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A duplicated pair of Arabidopsis RING-finger E3 ligases contribute to the RPM1- and RPS2-mediated hypersensitive response

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Summary

The Arabidopsis RPM1 protein confers resistance to disease caused by *Pseudomonas syringae* strains delivering either the AvrRpm1 or AvrB type III effector proteins into host cells. We characterized two closely related RPM1-interacting proteins, RIN2 and RIN3. RIN2 and RIN3 encode RING-finger type ubiquitin ligases with six apparent transmembrane domains and an ubiquitin-binding CUE domain. RIN2 and RIN3 are orthologs of the mammalian autocrine motility factor receptor, a cytokine receptor localized in both plasma membrane caveolae and the endoplasmic reticulum. RIN2 is predominantly localized to the plasma membrane, as are RPM1 and RPS2. The C-terminal regions of RIN2 and RIN3, including the CUE domain, interact strongly with an RPM1 N-terminal fragment and weakly with a similar domain from the Arabidopsis RPS2 protein. RIN2 and RIN3 can dimerize through their C-terminal regions. The RING-finger domains of RIN2 and RIN3 encode ubiquitin ligases. Inoculation with *P. syringae* DC3000(*avrRpm1*) or *P. syringae* DC3000(*avrRpt2*) induces differential decreases of RIN2 mobility in SDS-PAGE and disappearance of the majority of RIN2. A *rin2 rin3* double mutant expresses diminished RPM1- and RPS2-dependent hypersensitive response (HR), but no alteration of pathogen growth. Thus, the RIN2/RIN3 RING E3 ligases apparently act on a substrate that regulates RPM1- and RPS2-dependent HR.

Keywords: RING-finger ubiquitin ligase, disease resistance, hypersensitive response, RPM1, RPS2.

Introduction

The plant immune system responds to infection using both general recognition of microbe-associated molecules (Gomez-Gomez and Boller, 2000) and specific recognition of particular pathogen-encoded molecules through the action of plant disease resistance (R) proteins (Belkhadir *et al.*, 2004; Dangl and Jones, 2001). The majority of R proteins defined to date have a central nucleotide binding site (NB) domain and C-terminal leucine-rich repeats (LRRs). One subclass of NB-LRR proteins contains a coiled-coil (CC) motif at the N-terminus (CC-NB-LRR), while a second carries an N-terminal motif homologous to the cytoplasmic domain of *Drosophila* Toll and mammalian interleukin (IL-1) receptors (TIR-NB-LRR) (Dangl and Jones, 2001; Martin *et al.*, 2003). Recognition of pathogens mediated by NB-LRR results in rapid ion fluxes, a sustained oxidative burst, the enhanced transcriptional induction of a basal defense transcriptome, production of antimicrobial compounds and induction of systemic acquired disease resistance at distal sites (Dangl and Jones, 2001; Durrant and Dong, 2004; Martin *et al.*, 2003). Defense responses are often accompanied by a localized programmed cell death at the infection site, called the

hypersensitive response (HR) (Dangl *et al.*, 1996; Greenberg and Yao, 2004).

Plant NB-LRR proteins directed against phytopathogenic bacteria recognize the type III effector proteins that these pathogens deliver into the host cell. Type III effector proteins can act as virulence factors, and are delivered to the host cell by the evolutionarily conserved type III secretion system (Alfano and Collmer, 2004; Chang *et al.*, 2004; Nimchuk *et al.*, 2003). It is increasingly likely that the recognition of many type III effector proteins is indirect, and that NB-LRR proteins have evolved to monitor the activity of these virulence factors on host targets (Axtell and Staskawicz, 2003; Dangl and Jones, 2001; Mackey *et al.*, 2003; Shao *et al.*, 2003). However, the mechanisms by which NB-LRR proteins transduce signals leading to successful disease resistance and the associated HR remain unclear.

