrRpm1*AAA). Immunoprecipitations with anti-RIN4 were performed with tissue samples collected 12 hours post infection followed by immunoblots with anti-RIN4, anti-panADPR and anti-pT166. Similar results were obtained from three independent experiments.

ADP-ribosylation activity is required for recognition of AvrRpm1 by soybean. The indicated proteins were delivered by *P. syringae* pv. *glycinea* Race 4 into unifoliate leaves of soybean cultivar Flambeau. The leaf was imaged under ultraviolet light to highlight areas of cell death. Both unifoliate leaves on three plants were inoculated, and all leaves showed the same pattern.
Figure 4. ADP-ribosylation of AtRIN4 by AvrRpm1 is Required for AtRIN4 T166 Phosphorylation.

(A) The proposed AvrRpm1 ADP-ribosyl transferase catalytic residues are required for activation of RPM1 in N. benthamiana. The indicated AvrRpm1 mutants were co-expressed with genomic RPM1-myc and AtRIN4. The AtRIN4 T166A mutant was used as a control as it is known to partially suppress RPM1 activation. Cell death induced by RPM1 activation was monitored by conductivity measurements following induction of AvrRpm1 expression with 20 μM estradiol. Statistical analyses were performed by using a one-way ANOVA at 95% confidence comparing the values for the indicated AvrRpm1 and RIN4 mutant derivatives to the wild-type AvrRpm1-RIN4-RPM1 combination at 6 hours-post-induction. Asterisks represent statistical differences with the indicated P-values (****: P<0.0001; ***: P<0.005; **: P<0.001; *: P<0.05)

(B) The D153 ribosylation site of AtRIN4 contributes to full AvrRpm1-mediated RPM1 activation in N. benthamiana. Wild type AtRIN4, AtRIN4{T166A} (T166A), or AtRIN4{D153A} (D153A) were co-infiltrated with RPM1-myc and wild-type AvrRpm1. Cell death was quantified by conductivity measurements as described in panel A. Statistical analyses were performed as in panel A, but using wild-type RPM1-RIN4 in the absence of AvrRpm1 as the reference.

(C) Cell death mediated by the AtRIN4{T166D} (T166D) phospho-mimic is epistatic to the loss of ribosylation at AtRIN4{D153A} (D153A). The quantification of cell death was monitored as in (A) and (B). Statistical analyses were performed as in panel B.

(D) Ribosylation at AtRIN4{D153} and phosphorylation of AtRIN4{T166} contribute additively to AvrRpm1-mediated activation of RPM1 in transgenic Arabidopsis plants. 5 x 10^7 cfu/mL of P. syringae strain DC3000 (avrRpm1) were inoculated into leaves of transgenic Arabidopsis lines expressing genomic versions of AtRIN4 wild type (gRIN4), AtRIN4{T166A} (gT166A), AtRIN4{D153A} (gD153A), and AtRIN4{N11AD153AT166A} (gN11AD153AT166A) mutant plants. The rpm1-3 null mutant was used as a negative control. The quantification of cell death was monitored as in (A) and (B). Statistical analyses were performed as in panel A, but using rpm1-3 as the reference.
The ADP-ribosylation activity of AvrRpm1 is required for full phosphorylation of AtRIN4\(^{T166}\). The ADP-ribosylation and phosphorylation of AtRIN4 in \textit{N. benthamiana} were monitored by immunoprecipitation of AtRIN4 with anti-T7 followed by immunoblots with anti-T7, anti-panADPR, or anti-pT166. Co-infiltration of AvrRpm1 alleles and AtRIN4 was performed as in (A). Samples were collected 4 hours post treatment with 20\(\mu\)M of estradiol. This result represents one of four independent experiments.

