Membrane Release and Destabilization of *Arabidopsis* RIN4 Following Cleavage by *Pseudomonas syringae* AvrRpt2

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The *Arabidopsis* RIN4 protein mediates interaction between the *Pseudomonas syringae* type III effector proteins AvrB, AvrRpm1, and AvrRpt2 and the *Arabidopsis* disease-resistance proteins RPM1 and RPS2. Confocal laser-scanning fluorescence microscopy following particle bombardment of tobacco leaf epidermal cells was used to examine the subcellular localization of fusions between GFP and RIN4 or several of its homologs and to examine the effects of co-bombardment with AvrRpt2 or AvrRpm1. This study showed that RIN4 was attached to the plasma membrane at its carboxyl terminus and that a carboxyl-terminal CCCFxFxxx prenylation or acylation (typically palmitoylation) motif, or both, was essential for this attachment. RIN4 was cleaved by AvrRpt2 at two PxFGxW motifs, one releasing a large portion of RIN4 from the plasma membrane and both exposing amino-terminal residues that destabilized the carboxyl-terminal cleavage products by targeting them for N-end ubiquitylation and proteasomal degradation. Plasma-membrane localization of RIN4 was not affected by AvrRpm1. RIN4 was found to be part of a protein family comprising two full-length homologs and at least 11 short carboxyl-terminal homologs. Representatives of this family, comprising a full-length RIN4 homolog and two short carboxyl-terminal RIN4 homologs, were also attached to the plasma membrane and cleaved near their amino termini by AvrRpt2, but in contrast to RIN4, the major portions of these proteins remained on the plasma membrane. N-end degradation may play a minor role in RIN4 degradation but probably plays a major role in the degradation of RIN4 homologs and is, therefore, a major pathogenic consequence of AvrRpt2 cleavage.

Additional keywords: guard hypothesis, plant disease resistance, virulence effector.

Many pathogenic bacteria attack animals or plants with effector proteins delivered into host cells by type III secretion mechanisms (Galán and Collmer 1999). While animals have evolved elaborate immune systems to combat bacterial pathogens, plants

are often dependent on direct or indirect recognition of bacterial type III effector proteins by disease resistance proteins, which, in effect, convert virulence effector proteins into avirulence effector proteins (Jones and Takemoto 2004). Animal- and plant-pathogenic bacteria show significant similarities in their type III secretion mechanisms (Galán and Collmer 1999) and in the effectors they deliver, including proteases targeted against specific host proteins (Xia 2004). An example is the cysteine protease encoded by the *Yersinia yopT* gene, which targets rho GTPases in animal cells, and a closely related cysteine protease encoded by the *Pseudomonas syringae* AvrPphB gene, which targets the membrane-anchored PBS1 serine/threonine protein kinase in *Arabidopsis* (Shao et al. 2002, 2003; Swiderski and Innes 2001). After delivery into the host cell, AvrPphB undergoes self-cleavage at a GDK motif to reveal a cryptic amino-terminal myristoylation site that enables mature AvrPphB to become attached to the host plasma membrane (Nimchuk et al. 2000; Puri et al. 1997). AvrPphB cleaves PBS1 at a GDK motif in its kinase activation loop motif and remains attached to the carboxyl-terminal fragment of PBS1 (Shao et al. 2003). PBS1 is monitored by the *Arabidopsis* RPS5 disease-resistance protein (Warren et al. 1998, 1999), and the cleavage and autophosphorylation of PBS1 are both required to trigger RPS5-dependent activation of plant defenses (Shao et al. 2003). This three-way interaction between bacterial effector, host target, and plant disease-resistance proteins provides strong evidence to support the ‘guard’ or ‘surveillance’ hypothesis, which proposes that, in some plant-disease interactions, plant disease resistance proteins monitor the status of host proteins that are targets for manipulation by pathogen effector molecules (Dangl and Jones 2001; Van Der Biezen and Jones 1998).

Recently, the *P. syringae* AvrRpt2 gene has also been shown to encode a cysteine protease that targets the RIN4 protein of *Arabidopsis* (Axtell et al. 2003). RIN4 is a target for multiple bacterial effectors and is monitored by the *Arabidopsis* RPM1 and RPS2 disease-resistance proteins (Mackey et al. 2002, 2003). The *P. syringae* AvrB and AvrRpm1 type III effector proteins trigger the phosphorylation of RIN4 and, consequently, the RPM1-dependent activation of plant defense responses (Mackey et al. 2002). The otherwise sequence-unrelated AvrB and AvrRpm1 proteins both possess amino-terminal myristoylation sites and are attached to the host plasma membrane via myristoylated amino termini (Nimchuk et al. 2000). The mechanism by which AvrB and AvrRpm1 effect the phosphorylation of RIN4 is not understood, as neither appears to be a protein kinase, but could involve the recruitment of host kinases. Cleavage of RIN4 by AvrRpt2 interferes with RPM1 function, thereby making the host cell unresponsive to AvrB or

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AvrRpm1 (Mackey et al. 2003). Thus, in the absence of RPS2, one of the virulence effector functions of AvrRpt2 may be considered the suppression of the host's ability to respond to AvrB or AvrRpm1. However, in the presence of RPS2, the loss of RIN4 following cleavage by AvrRpt2 triggers RPS2-dependent activation of plant defense responses (Axtell and Staskawicz 2003; Mackey et al. 2003). Together, this complex six-way interaction between bacterial effector proteins, host target protein, and disease resistance proteins provides further strong support for the surveillance hypothesis.

After delivery into the host cell, AvrRpt2 is activated by one or more host cyclophilins (Coaker et al. 2005), undergoes self-cleavage at a VPAFGGW motif, and becomes associated with the host plasma membrane (Axtell and Staskawicz 2003; Axtell et al. 2003). Although self-cleavage of AvrRpt2 reveals an amino-terminal glycine residue, which would otherwise be required for myristoylation, it does not seem to be myristoylated and attached to the plasma membrane by its amino terminus (Jin et al. 2003). The molecular basis for the association of AvrRpt2 with the plasma membrane remains to be determined. RIN4 is also a membrane-associated protein (Mackey et al. 2002) and has two sequence motifs, VPKFGNW and VPKFGDW, homologous to the self-cleavage site of AvrRpt2 (Fig. 1A and B) (Jones and Takemoto 2004). During preparation of this paper, AvrRpt2 was shown to cleave both of these motifs (Chisholm et al. 2005; Coaker et al. 2005), but the molecular basis for AvrRpt2-dependent disappearance of RIN4 following cleavage by AvrRpt2 remains poorly understood.