The Arabidopsis *RPM1* and *RPS2* genes encode CC-NB-LRR proteins that confer resistance to *Pseudomonas syringae* strains expressing either of two different type III effectors, *avrRpm1* or *avrB* (Grant *et al.*, 1995) or *avrRpt2* (*RPS2*; Bent *et al.*, 1994; Mindrinos *et al.*, 1994). RPM1 is a peripheral plasma membrane protein, and it disappears via an unknown mechanism coincident with the onset of the HR (Boyes *et al.*, 1998). Degradation of diverse receptors has been investigated in various animal systems. For example, some Toll-like receptors (TLRs) that function in the innate animal immune response are degraded via a ubiquitination pathway, thus limiting TLR signaling to prevent undesirable amplification of host responses (Chuang and Ulevitch, 2004). By contrast, RPS2 is also membrane associated, but it does not disappear following stimulation(Axtell and Staskawicz, 2003).

Protein degradation mediated by the ubiquitination pathway is performed by a sequential reaction of three enzymes (Smalle and Vierstra, 2004). First, a ubiquitin-activating enzyme (E1) activates ubiquitin by the formation of a thioester bond between ubiquitin and a cysteine at the E1 active site. The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2) to form a thioester linkage. A ubiquitin–protein ligase (E3) recognizes the target protein as a substrate, and promotes the formation of an isopeptide bond between ubiquitin and lysine residues on the target protein. The ubiquitinated protein is then rapidly degraded via the proteasome (Vierstra, 2003). Ubiquitination can regulate target protein function independent of the proteasome. In these cases, ubiquitination is required for various subsequent regulatory responses including endocytosis and relocalization of the target protein (Prag *et al.*, 2003).

In Arabidopsis, E3 ubiquitin ligases are classified into various groups based on the presence of RING, HECT, or U-box domains (Vierstra, 2003). There are 469 predicted RING-proteins in Arabidopsis (Stone *et al.*, 2005). The RING domains in some of these proteins have demonstrable ubiquitin ligase activity, and function in hormone, light and various developmental processes (Vierstra, 2003). Several targets for RING E3 ligases, including COP1, SINAT5 and RMA1, have been identified (Matsuda *et al.*, 2001; Saijo *et al.*, 2003; Seo *et al.*, 2003; Xie *et al.*, 2002).

Participants in NB-LRR protein function have been characterized by genetic screens for mutants that exhibit defects in disease resistance, HR or both, and by yeast two-hybrid screens. For example, *PBS1*, *NDR1*, *EDS1*, *PAD4, RAR1*, *SGT1b* and *HSP90* encode proteins that are required for the activity of some or all NB-LRR proteins. (Dangl and Jones, 2001; Muskett and Parker, 2003; Schulze-Lefert, 2004; Shirasu and Schulze-Lefert, 2003; Swiderski and Innes, 2001; Tornero *et al.*, 2002). Some of these, like RAR1 and HSP90, function in the stabilization and/or accumulation of signal competent NB-LRR proteins (Hubert *et al.*, 2003; Tornero *et al.*, 2002), while others might function in signal transduction (e.g. PAD4 and EDS1; Hammond-Kosack and Parker, 2003).

Yeast two-hybrid screening using RPM1 fragments as baits has identified two other proteins that influence RPM1 function, RIN1 and RIN13 (Al-Daoude *et al.*, 2005; Holt *et al.*, 2002). *RIN1* encodes an ortholog of TIP49a that interacts with TATA binding protein. RIN1 is a negative regulator of disease resistance responses and is also required for normal plant development (Holt *et al.*, 2002). The function of RIN13 is not predicted from its sequence. Overexpression of *RIN13* enhances resistance to *P. syringae* (*Pto*) expressing *avrRpm1* without triggering HR (Al-Daoude *et al.*, 2005). Resistance mediated by RPM1 is inhibited by suppression of *RIN13* expression, suggesting that RIN13 may function as a positive regulator of RPM1. However, the molecular basis of how RIN13 regulates the RPM1-mediated response is unknown.