ADP-ribosylation at AtRIN4\(^{D153}\) is required for full phosphorylation of T166. \textit{N. benthamiana} leaves were co-infiltrated with AvrRpm1 and AtRIN4 or AtRIN4\(^{D153A}\), as in (A). Samples were taken 12 hours post induction of AvrRpm1 with 20\(\mu\)M estradiol. The ADP-ribosylation and phosphorylation of AtRIN4 in \textit{N. benthamiana} were monitored by immunoprecipitation of AtRIN4 with anti-T7 followed by immunoblots with anti-T7, anti-panADPR, or anti-pT166. This result represents one of five independent experiments.

ADP-ribosylation at AtRIN4\(^{D153}\) is necessary for full phosphorylation of T166 in Arabidopsis. Transgenic plants expressing wild type AtRIN4 (\textit{gRIN4}) and two independent T2 plants expressing AtRIN4\(^{D153A}\) (\textit{gD153A} #10 and #27) were inoculated with DC3000(\textit{avrRpm1}) as in (D). Samples were collected 4 hours post-infection before HR developed. Immunoprecipitation of RIN4 proteins with anti-T7 was followed by immunoblots with anti-T7, anti-panADPR and anti-pT166.

AvrRpm1 ADP-ribosylation catalytic residues are required for a virulence function of AvrRpm1 in Arabidopsis. Arabidopsis \textit{rpm1-3} mutant plants were inoculated with 5 \times 10^7 cfu/mL of \textit{P. syringae} strains DC3000(EV), DC3000(\textit{avrRpm1}), DC3000(\textit{avrRpm1}\(^{D185A}\)), or DC3000(\textit{avrRpm1}\(^{AAA}\)). Callose accumulation from Arabidopsis leaves 18 hours post infection was analyzed by aniline blue staining. The box plot indicates mean (X), median (inner line), inner quartiles (box), outer quartiles (whiskers), and outliers (dots) for composite data from 16 samples (\(n = 16\)). Statistical significances compared to DC3000(EV)-infected samples were determined by Student’s T-test; * indicates \(P < 0.05\). This result was confirmed in three independent experiments. See also Supplemental Figure 2.
Figure 5. RIN4 Proteins Interact with EXO70 Proteins Via the C-NOI Domain.
(A) AtRIN4 interacts with multiple Arabidopsis EXO70 family members in yeast. Shown are the results of a yeast two-hybrid assay, with yeast expressing the indicated protein fusions. The AvrB protein was used as a positive control for interaction with AtRIN4. Growth on −His and −Ura plates indicate positive interactions. The immunoblot on the right confirms protein expression of each bait and prey construct.
(B) The C-NOI domain of AtRIN4 is required for interaction with Arabidopsis EXO70 proteins. \(1\Delta141\) indicates deletion of AtRIN4 amino acids 1-141, which contains the N-NOI domain. \(149\Delta176\) indicates deletion of amino acids 149-176, which contains the C-NOI domain. The anti-RIN4 immunoblot shows that all three RIN4 protein fusions were expressed in yeast.
(C) AvrRpm1 inhibits association of Arabidopsis EXO70B1 with Arabidopsis and soybean RIN4 proteins. The indicated proteins were co-expressed in N. benthamiana and then RIN4 proteins were immunoprecipitated with anti-Myc antibody. EXO70B1 co-immunoprecipitated in the absence of AvrRpm1, but not in its presence. \(D185A\) indicates AvrRpm1 with the D185A substitution. This result represents one of three independent experiments.
(D) Leaves from Arabidopsis plants were infiltrated with water or 100 μM flg22 and callose deposits were enumerated after 16 hours. Shown is a box plot indicating mean (X), median (inner line), inner quartiles (box), outer quartiles (whiskers), and outliers (dots) for composite data from 13 independent experiments [for flg22 treatments, N = 13 (Col-0), 6 (fls2, exo70e1, exo70 f1-1), 8 (exo70e1exo70e2-2 and exo70e1exo70e2-2exo70f1-1), and 3 (exo70e2-2 and exo70b1-3)] with the number of callose deposits detected in flg22-infiltrated Col-0 normalized to 100 within each experiment. Significance for flg22-treated mutant plants compared to flg22-treated Col-0 plants were determined by Student’s T-test; * indicates P < 0.05 and ** indicates P < 0.005.
See also Supplemental Figures 3 and 4.