Examination of the RIN4 protein sequence reveals the presence of a carboxyl-terminal CCC motif similar to the carboxyl-terminal prenylation motifs CC, CxC, or CCxx (Crowell 2000) but more distant from the carboxyl terminus than is usual for such motifs (Fig. 1A). Internal cysteine residues can also be a substrate for acylation (Smotrys and Linder 2004). Prenylation or acylation of cysteine residues, or both, near the carboxyl terminus of RIN4 could anchor RIN4 to a membrane. During preparation of this paper, data were published to suggest that palmitoylation of the CCC motif was required for membrane attachment of RIN4 (Kim et al. 2005).

Here, we have used green fluorescent protein (GFP) fusions to RIN4 to show that it does indeed contain a carboxyl-terminal signal for plasma membrane association and that AvrRpt2 cleaves both of the predicted cleavage sites. We used alanine-scanning site-directed mutagenesis to show that each cysteine residue in the CCC motif and each of two adjacent phenylalanine residues are essential for plasma membrane association. We also used an alanine scan to show that a conserved phenylalanine residue of the predicted VPxFGxW cleavage sites is essential for cleavage by AvrRpt2 and that the conserved proline, glycine, and tryptophan residues also play an important role. In addition, we used GFP fusions and site-directed mutagenesis to show that cleavage of RIN4 by AvrRpt2 exposes amino-terminal amino acid residues that destabilize the cleavage products, leading to their proteasomal degradation according to the N-end rule. Finally, we used GFP fusions to show that a representative full-length homolog (FLH) of RIN4 and two representative short carboxyl-terminal homologs (CTH) of RIN4 are also attached to the plasma membrane via their carboxyl termini and cleaved near their amino termini by AvrRpt2.

RESULTS

RIN4 is attached to the plasma membrane at its carboxyl terminus.

A DNA construct, GFP:RIN4-70C, encoding the 70 carboxyl-terminal amino acid residues of RIN4 fused to the carboxyl-terminus of GFP and expressed under the control of the caulim-

flower mosaic virus (CaMV) 35S promoter, was used to produce transgenic *Arabidopsis* plants. These were examined by confocal laser-scanning fluorescence microscopy and showed a pattern of GFP fluorescence consistent with plasma-membrane localization (Fig. 1D). Epidermal cells of tobacco leaves showed the same pattern of fluorescence (Fig. 1D) after particle bombardment with GFP:RIN4-70C. Given that bombardment enabled rapid generation of localization data reflecting those observed in transgenic plants, all subsequent RIN4 localization experiments were carried out using particle bombardment of tobacco leaves, except where otherwise indicated. In addition to its rapidity, particle bombardment also offered other advantages over the use of transgenic plants expressing GFP fusion proteins under the control of a strong constitutive promoter. One advantage was the ability to image single fluorescent cells against a background of nonfluorescent cells. This enabled very striking images of plasma-membrane labeling to be obtained without interference from adjacent cells. Another advantage, given that expression of the GFP fusions starts from zero and rises over a period of several hours, was the ability to visualize bombarded cells at the lowest levels of GFP expression compatible with imaging. This allowed confirmation that the pattern of GFP fluorescence did not alter at higher levels of expression suitable for optimal imaging.

GFP:RIN4-16C, encoding a GFP fusion to the 16 carboxyl-terminal amino acid residues of RIN4, which contain the CCC motif, showed the same pattern of fluorescence as GFP:RIN4-70C (Fig. 1D), but the equivalent fusion with an AAA mutation of the CCC motif showed a pattern of fluorescence consistent with cytosolic localization (Fig. 1D). Individual C to A mutations in the CCC motif of GFP:RIN4-70C produced the same result (Fig. 1E), indicating that each of the cysteine residues plays an important role in plasma-membrane localization. This result suggests that at least one of these cysteine residues is prenylated or acylated, resulting in attachment of RIN4 to the plasma membrane via its carboxyl-terminus, while the others may be prenylated or acylated or contribute to a hydrophobic sequence context (Fig. 1C), which may be part of a prenyl- or acyl-transferase recognition motif. Mutation of the downstream phenylalanine residues adjacent to the CCC motif also showed a cytosolic pattern of fluorescence (Fig. 1E), indicating that other residues adjacent to the cysteines are also important for membrane association. These phenylalanine residues contribute to the hydrophobic sequence context provided by the CCC motif (Fig. 1C) and may form part of an extended prenylation or acylation motif. However, individual alanine mutations of other residues flanking the CCC motif had no effect on plasma membrane localization (Fig. 1E).

RIN4 is released from the plasma membrane following cleavage by AvrRpt2.

To investigate the cleavage of RIN4 by AvrRpt2, a DNA construct encoding a start methionine followed by the 184 carboxyl-terminal amino acid residues of AvrRpt2, which comprise the mature protein (Mudgett and Staskawicz 1999), was cobombarded together with either GFP:RIN4-70C or GFP:RIN4-16C. Cobombardment with GFP:RIN4-70C and AvrRpt2 caused a cytosolic pattern of fluorescence consistent with the release of GFP from the plasma membrane (Fig. 2A). Cobombardment with an equivalent AvrRpt2 construct carrying a C122A mutation, which disrupts the cysteine protease activity of AvrRpt2 (Axtell et al. 2003), had no effect on the membrane localization of the GFP:RIN4-70C fusion, indicating that the release of GFP was dependent on the proteolytic activity of AvrRpt2 (Fig. 2A). Cobombardment with GFP:RIN4-16C and AvrRpt2 had no effect on the membrane localization of the GFP:RIN4-16C

fusion, indicating that the release of GFP was dependent on cleavage in the RIN4 segment carrying the predicted carboxyl-terminal cleavage site (Fig. 2A). Alanine-scanning mutagenesis of the conserved amino acid residues of the predicted carboxyl-

terminal cleavage site in GFP:RIN4-70C (VPxFGxW; Fig. 1A) showed that the conserved phenylalanine residue and, to a lesser extent, the conserved proline residue but none of the other conserved residues were essential for AvrRpt2 cleavage (Fig. 2B).