A similar screen using AvrB as a bait led to isolation of the RIN4 protein, a small protein of unknown function that is acylated into the plasma membrane (H.S. Kim *et al.*, 2005; M.G. Kim *et al.*, 2005). RIN4 co-immunoprecipitates with RPM1 and RPS2, and is required for their activation (Axtell and Staskawicz, 2003; Mackey *et al.*, 2002, 2003). RIN4 is a target for AvrB, AvrRpm1 and AvrRpt2 and its manipulation by these unrelated type III effectors drives activation of RPM1 or RPS2 (Axtell and Staskawicz, 2003; Day *et al.*, 2005; H.S. Kim *et al.*, 2005; M.G. Kim *et al.*, 2005; Mackey *et al.*, 2002, 2003). These examples suggest that yeast two-hybrid screens followed by reverse genetics and biochemical analyses can contribute to our understanding of NB-LRR regulation.

We characterize here two new RPM1-interacting proteins, RIN2 and RIN3. RIN2 and RIN3 are highly related plasma-membrane localized E3 ubiquitin ligases that contain RING-finger domains in association with a CUE domain (Prag *et al.*, 2003). Inoculation of *Pto* DC3000(*avrRpm1*) induces both a gradual decrease of RIN2 mobility in SDS-PAGE before HR and its degradation at the time of HR. A similar mobility shift of RIN2 is induced by inoculation of *Pto*

DC3000(*avrRpt2*), concomitant with the RPS2-dependent HR. A *rin2 rin3* double mutant exhibits reduced RPM1- and RPS2-dependent HR. However, no alteration of pathogen growth was found in the *rin2 rin3* mutant. Thus, the RIN2/RIN3 RING E3 ligases contribute positively to RPM1- and RPS2-dependent HR.

Results

Two closely related Arabidopsis RING-finger proteins interact with RPM1

We isolated RIN2 (RPM1 interacting protein 2; At4g25230) from a yeast two-hybrid screen using an RPM1 domain lacking the CC but containing most of the NB domain (amino acids 55–341; pEG10 in Figure 2; Holt *et al.*, 2002). Homology searches indicated that the Arabidopsis genome encodes another protein 83% identical to RIN2; we designated it RIN3 (At5g51450; Figure 1a). The deduced RIN2 and RIN3 proteins are 578 and 577 amino acid residues, respectively, and their estimated molecular weights are 64 567 and 64 516 Da, respectively. RIN2 and RIN3 each possess six probable transmembrane domains, a RING-finger domain and a CUE domain, recently implicated in ubiquitin binding (Figure 1b; Prag *et al.*, 2003; Shih *et al.*, 2003). RIN2 and RIN3 each contain a RING-finger consensus sequence composed of eight conserved cysteines and histidines that normally coordinate two zinc ions in a cross-braced fashion