Figure 6. A T166D Phosphomimic Mutation in AtRIN4 Enhances RIN4 Interaction with EXO70E2.
A yeast two hybrid assay was used to assess the impact of the T166D substitution on the interaction between AtRIN4 and EXO70B1 and EXO70E2. The enhanced growth on uracil plates indicates that the T166D substitution stabilizes the interaction (top panel). The bottom panel shows immunoblots of protein extracts from the yeast strains growing on –Leu-Trp media, establishing equivalent expression levels in all four strains. Data shown were replicated with three independent yeast transformations for each combination of plasmids.

**Figure 7.** Model for How ADP-Ribosylation of RIN4 Affects Resistance and Susceptibility.

Perception of pathogens by a cell surface receptor promotes phosphorylation of AtRIN4 S141 (Chung et al., 2014), which decreases the affinity of AtRIN4 for EXO70. Release of EXO70 promotes secretion of defense compounds such as callose. To combat this, *P. syringae* injects AvrRpm1, which phosphorylates AtRIN4 on D153, which promotes a conformational change in AtRIN4. This then enables phosphorylation on T166, which enhances the affinity of AtRIN4 for EXO70, thus suppressing secretion of defense compounds. To guard against such suppression, plants have evolved NLR proteins that sense RIN4 modification. In this example, phosphorylation on T166 activates RPM1.
Figure 1. AvrRpm1 Induces a Size Shift in GmRIN4b that Is Insensitive to Protein Phosphatase Treatment.

(A) 5xMyc-GmRIN4b was transiently expressed in N. benthamiana in the presence of AvrB, AvrRpm1, or empty vector (EV). Total protein lysate was incubated with (+) or without (-) Lambda Protein Phosphatase (PP) and separated on an 8% poly-acrylamide gel supplemented with 50 μM Phos-tag reagent. Proteins were detected by immunoblot using anti-Myc antibodies. Similar results were obtained from three independent experiments.

(B) AvrRpm1-dependent modification of GmRIN4b occurs at a novel location. Wild-type (WT), phospho-deficient (T22A T198A), or phospho-mimic (T22D, T198D) alleles of GmRIN4b were transiently expressed in N. benthamiana in the presence of AvrRpm1. Total protein lysate was separated for 16 h on a 10 cm 8% poly-acrylamide gel at low voltage (top), or separated on a 8% poly-acrylamide gel supplemented with 50 μM Phos-tag reagent (bottom). Proteins were detected by immunoblot using anti-Myc antibodies. Similar results were obtained from three independent experiments.

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sYFP-GmRIN4b was transiently expressed with AvrRpm1 or an empty vector control in N. benthamiana and purified YFP-GmRIN4b protein was subjected to trypsin digestion prior to analysis by LC-MS/MS.

(A) GmRIN4b coverage map. Peptides detected in the negative control empty vector (EV) samples are underlined in blue. Peptides detected in the AvrRpm1 treated samples are underlined in red with red stars indicating potentially ADP-ribosylated residues (only found in the AvrRpm1 samples; Supplemental Dataset 1). Blue stars indicate phosphorylated residues found in both EV and AvrRpm1 treated samples. The highly conserved PxFGxWD and F/YTxxFxK motifs of the NOI domains are highlighted in green.

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(C) Mass spectra resulting from fragmentation of the GmRIN4b N-terminal NOI-domain peptide, FGNWDSGENVPYTAYFDK, which was expressed in the absence of AvrRpm1.

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Figure 3. AvrRpm1 ADP-ribosylates GmRIN4a, ATRIN4 and at least 10 Other Arabidopsis NOI Domain-containing Proteins.