Fig. 1. A, Protein sequence of RIN4 showing the amino acid residues (underlined) expressed in the GFP:RIN4-30N, GFP:RIN4-70C, and GFP:RIN4-16C fusion constructs, the AvrRpt2 cleavage-site motifs (highlighted in blue), and the carboxyl-terminal membrane-attachment motif (highlighted in red). **B**, Alignment of the cleavage-site motifs of RIN4 and AvrRpt2, with an arrow indicating the experimentally confirmed cleavage site in AvrRpt2 and the inferred cleavage sites in RIN4. **C**, Hydrophobicity plot of the carboxyl terminus of RIN4, showing a hydrophobic domain corresponding to the CCCFx membrane-attachment motif. The plot was generated using the Kyte and Doolittle algorithm at the ExPASy website, with a window size of five amino acid residues. **D**, Left panel: Micrograph showing a membrane-localized pattern of green fluorescent protein (GFP) fluorescence in leaf epidermal cells of a transgenic *Arabidopsis* plant expressing the GFP:RIN4-70C fusion protein. Right panels: Micrographs showing membrane-localized patterns of GFP fluorescence in epidermal cells of tobacco leaves bombarded with GFP:RIN4-70C or GFP:RIN4-16C but a cytosolic pattern of GFP fluorescence for a CCC to AAA mutation of the GFP:RIN4-16C fusion protein. **E**, On the left: A summary of plasma-membrane localization patterns for mutant variants of GFP:RIN4-70C, representing an alanine scan across the region containing the carboxyl-terminal membrane-attachment motif of RIN4. The unmodified RIN4 sequence is shown at the top, and mutated RIN4 sequences are aligned beneath with the mutant alanine residue highlighted in blue. + = plasma membrane-localized, - = cytosolic. On the right: Micrographs showing examples of the GFP localization patterns in epidermal cells of tobacco leaves bombarded with mutant variants of GFP:RIN4-70C, with the mutations shown in brackets.

However, combined mutation of the glycine and tryptophan residues flanking the presumed cleavage site abolished cleavage suggesting that these conserved residues do play a role in cleavage site recognition (Fig. 2B). In contrast to the combined glycine and tryptophan mutation, combined mutation of the valine and proline residues upstream of the presumed cleavage site had a similar effect on cleavage as did mutation of the proline residue alone, suggesting that the valine residue has little or no specific role in cleavage (Fig. 2B).

Cleavage of RIN4 by AvrRpt2 destabilizes RIN4 in a manner consistent with N-end ubiquitylation and subsequent proteasomal degradation.

AvrRpt2 is predicted to expose different amino-terminal amino acids on the carboxyl-terminal products of self-cleavage (glycine) or cleavage of RIN4 at the amino-terminal (asparagine) or carboxyl-terminal (aspartic acid) cleavage sites (Fig. 1B). Based on the N-end rule (Bachmair et al. 1986), these carboxyl-terminal products would be expected to display different rates of amino-terminal ubiquitylation and protein degradation, with both amino-terminal asparagine and aspartic acid residues conferring reduced protein stability compared with amino-terminal methionine or glycine residues.

To test this idea, a DNA construct, RIN4-30N:GFP, encoding the 30 amino-terminal amino acid residues of RIN4 fused

to the amino terminus of GFP was cobombarded with AvrRpt2 or AvrRpt2(C122A) and the level of fluorescence was assessed over time (Fig. 3). Bombardment with RIN4-30N:GFP alone resulted in a cytosolic pattern of fluorescence, consistent with the absence of any predicted amino-terminal membrane anchoring motifs (data not shown). Control bombardments with GFP and AvrRpt2 or RIN4-30N:GFP and AvrRpt2(C122A) showed that GFP fluorescence is apparent by 8 h after bombardment and decreases gradually over the next 16 h (Fig. 3A and C). In contrast, cobombardment with RIN4-30N:GFP and AvrRpt2 showed a much more rapid decrease in GFP fluorescence, consistent with degradation of the GFP fusion protein (Fig. 3A and C). The AvrRpt2(C122A) control shows that this rapid loss of GFP fluorescence is dependent on AvrRpt2 protease activity, and the GFP control shows that it is dependent on the presence of the amino terminus of RIN4. Moreover, cobombardment with GFP:RIN4-70C and AvrRpt2 did not show a rapid decrease in GFP fluorescence (data not shown), indicating that, although the carboxyl terminal product of AvrRpt2 cleavage is unstable, the amino-terminal product of cleavage by AvrRpt2 is relatively stable.

The hypothesis of N-end rule degradation was further tested by introducing an N11G mutation into RIN4-30N:GFP, to expose an amino-terminal glycine residue instead of an asparagine residue after cleavage by AvrRpt2, thus mimicking the

Fig. 2. A, Micrographs showing a cytosolic pattern of green fluorescent protein (GFP) fluorescence in epidermal cells of tobacco leaves cobombarded with GFP:RIN4-70C and AvrRpt2 (left panel) but membrane-localized patterns of GFP fluorescence for cells cobombarded with GFP:RIN4-70C and a protease-defective C122A mutant of AvrRpt2 (center panel) or GFP:RIN4-16C and AvrRpt2 (right panel). **B**, On the left, a summary of cleavage competence for mutant variants of GFP:RIN4-70C, representing an alanine scan across the region containing the carboxyl-terminal cleavage motif PxFGxW of RIN4 and some double alanine mutants. The unmodified RIN4 sequence is shown at the top and mutated RIN4 sequences are aligned beneath. + = cleaved by AvrRpt2, - = not cleaved by AvrRpt2, +/- = partial cleavage by AvrRpt2. On the right, micrographs showing examples of the GFP-localization patterns in epidermal cells of tobacco leaves cobombarded with AvrRpt2 and mutant variants of GFP:RIN4-70C, with the mutations shown in brackets. A cytosolic pattern of GFP fluorescence (top left panel) indicates cleavage of GFP:RIN4-70C by AvrRpt2, whereas a plasma membrane-localized pattern of fluorescence (bottom panels) indicates lack of cleavage by AvrRpt2. An intermediate pattern of fluorescence (top right panel) indicates partial cleavage by AvrRpt2.

product of AvrRpt2 self-cleavage (Fig. 1B). Cobombardment with RIN4-30N(N11G):GFP and AvrRpt2 showed a slower rate of decrease in GFP fluorescence than for RIN4-30N:GFP, consistent with greater stability of RIN4-30N(N11G):GFP after cleavage by AvrRpt2 (Fig. 3C).