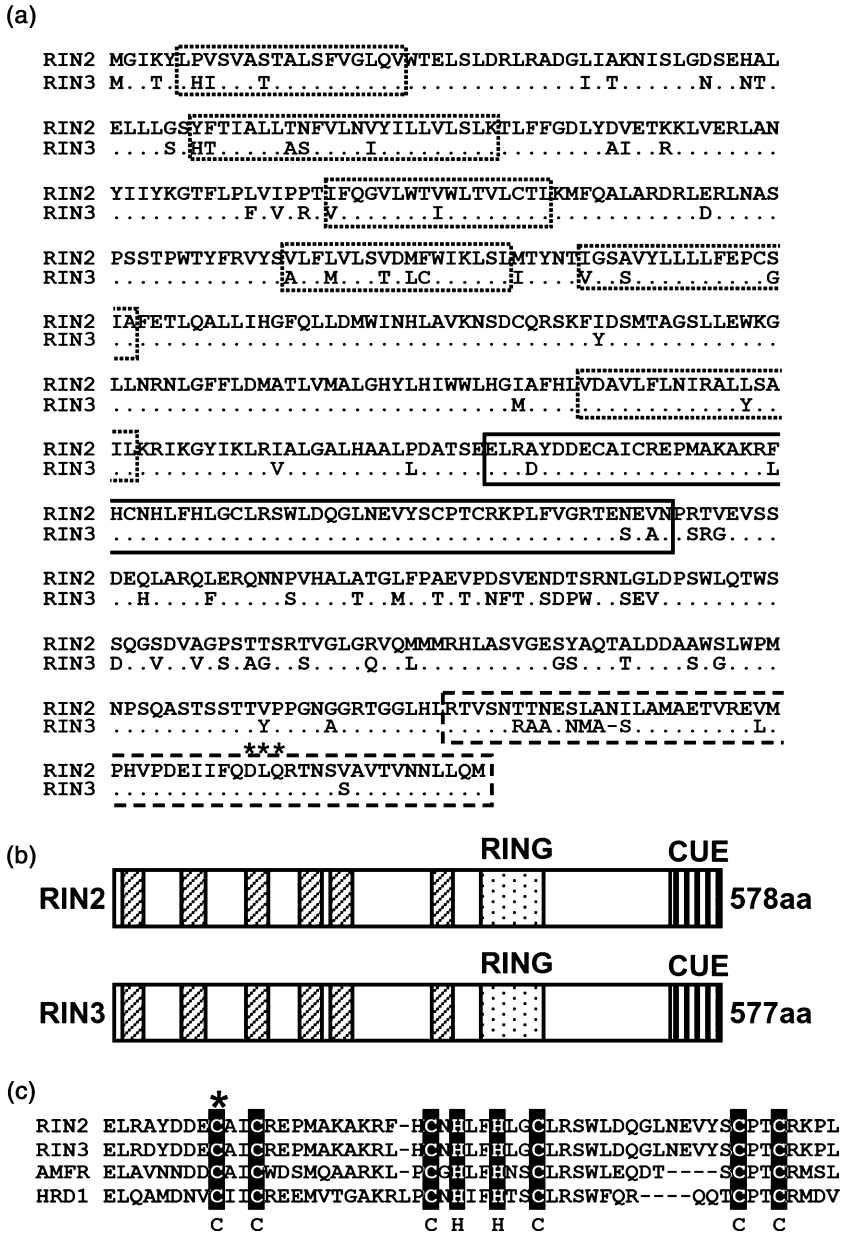

Figure 1. RIN2 and RIN3 encode multi-TM RING-finger CUE domain proteins.
(a) Comparison of the deduced amino acid sequences of RIN2 (At4g25230) and RIN3 (At5g51450). The putative transmembrane domains and CUE domain are boxed by dotted and dashed lines, respectively. The RING-finger domain is boxed by a solid line. The conserved DLQ sequence is indicated by asterisks.
(b) Protein domains of RIN2 and RIN3 showing six putative transmembrane helices (hatched boxes), the RING-finger motif (dotted box) and the CUE domain (vertical lines).
(c) RING-finger motif alignment of RIN2 and RIN3 with mammalian orthologs. The conserved Cys and His residues are boxed in black. Human AMFR (autocrine motility factor receptor; GenBank accession no AF124145) and human HRD1 (AF317634) are shown for comparison. An asterisk indicates the conserved Cys residue used for the RING-finger mutant RIN2(C337A).