(A) AvrRpm1 ADP-ribosylates GmRIN4a, GmRIN4b, and ATRIN4. The indicated RIN4 proteins were fused to sYFP on their N-termini and transiently expressed in N. benthamiana in the presence and absence of AvrRpm1. Total protein lysates were then analyzed by immunoblot using anti-GFP antibody or an anti-pan ADP-ribosylation reagent. Similar results were obtained from three independent experiments.

(B) The sequences of the N-NOI domains of GmRIN4c and GmRIN4d are poorly conserved. The asterisk indicates the position of ADP-ribosylation in GmRIN4b. Note, in particular, the lack of a tryptophan adjacent to the predicted ribosylation site. The C-NOI domains, in contrast, are well-conserved across all five RIN4 proteins.

(C) AvrRpm1 ADP-ribosylates the majority of Arabidopsis NOI-domain containing proteins. ATRIN4 and fourteen Arabidopsis NOI proteins, epitope tagged with the T7 peptide, were transiently expressed in N. benthamiana in the presence or absence of AvrRpm1. Immunoprecipitation with anti-T7 was performed followed by immunoblotting with anti-T7, anti-panADPR, or anti-HA. The expression of AvrRpm1 was confirmed in the input samples by immunoblot with anti-HA. All images in a single row are extracted from a single blot. The molecular weight of NOI-domain containing proteins ranges from 10 kDa to 30 kDa. Similar results were obtained from two independent experiments.

(D) Mutation of putative catalytic site residues in AvrRpm1 inhibit ADP-ribosylation activity. HA-tagged GmRIN4b was transiently co-expressed with the indicated Myc-tagged AvrRpm1 mutant proteins in N. benthamiana. Total protein lysates were then analyzed by immunoblot to detect AvrRpm1, GmRIN4b, and anti-RIN4 were performed with tissue samples collected 12 hours post infection. Similar results were obtained from two independent experiments.

(E) AvrB does not possess ADP-ribosylase activity. ATRIN4 was transiently expressed in N. benthamiana with the indicated proteins. Total protein lysates were then analyzed by immunoblot using anti-Myc antibody or an anti-pan ADP-ribosylation reagent. Similar results were obtained from two independent experiments.

(F) Delivery of AvrRpm1 from P. syringae pv. glycinea Race 4 into unifoliate leaves of soybean cultivar Flambeau. The indicated proteins were delivered by P. syringae pv. glycinea Race 4 into unifoliate leaves of soybean cultivar Flambeau. The leaf was imaged under ultraviolet light to highlight areas of cell death. Both unifoliate leaves on three plants were inoculated, and all leaves showed the same pattern.
Figure 4. ADP-ribosylation of AtRIN4 by AvrRpm1 is Required for AtRIN4 T166 Phosphorylation.

(A) The proposed AvrRpm1 ADP-ribosyl transferase catalytic residues are required for activation of RPM1 in *N. benthamiana*. The indicated AvrRpm1 mutants were co-expressed with genomic RPM1-myc and AtRIN4. The AtRIN4 T166A mutant was used as a control as it is known to partially suppress RPM1 activation. Cell death induced by RPM1 activation was monitored by conductivity measurements following induction of AvrRpm1 expression with 20 µM estradiol. Statistical analyses were performed by using a one-way ANOVA at 95% confidence comparing the values for the indicated AvrRpm1 and RIN4 mutant derivatives to the wild-type AvrRpm1-RIN4-RPM1 combination at 6 hours-post-induction. Asterisks represent statistical differences with the indicated P-values (**:** P<0.0001; ***: P<0.001; **: P<0.01; *: P<0.05)

(B) The D153 ribosylation site of AtRIN4 contributes to full AvrRpm1-mediated RPM1 activation in *N. benthamiana*. Wild type AtRIN4, AtRIN4 T166A (T166A), or AtRIN4 D153A (D153A) were co-infiltrated with RPM1-myc and wild-type AvrRpm1. Cell death was quantified by conductivity measurements as described in panel A. Statistical analyses were performed as in panel A, but using wild-type RPM1-RIN4 in the absence of AvrRpm1 as the reference.