AvrRpm1 does not affect the membrane localization of RIN4.

RIN4 is also a target for attack by the *P. syringae* AvrB and AvrRpm1 type III effector proteins, which cause the phosphorylation of RIN4 and the activation of plant defense re-

sponses by the RPM1 disease resistance protein (Mackey et al. 2002). Phosphorylation of serine or threonine residues near the carboxyl-terminus of RIN4 could potentially alter its membrane attachment properties by increasing the amount of negative charge adjacent to the CCC motif or, given that palmitoylation is a reversible process (Smotrys and Linder 2004), by triggering depalmitoylation of any palmitoylated cysteine residues present in the CCC motif. To test this possibility, the TSS motif immediately upstream of the CCC motif in GFP-RIN4-16C (Fig. 1A) was mutated to DDD to mimic the effect of phosphorylation of these residues. An AAA mutation of the TSS motif did not affect plasma membrane localization of GFP-RIN4-16C, whereas the DDD mutation resulted in cytoplasmic localization of GFP-RIN4-16C (Fig. 4A), suggesting that phosphorylation of RIN4 might alter its location. To further test this idea, a DNA construct, GFP:RIN4, encoding full-length RIN4 fused to the carboxyl terminus of GFP was cobombarded with AvrRpm1. GFP:RIN4 showed membrane localization when bombarded on its own and when cobombarded with AvrRpm1 (Fig. 4B), suggesting that AvrRpm1-induced phosphorylation does not abolish the association of RIN4 with the plasma membrane. To exclude the possibility that tobacco lacks the requisite cofactors to effect AvrRpm1-induced phosphorylation of RIN4, this experiment was repeated in *Arabi-*

dopsis, with the same results (data not shown). Assuming that the GFP tag does not hinder the phosphorylation of RIN4, these data suggest that a change in the physical properties of RIN4 is the trigger for activation of RPM1 rather than a change in its subcellular location.

A FLH of RIN4 is also anchored to the plasma membrane at its carboxyl terminus but lacks a carboxyl-terminal AvrRpt2 cleavage site.

There are two FLH of RIN4 in the *Arabidopsis* genome (At3g07195 and At5g48657), and these have variations of the carboxyl-terminal membrane attachment and AvrRpt2 cleavage-site motifs (Fig. 5A and B). To test the functionality of these motifs, similar bombardment experiments to those performed with GFP:RIN4-70C were performed with an equivalent construct, GFP:RIN4-FLH1-80C, derived from the FLH here designated FLH1 (corresponding to At3g07195). GFP:RIN4-FLH1-80C showed a plasma-membrane pattern of fluorescence suggesting that the RIN4 homologs are also plasma-membrane localized (Fig. 5C). The RIN4 homolog carries a GCC variant of the CCC motif present in RIN4 (Fig. 5A) but still allows plasma membrane-attachment. This result is surprising, given that mutation of the first cysteine of the CCC motif of RIN4 to alanine abolished membrane localiza-

Fig. 3. A, Low-magnification fluorescence micrographs of tobacco leaf tissue 24 h after cobombardment with RIN4-30N:GFP and AvrRpt2 (left) or RIN4-30N:GFP and AvrRpt2 (C122A) (right), showing a consistent difference in green fluorescent protein (GFP) fluorescence. Bombarded epidermal cells expressing GFP (green) are seen against a background of chlorophyll fluorescence (red). Images shown are representative of three separate experiments, each with two bombardments. **B**, Reduction in the percentage of cells retaining detectable GFP fluorescence 16, 24, and 30 h after cobombardment with GFP and AvrRpt2 (squares) or RIN4-30N:GFP and AvrRpt2 (circles), relative to the number of fluorescent cells present 8 h after cobombardment. Data are representative of three separate experiments and were collected from four defined areas from two separate bombardments. **C**, Fluorescence micrographs of tobacco leaf epidermal cells cobombarded with RIN4-30N:GFP and AvrRpt2 (panels 1 and 2) or GFP and AvrRpt2 (panels 3 and 4) showing differences in GFP fluorescence intensity representative of the four intensity classifications used in D. **D**, Plots showing percentages of tobacco leaf epidermal cells with GFP fluorescence intensities in each of the four classes represented in C, for cells cobombarded with RIN4-30N:GFP and AvrRpt2 (C122A) (top left), RIN4-30N:GFP and AvrRpt2 (top right), GFP and AvrRpt2 (bottom left), or RIN4-30N:GFP (N11G) and AvrRpt2 (bottom right) at 8, 16, and 24 h after bombardment. At least 300 bombarded cells from at least two experiments each, with two bombardments for each combination of constructs, were counted for each timepoint. The data was collected from experiments different from those in B.

Fig. 4. A, On the left, a summary of plasma-membrane localization patterns for TSS to AAA or DDD mutations of GFP:RIN4-70C, indicating that the TSS sequence probably does not contribute to a prenylation or acylation motif per se, but does provide an appropriate sequence context for prenylation, acylation, or membrane attachment. The unmodified RIN4 sequence is shown at the top and mutated RIN4 sequences are aligned beneath. + = plasma membrane localized, - = cytosolic. On the right, micrographs showing examples of the green fluorescent protein (GFP) localization patterns in epidermal cells of tobacco leaves bombarded with constructs carrying TSS to AAA or DDD mutations (shown in brackets) of GFP:RIN4-70C. **B**, Micrographs showing GFP localization patterns in epidermal cells of tobacco leaves bombarded with GFP:RIN4 alone (left) or cobombarded with AvrRpm1 (middle) or AvrRpt2 (right).

tion (Fig. 1E). Moreover, GFP:RIN4-16C carrying a GCC variant of the CCC motif did not show plasma membrane localization either (data not shown), suggesting that other residues flanking the GCC motif in the two FLHs may compensate for the C to G substitution. Cobombardment with AvrRpt2 did not alter the GFP:RIN4-FLH1-80C plasma-membrane pattern of fluorescence, suggesting that sequence differences in the homologs prevent cleavage by AvrRpt2 in the region corresponding to the carboxyl-terminal cleavage site motif of RIN4 (Fig. 5B).