(C) Cell death mediated by the AtRIN4 T166D (T166D) phospho-mimic is epistatic to the loss of ribosylation at AtRIN4 D153A (D153A). The quantification of cell death was monitored as in (A) and (B). Statistical analyses were performed as in panel B.

(D) Ribosylation at AtRIN4 D153 and phosphorylation of AtRIN4 T166 contribute additively to AvrRpm1-mediated activation of RPM1 in transgenic Arabidopsis plants. 5 x 10^7 cfu/mL of *P. syringae* strain DC3000 (avrRpm1) were inoculated into leaves of transgenic Arabidopsis lines expressing genomic versions of AtRIN4 wild type (gRIN4), AtRIN4 T166A (gT166A), AtRIN4 D153A (gD153A), and AtRIN4 N11AD153AT166A (gN11AD153AT166A) mutant plants. The rpm1-3 null mutant was used as a negative control. The quantification of cell death was monitored as in (A) and (B). Statistical analyses were performed as in panel A, but using rpm1-3 as the reference.

(E) The ADP-ribosylation activity of AvrRpm1 is required for full phosphorylation of AtRIN4 T166. The ADP-ribosylation and phosphorylation of AtRIN4 in *N. benthamiana* were monitored by immunoprecipitation of AtRIN4 with anti-T7 followed by immunoblots with anti-T7, anti-panADPR, or anti-pT166. Co-infiltration of AvrRpm1 alleles and AtRIN4 was performed as in (A). Samples were collected 4 hours post treatment with 20µM of estradiol. This result represents one of four independent experiments.

(F) ADP-ribosylation at AtRIN4 D153 is required for full phosphorylation of T166. *N. benthamiana* leaves were co-infiltrated with AvrRpm1 and AtRIN4 or AtRIN4 D153A, as in (A). Samples were taken 12 hours post induction of AvrRpm1 with 20µM estradiol. The ADP-ribosylation and phosphorylation of AtRIN4 in *N. benthamiana* were monitored by immunoprecipitation of AtRIN4 with anti-T7 followed by immunoblots with anti-T7, anti-panADPR, or anti-pT166. This result represents one of five independent experiments.

(G) ADP-ribosylation at AtRIN4 D153 is necessary for full phosphorylation of T166 in Arabidopsis. Transgenic plants expressing wild type AtRIN4 (gRIN4) and two independent T2 plants expressing AtRIN4 D153A (gD153A #10 and #27) were inoculated with DC3000 (avrRpm1) as in (D). Samples were collected 4 hours post-infection before HR developed. Immunoprecipitation of RIN4 proteins with anti-T7 was followed by immunoblots with anti-T7, anti-panADPR, and anti-pT166. This result represents one of five independent experiments.

(H) AvrRpm1 ADP-ribosylation catalytic residues are required for a virulence function of AvrRpm1 in Arabidopsis. Arabidopsis rpm1-3 mutant plants were inoculated with 5 x 10^7 cfu/mL of *P. syringae* strains DC3000(EV), DC3000(avrRpm1), DC3000 (avrRpm1 D185A) or DC3000 (avrRpm1 AAA). Callose accumulation from Arabidopsis leaves 18 hours post infection was analyzed by aniline blue staining. The box plot indicates mean (X), median (inner line), inner quartiles (box), outer quartiles (whiskers), and outliers (dots) for composite data from 16 samples (n = 16). Statistical significances compared to DC3000(EV)-infected samples were determined by Student’s T-test; * indicates P < 0.05. This result was confirmed in three independent experiments. See also Supplemental Figure 2.
Figure 5. RIN4 Proteins Interact with EXO70 Proteins Via the C-NOI Domain.

(A) AtRIN4 interacts with multiple Arabidopsis EXO70 family members in yeast. Shown are the results of a yeast two-hybrid assay, with yeast expressing the indicated protein fusions. The AvrB protein was used as a positive control for interaction with AtRIN4. Growth on –His and –Ura plates indicate positive interactions. The immunoblot on the right confirms protein expression of each bait and prey construct.