Short CTH of RIN4 are also anchored to the plasma membrane at their carboxyl termini and have amino-terminal AvrRpt2 cleavage sites.

There are several close homologs of the carboxyl-terminal portion of RIN4 in the *Arabidopsis* genome (At2g04410, At2g17660, At3g48450, At4g35655, At5g09960, At5g40645, At5g55850, At5g63270, and At5g64850). These carry highly-conserved AvrRpt2 cleavage-site motifs (VPQFGDW or LPKFGEW) near their amino termini, suggesting they are also targets for cleavage by AvrRpt2, and all have at least one conserved cysteine residue near their carboxyl termini, suggesting they may also be either prenylated, acylated, or both and anchored to the plasma membrane (Fig. 6A and B). To test the functionality of these motifs, similar bombardment experiments to those performed with GFP:RIN4-70C were performed

with equivalent constructs, GFP:RIN4-CTH1 and GFP:RIN4-CTH2, containing entire CTH, here designated CTH1 (corresponding to At2g17660) and CTH2 (corresponding to At5g09960), representative of two distinct subclasses of CTH.

Fig. 5. **A**, Alignment of the carboxyl-terminal membrane-attachment motifs of RIN4 and its full-length homologs (FLH). The putative plasma membrane-attachment motif KGCCF of FLH1 and FLH2 is underlined. *Arabidopsis* gene identifiers are shown to the left of the alignment. **B**, Alignment of the amino- and carboxyl-terminal AvrRpt2 cleavage sites and downstream regions of RIN4 and its FLH. FLH1 and FLH2 have no conserved carboxyl-terminal cleavage sites but do have conserved carboxyl-terminal downstream regions. The cleavage-site motif PxFGxW and the conserved downstream TxxFxK motif are underlined. Amino-acid identities are highlighted in black and similarities in gray. Alignments were produced using ClustalW and were edited manually. **C**, Micrographs showing membrane-localized patterns of green fluorescent protein (GFP) fluorescence in epidermal cells of tobacco leaves bombarded with GFP:RIN4-FLH1-80C alone (left) or cobombarded with AvrRpt2 (right), indicating lack of carboxyl-terminal cleavage by AvrRpt2.

Fig. 6. **A**, Alignment of the carboxyl-terminal membrane-attachment motifs of RIN4 and its short carboxyl-terminal homologs (CTH). The putative plasma membrane-attachment motifs WLCF and CCVK are underlined. **B**, Alignment of the amino- and carboxyl-terminal AvrRpt2 cleavage sites (or corresponding regions) and downstream regions of RIN4 and its short CTH. The cleavage-site motif PxFGxW and the conserved downstream TxxFxK motif are underlined. Amino acid identities are highlighted in black and similarities in gray. Alignments were produced using ClustalW and were edited manually. *Arabidopsis* gene identifiers are shown to the left of the alignments. The conceptual translation for At2g04410 published as GenBank accessions AY058877 and AAL24264 is incorrect, and we have submitted a revised conceptual translation to The Arabidopsis Information Resource (TAIR) database. At4g33655 is a previously unrecognized gene located between At4g33650 and At4g33660 in GenBank accession AL161583, and we have submitted the new conceptual translation to the TAIR database. **C**, Micrographs showing membrane-localized patterns of green fluorescent protein (GFP) fluorescence in epidermal cells of tobacco leaves bombarded with GFP:RIN4-CTH1 (top row) or GFP:RIN4-CTH2 (bottom row) alone (left panels) or cytosolic patterns of GFP fluorescence when cobombarded with AvrRpt2 (right panels), indicating cleavage by AvrRpt2.

Both constructs showed membrane-localized patterns of fluorescence, indicating that these homologs of RIN4 are also plasma-membrane localized (Fig. 6C). Cobombardment of GFP:RIN4-CTH1 or GFP:RIN4-CTH2 with AvrRpt2 resulted in a cytosolic pattern of fluorescence, indicating that AvrRpt2 cleaved both homologs (Fig. 6C).

DISCUSSION

RIN4 and its homologs are attached to the plasma membrane at their carboxyl termini.

We have shown that RIN4 is attached to the plasma membrane at its carboxyl terminus and carries a carboxyl-terminal CCCFxF motif essential for plasma-membrane attachment (Fig. 1). These data are consistent with either prenylation, acylation, or both of one or more of the three cysteines in this motif. Consistent with this interpretation, Kim and associates (2005) used a CCC to AAA mutation to show that the CCC motif is required for membrane attachment and used a palmitoylation inhibitor to show that RIN4 is probably palmitoylated on one or more of the three cysteine residues.

In contrast to the CCCFxF motif of RIN4, the FLH of RIN4 have a carboxyl-terminal KGCCF motif (Fig. 5A) and most of the short CTH have only a conserved carboxyl-terminal CF motif (Fig. 6A). Representative homologs FLH1, CTH1, and CTH2 were all shown to be plasma-membrane attached (Fig. 5C and 6C), suggesting that a single cysteine may be sufficient for membrane attachment and, therefore, that prenylation or acylation may not occur on the first or second cysteines of the CCCF motif in RIN4. These residues might instead contribute to a prenylation or acylation signal by providing an appropriate hydrophobic sequence context for the prenylated or acylated cysteine (Fig. 1C). However, lysine can be an additional substrate for acylation (Smotrys and Linder 2004), so acylation of the lysine in the KGCCF motif could potentially compensate for the absence of a third cysteine in the FLH. In the CTH subclass represented by CTH1, the CF motif is often preceded by a WL motif or other hydrophobic amino acid residues, suggesting that a hydrophobic sequence context (Fig. 6A) may also be important for prenylation or acylation of the single cysteine. However, the CF motif is closer to the carboxyl terminus in most of these CTH than the CCCF motif of RIN4 (Fig. 6A), perhaps enhancing the possible recognition of a single cysteine as a substrate for prenylation or acylation. Moreover, the WLCF motif is preceded by two or three lysine residues in a KxK, xKK, or KKK configuration (Fig. 6A), which may provide additional substrates for acylation or help stabilize a weak membrane association effected by a single prenylated or acylated cysteine. In contrast, the two homologs represented by CTH2 have a CF motif slightly more distant from the carboxyl terminus than the CCCF motif of RIN4 (Fig. 6A). However, these homologs each have a CCVK motif downstream of the CF motif (Fig. 6A) that may provide alternative sites for prenylation or acylation. From this variation in the number, arrangement, and context of the cysteines in the carboxyl termini of RIN4 and its homologs, it seems likely that there will be considerable variation between RIN4 and its homologs in the patterns of prenylation or acylation and in the strength of plasma-membrane association. Nevertheless, the conservation of cysteine-based membrane-attachment motifs across the protein family suggests that they are functionally important.