(B) The C-NOI domain of AtRIN4 is required for interaction with Arabidopsis EXO70 proteins. 1△141 indicates deletion of AtRIN4 amino acids 1-141, which contains the N-NOI domain. 149△176 indicates deletion of amino acids 149-176, which contains the C-NOI domain. The anti-RIN4 immunoblot shows that all three RIN4 protein fusions were expressed in yeast.

(C) AvrRpm1 inhibits association of Arabidopsis EXO70B1 with Arabidopsis and soybean RIN4 proteins. The indicated proteins were co-expressed in N. benthamiana and then RIN4 proteins were immunoprecipitated with anti-Myc antibody. EXO70B1 co-immunoprecipitated in the absence of AvrRpm1, but not in its presence. D185A indicates AvrRpm1 with the D185A substitution. This result represents one of three independent experiments.

(D) Leaves from Arabidopsis plants were infiltrated with water or 100 μM flg22 and callose deposits were enumerated after 16 hours. Shown is a box plot indicating mean (X), median (inner line), inner quartiles (box), outer quartiles (whiskers), and outliers (dots) for composite data from 13 independent experiments [for flg22 treatments, N = 13 (Col-0), 6 (fls2, exo70e1, exo70 f1-1), 8 (exo70e1exo70e2-2 and exo70e1exo70e2-2exo70f1-1), and 3 (exo70e2-2 and exo70b1-3)] with the number of callose deposits detected in flg22-infiltrated Col-0 normalized to 100 within each experiment. Significance for flg22-treated mutant plants compared to flg22-treated Col-0 plants were determined by Student’s T-test; * indicates P < 0.05 and ** indicates P < 0.005. See also Supplemental Figures 3 and 4.
Figure 6. A T166D Phosphomimic Mutation in AtRIN4 Enhances RIN4 Interaction with EXO70E2.

A yeast two hybrid assay was used to assess the impact of the T166D substitution on the interaction between AtRIN4 and EXO70B1 and EXO70E2. The enhanced growth on –uracil plates indicates that the T166D substitution stabilizes the interaction (top panel). The bottom panel shows immunoblots of protein extracts from the yeast strains growing on –Leu -Trp media, establishing equivalent expression levels in all four strains. Data shown were replicated with three independent yeast transformations for each combination of plasmids.
Figure 7. Model for How ADP-Ribosylation of RIN4 Affects Resistance and Susceptibility.

Perception of pathogens by a cell surface receptor promotes phosphorylation of AtRIN4 S141 (Chung et al., 2014), which decreases the affinity of AtRIN4 for EXO70. Release of EXO70 promotes secretion of defense compounds such as callose. To combat this, P. syringae injects AvrRpm1, which phosphorylates AtRIN4 on D153, which promotes a conformational change and phosphorylation on T166 (red circle), which promotes RIN4 association with EXO70, inhibiting secretion.

In the absence of pathogen signals, RIN4 associates with EXO70s to suppress secretion of defense compounds.

PRR activation induces RIN4 S141 phosphorylation, releasing EXO70 from inhibition by RIN4 and thus promoting secretion of defense compounds.

ADP-ribosylation on D153 (green diamond) by AvrRpm1 induces a conformational change and phosphorylation on T166 (red circle), which promotes RIN4 association with EXO70, inhibiting secretion.

RPM1 forms a pre-activation complex with RIN4 that remains in the off state in the absence of RIN4 modification.

Phosphorylation of RIN4 S141 does NOT activate RPM1.

Phosphorylation of RIN4 T166 activates RPM1, inducing a conformational change that signals defense induction, including cell death.


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Plant Cell; originally published online September 23, 2019;
DOI 10.1105/tpc.19.00020

This information is current as of February 11, 2021

Supplemental Data
/content/suppl/2019/09/23/tpc.19.00020.DC1.html
/content/suppl/2019/11/09/tpc.19.00020.DC2.html

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