The functional importance of the RIN4 membrane-attachment motif has been demonstrated by two studies of RIN4 published while this paper was in preparation. Day and associates (2005) showed that deletion of the carboxyl-terminal two, six, or nine amino acids of RIN4 had a stepwise effect on the ability of

RIN4 to negatively repress RPS2 autoactivity in *Nicotiana benthamiana*, with the two-amino acid deletion having a minor effect and the six- and nine-amino acid deletions having a major effect (Day et al. 2005). The latter deletions encompass all or part of the CCCFxF motif that we have shown to be important for plasma-membrane attachment and, consistent with our results, the study by Day and associates (2005) provides biochemical evidence for a stepwise loss of membrane association in the deletion mutants and also showed that a C203R mutant (affecting the first cysteine of the CCCFxF motif) was no longer able to interact with RPS2 or repress its function. We have shown that a C203A mutation abolishes plasma-membrane association, so it is reasonable that the C203R mutation will have the same effect. Thus, membrane attachment of RIN4 appears to be critically important for both interaction with RPS2 and negative regulation of RPS2. Kim and associates (2005) showed that either a CCC to AAA mutation of RIN4 or treatment with a palmitoylation inhibitor resulted in the degradation of RIN4 in *Arabidopsis*, indicating that membrane attachment is also required for RIN4 stability.

RIN4 is cleaved by AvrRpt2 at a conserved PxFGxW motif.

Our data show that RIN4 is cleaved by AvrRpt2 at both the amino-terminal VPKFGNW (Fig. 3) and carboxyl-terminal VPKFGDW (Fig. 2) motifs. Studies of RIN4 cleavage by AvrRpt2 published while this paper was in preparation also showed cleavage at these two sites (Chisholm et al. 2005; Coaker et al. 2005; Kim et al. 2005). Alanine-scanning mutagenesis of the carboxyl-terminal cleavage site of RIN4 showed an essential role for the phenylalanine residue, an important role for the proline residue and a less important role for the glycine and tryptophan residues in AvrRpt2 cleavage-site recognition (Fig. 2). Evidence for an essential role of the phenylalanine residue in AvrRpt2 cleavage-site recognition was also obtained in two studies of AvrRpt2 self-cleavage (Chisholm et al. 2005; Jin et al. 2003). These studies also showed an important role for the glycine residue in self-cleavage, but the earlier study suggested a more important role than the latter. Evidence of a less-important role for the proline and tryptophan residues in self-cleavage was also obtained in the latter study (Chisholm et al. 2005). These results are broadly consistent with our data for recognition by AvrRpt2 of the carboxyl-terminal cleavage site of RIN4. The phenylalanine residue was also shown to be essential for recognition by AvrRpt2 of both the amino-terminal and carboxyl-terminal cleavage sites of RIN4 (Chisholm et al. 2005; Coaker et al. 2005) and the amino-terminal cleavage sites of several RIN4 homologs (Chisholm et al. 2005), in agreement with our data. Determination of the residues important for cleavage-site recognition by AvrRpt2 has enabled the identification of a large number of potential targets for AvrRpt2 action in the *Arabidopsis* proteome (Chisholm et al. 2005), including all but two of the homologs identified in this study.

RIN4 is released from the plasma membrane following cleavage by AvrRpt2, but its homologs are not.

We have generated data indicating that a large portion of the RIN4 protein is released from the plasma membrane by cleavage at the carboxyl-terminal AvrRpt2 cleavage site (Fig. 2), and while this paper was in preparation, similar data were published by Kim and associates (2005). We have also shown that one of the FLHs of RIN4, FLH1, lacks a carboxyl-terminal AvrRpt2 cleavage-site motif (Fig. 5B) and is not released from the plasma membrane by AvrRpt2 cleavage (Fig. 5C). The other FLH (corresponding to At5g48657), here designated FLH2, also lacks a carboxyl-terminal cleavage-site motif (Fig. 5B) and is unlikely to be released from the plasma membrane by AvrRpt2 cleavage.

The presence of two AvrRpt2 cleavage sites in RIN4 probably reflects an ancient coding-region duplication of a CTH with a single amino-terminal cleavage site. This possibility is supported by homology between regions downstream of the amino-terminal and carboxyl-terminal cleavage sites (or corresponding regions) of RIN4 and its two FLHs (Fig. 5B). In particular, a (F/Y)TxxFxK motif is highly conserved in RIN4 and its homologs (Figs. 5B and 6B), and the (F/Y)TxxF motif is conserved even in two distant homologs of RIN4 (At5g18310 and At5g48500). The retention of the carboxyl-terminal cleavage site in RIN4 but its loss in the FLH, as opposed to conservation of the adjacent downstream sequences in RIN4 and the FLH, is remarkable because it suggests that the carboxyl-terminal cleavage site and the adjacent downstream sequences have evolved independently of one another and, therefore, that the adjacent downstream sequences probably serve a different function to that of the carboxyl-terminal cleavage site. Kim and associates (2005) have shown that AvrB binds to a region of RIN4 defined by amino acid residues 142 to 179. This includes the carboxyl-terminal AvrRpt2 cleavage site and the conserved (F/Y)TxxFxK motif. However, mutation of the AvrRpt2 cleavage site from VPKFGNW to APKAANA did not abolish RPM1 activation by AvrB (Kim et al. 2005), suggesting that amino acid residues in the cleavage site probably do not play a key role in AvrB binding. The failure of AvrB to bind to the amino-terminal region of RIN4 (Kim et al. 2005) also suggests that neither the cleavage-site motif nor the conserved (F/Y)TxxFxK motif play a major role in AvrB binding. Interestingly, the intervening sequences between these two motifs are not conserved between the duplications within RIN4 or between RIN4 and the FLH (Fig. 5B) but are strongly conserved between RIN4 and the seven CTH in the subclass represented by CTH1 (Fig. 6B). It is therefore possible that a subset of the CTHs bind AvrB and are targets for the pathogenicity function of AvrB. The (F/Y)TxxFxK motif may contribute to AvrB binding or it could be a target for AvrRpm1 action or it may interact with other pathogen or plant proteins or even serve an innate function. Its conservation in RIN4 and its homologs suggests that it probably has an important role to play, which makes it an interesting target for site-directed mutagenesis.

The amino-terminal AvrRpt2 cleavage site is conserved throughout the RIN4 protein family.

In contrast to the absence of a carboxyl-terminal AvrRpt2 cleavage motif in the FLHs of RIN4, the amino-terminal AvrRpt2 cleavage site is highly conserved (Fig. 5B and B), suggesting that the full-length RIN4 homologs are also likely to be cleaved by AvrRpt2. Consistent with this expectation, cleavage of the FLH designated FLH2 has been shown by Chisholm and associates (2005). Similarly, the short carboxyl-terminal RIN4 homologs are also likely to be cleaved near their amino termini by AvrRpt2. We have shown that CTH1 and CTH2 are cleaved by AvrRpt2, and Chisholm and associates (2005) have shown that three others (encoded by At5g18310, At5g48500, and At5g55850) are also cleaved by AvrRpt2. Thus, there is a family of three full-length proteins, comprising RIN4 and two homologs, and at least 11 shorter RIN4 homologs in *Arabidopsis* all of which are likely to be attached to the plasma membrane by their carboxyl termini, and all of which are likely to be targets for cleavage by AvrRpt2 and exposure of destabilizing amino-terminal residues. The strong conservation of the amino-terminal cleavage sites across different members of the RIN4 protein family suggests that AvrRpt2 may be an exogenous mimic of an endogenous plant protease involved in regulation of these proteins. However, the function of these proteins and the consequences of

their degradation following cleavage by either an endogenous protease or by AvrRpt2 have yet to be determined. Potentially, AvrRpt2 has tapped into a regulatory system whereby cleavage and exposure of amino-terminal residues with different N-end rule properties allows the fine-tuning of protein levels by differential degradation of the various cleavage targets.

Cleavage by AvrRpt2 destabilizes RIN4 and its homologs in a manner consistent with the N-end ubiquitylation and subsequent proteasomal degradation.

Our data suggest that cleavage of RIN4 by AvrRpt2 has a destabilizing effect consistent with the N-end rule for protein ubiquitylation and proteasomal degradation (Fig. 3). In N-end rule degradation destabilizing amino-terminal amino acid residues are classified as primary, secondary, or tertiary destabilizing residues (Spremulli 2000). Basic residues (arginine, lysine, or histidine) or bulky hydrophobic residues (leucine, isoleucine, phenylalanine, tyrosine, and tryptophan) are primary destabilizing residues that require no modification before ubiquitylation and proteasomal degradation. The secondary destabilizing residues aspartic acid and glutamic acid are conjugated to arginine, a primary destabilizing residue, by an arginyl-tRNA:protein arginyltransferase (Spremulli 2000). The tertiary destabilizing residues asparagine and glutamine are deamidated by an N-terminal deamidase converting them into the secondary destabilizing residues aspartic acid and glutamic acid (Spremulli 2000).

Cleavage of RIN4 and its homologs reveals the secondary destabilizing residues aspartic acid or glutamic acid, or the tertiary destabilizing residue asparagine (Figs. 1B, 5B, and 6B), suggesting that potentially all of these proteins are ubiquitylated and degraded by the proteasome following cleavage by AvrRpt2. However, our evidence for destabilization following cleavage by AvrRpt2 was obtained using a soluble RIN4-30N:GFP fusion conceptually similar to the large soluble RIN4 fragment released from the plasma membrane by cleavage at the amino- and carboxyl-terminal cleavage sites and does not necessarily extrapolate to the RIN4 homologs, which remain membrane-anchored after cleavage. It is conceivable that membrane release and exposure of destabilizing amino-terminal amino acids are both required for efficient degradation by the proteasome, and this could explain why RIN4 has two cleavage sites.

Membrane release following cleavage at the carboxyl-terminal AvrRpt2 cleavage site alone results in degradation of RIN4 (Chisholm et al. 2005; Kim et al. 1005). Cleavage at the carboxyl-terminal cleavage site alone would result in the release of RIN4 but would not make it a substrate for N-end rule degradation. This suggests that another degradation mechanism must also be involved in the degradation of RIN4. Kim and associates (2005) have also shown that cleavage at the amino-terminal AvrRpt2 cleavage site alone did not result in degradation of RIN4 in *Arabidopsis*, but this conflicts with Chisholm and associates (2005), who have shown that cleavage at the amino-terminal AvrRpt2 cleavage site alone does result in degradation of RIN4 in *N. benthamiana*. The latter result is consistent with N-end rule degradation. Therefore it seems likely that both mechanisms operate in the degradation of RIN4, although their perceived importance may vary according to the assay system used.

Kim and associates (2005) have also shown that AvrB binds to the region of RIN4 that contains the carboxyl-terminal AvrRpt2 cleavage-site motif, downstream (F/Y)TxxFxK motif, and conserved intervening sequence (Figs. 5B and 6B) but not to the region that contains the amino-terminal AvrRpt2 cleavage-site motif and downstream (F/Y)TxxFxK motif but no conserved intervening sequence (Figs. 5B and 6B). It is con-

ceivable that AvrB could mask the carboxyl-terminal AvrRpt2 cleavage site, preventing cleavage by AvrRpt2. However, cleavage would still occur unhindered at the amino-terminal AvrRpt2 cleavage site allowing AvrRpt2 to interfere with detection of AvrB as observed previously (Ritter and Dangel 1996; Mackey et al. 2003) rather than the other way around. This scenario suggests that N-end rule degradation might play an important role in RIN4 degradation, depending on the repertoire of avirulence genes carried by the pathogen.

Chisholm and associates (2005) have also shown that FLH2, a CTH, and two CTH-like proteins with weak RIN4 homology are all rapidly degraded following cleavage at their solitary amino-terminal AvrRpt2 cleavage sites (Chisholm et al. 2005). These results are consistent with N-end rule degradation but cannot be explained by a degradation mechanism related to membrane release because we have shown that AvrRpt2 does not release these proteins from the plasma membrane. Thus a major pathogenic consequence of AvrRpt2 cleavage may be the N-end rule degradation of the homologs of RIN4.

Most work on N-end rule degradation has been carried out in *Escherichia coli*, yeast, or mammals, but there is ample evidence for the same process in plants. For example, the *ATE1* gene of *Arabidopsis* encodes an arginyl-tRNA:protein arginyl-transferase (Yoshida et al. 2002) and the *PRT1* gene of *Arabidopsis* encodes a ubiquitin protein ligase that targets aromatic amino-terminal residues (Stary et al. 2003). *ATE1*, but not *PRT1*, may therefore be required for degradation of proteins identified as potential pathogenicity targets of AvrRpt2. It would, therefore, be interesting to examine AvrRpt2 virulence effector function in *ate1* mutant plants or *ATE1*-silenced plants.

Concluding remarks.

RIN4 is a member of a family of proteins that are targets for cleavage by AvrRpt2. A major challenge of the future will be to understand the virulence effector function of AvrRpt2 in terms of its effect on the function of these targets. A second challenge will be to determine exactly how AvrB and AvrRpm1 interact with RIN4 and whether, if as seems likely for AvrB, they target the same protein family or have a different set of targets. RIN4 has two AvrRpt2 cleavage sites, compared with one in each of its homologs. Thus, in dramatic contrast to its homologs, a major portion of RIN4 is released from the plasma membrane by AvrRpt2 cleavage, so facilitating its degradation. In addition to more cleavage sites, RIN4 also has many more potential phosphorylation sites with 29 serines upstream of the CCC motif, including seven pairs of adjacent serine residues, compared with 13 and 16 in its FLH, with only one and two pairs of adjacent serine residues. Perhaps significantly, two of these pairs of serines flank the conserved (F/Y)TxxFxxK motif present in the region of RIN4 that binds AvrB. Thus, RIN4 is potentially more sensitive to both the cleavage caused by AvrRpt2 and the hyperphosphorylation induced by AvrB or AvrRpm1. This, combined with the potential for functional redundancy provided by other members of the protein family, leads us to question whether RIN4 retains a function, e.g., in plant metabolism, that makes other members of the family targets for virulence effectors or whether it has been recruited for a role in resistance and functions solely in this capacity. The RIN4-mediated interactions between RPS2 and AvrRpt2 or between RPM1 and AvrB or AvrRpm1 have generated strong support for the guard or surveillance hypothesis, which postulates that resistance proteins monitor host proteins whose functions are targets for manipulation by the pathogen (Dangel and Jones 2001; Van Der Biezen and Jones 1998). However, this hypothesis may require modification to include the possibility that some resistance proteins, rather than moni-

toring functional target proteins, may be monitoring bait proteins that do not retain their original function.

MATERIALS AND METHODS

Plant growth conditions and plant transformation.

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown under standard glasshouse conditions at 25°C by day and 18°C by night, and leaves were harvested from 4- to 8-week-old plants for particle bombardment experiments. *Arabidopsis* plants (*Arabidopsis thaliana* ecotype Columbia) were grown at 21°C with 16 h of light (50 μmol of photons $\text{m}^{-2} \text{s}^{-1}$) per day before transformation, pathogen inoculation, or particle bombardment. Transformation of *Arabidopsis* was performed by the floral-dip method (Clough and Bent 1998) using *Agrobacterium tumefaciens* AGL-1 (Lazo et al. 1991) containing pCBJ-RIN4-70C. Screening of transformants was performed as described previously (Takemoto et al. 2003).

Construction of vectors and particle bombardment.

RIN4 and *RIN4* homologs, *GFP*, *AvrRpt2*, and *AvrRpm1* sequences were obtained by polymerase chain reaction amplification, using mutagenic primers to introduce cloning sites and site-specific mutations as required. *Arabidopsis* genomic DNA was used as the template for *RIN4* components, and plasmid DNA carrying a derivative of *GFP* described previously (Benghezal et al. 2000; Takemoto et al. 2003) was used as a template for *GFP* components. *Pseudomonas syringae* pv. *tomato* DC3000 strains with plasmids carrying *AvrRpt2* or *AvrRpm1* were obtained from R. Innes (Indiana University, Bloomington, IN, U.S.A.). Plasmid DNA containing *AvrRpt2* or *AvrRpm1* was used as a template for amplification of *AvrRpt2* or *AvrRpm1* (Dangel et al. 1992; Innes et al. 1993). The C122A mutation of *AvrRpt2* was generated by amplification of *AvrRpt2* in two parts using mutagenic primers and reconstituting the full-length gene, using cloning sites introduced at the same time. All amplified components were cloned and authenticated by sequence analysis. *GFP* fusions were placed under the control of a CaMV 35S promoter and terminator in a binary plant transformation vector (pSLJ7292) for generation of transgenic plants or pBluescript SK(-) (Stratagene, La Jolla, CA, U.S.A.) for bombardment experiments. Bombardments were carried out using a BioRad PDS-1000/He biolistic particle delivery system (BioRad, Hercules, CA, U.S.A.) according to the manufacturer's instructions, and transformed epidermal cells were monitored by confocal laser-scanning microscopy 6 h after bombardments, except where otherwise indicated. For cobombardment experiments, 400 ng of each GFP-containing vector and plasmid with *Avr* gene were used to coat 1.25 mg of 1- μm diameter gold particles in a 25- μl suspension. For Figure 3, the numbers of cells with various levels of GFP fluorescence were monitored using a fluorescence stereo-microscope (model MZ FLIII; Leica, Heidelberg, Germany).

Confocal laser-scanning microscopy.

Confocal fluorescence images were recorded on a TCS SP2 confocal system (Leica) with a 63 $\times$ NA 1.2 water-immersion lens. Leaf sections were mounted between glass coverslips with the transformed surface facing the objective lens. A krypton-argon laser was used as a 488-nm excitation source, and GFP fluorescence was recorded between 495 and 520 nm. Images of GFP fluorescence shown in the figures are projections of optical sections taken at 1- μm intervals from the outer epidermal wall through to a position just above the cortical cytoplasm adjacent to the inner periclinal wall of the epidermal cell. The images were stored as TIF files and processed with Canvas 8 software (Deneba Systems Inc., Miami, FL, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

ExPASy Proteomics Server website: au.expasy.org
The *Arabidopsis* Information Resource (TAIR) website: www.arabidopsis.org