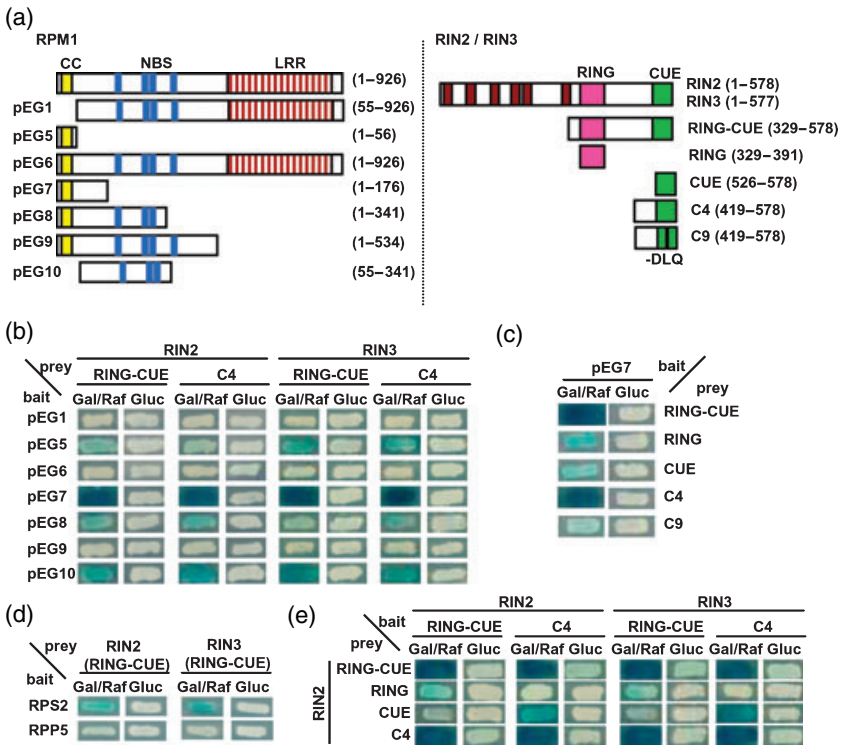

Figure 2. RIN2 and RIN3 interact with RPM1 in yeast.
(a) Constructs used for the yeast two-hybrid assay. Various RPM1 fragments (left) were inserted into a bait vector (pEG202), to give rise to fragments designated at the left of each. Their amino acid residue end-points are given to the right of each. The RIN2 and RIN3 fragments (right) were inserted into both a bait vector (pEG202) and a prey vector (pJG4-5). Their designations and amino acid residue end-points are shown at the right.
(b) RIN2 and RIN3 interact with the N-terminal region of RPM1. Columns under Gluc (glucose) and Gal/Raf (galactose/raffinose) show yeast colonies containing the indicated bait and prey vectors. The absence of β-galactosidase activation on the Gluc plates indicates lack of autoactivation. The blue color on the Gal/Raf plates indicates a positive interaction. All tests were repeated at least three times.
(c) RPM1 interacts with C-terminal domains of RIN2. Symbols as in (b).
(d) RIN2/RIN3 weakly interact with RPS2, but not RPP5. The RPS2 bait contains a domain (amino acids 1–159) including the coiled-coil region. The RPP5 bait contains a domain (amino acids 1–223) including the TIR region. Symbols as in (b).
(e) RIN2 dimerizes, and can interact with RIN3, via its C-terminal domain. Symbols as in (b).

(Figure 1c). The RING-finger domains of RIN2 and RIN3 have a histidine at the fifth zinc-coordinating residue, indicative of the RING-H2 subclass reported to have E3 ubiquitin ligase activity (Jackson *et al.*, 2000; Joazeiro and Weissman, 2000).

RIN2 and RIN3 display significant similarity to mammalian autocrine motility factor receptor (AMFR) throughout the coding region, including the order and relative spacing of all domains (not shown). AMFR is a cell surface cytokine receptor (Benlimame *et al.*, 1998; Parton and Richards, 2003; Shimizu *et al.*, 1999). Single-copy *AMFR*-like genes are present in many organisms including rice, *Drosophila*, *Caenorhabditis elegans*, zebrafish, *Xenopus*, mouse and rat. Figure 1(d) presents a phylogenetic tree of *AMFR*-like genes. RIN2 and RIN3 showed higher similarity to a rice ortholog (approximately 48% identity) compared with the animal orthologs [identities are: human (AF124145) 23%, *C. elegans* (NM_060201) 23%, zebrafish (NM_213163) 22%, *Xenopus* (BC072063) 23%, mouse (NM_011787) 23%, rat (XM_341644) 21%]. The human HRD1 protein (AF317634), which functions in protein quality control in the endoplasmic reticulum (ER) (Gardner *et al.*, 2001), also has similarity to AMFR-like proteins in the transmembrane and RING-finger domains (Figure 1c), but lacks the CUE domain.

We conducted further yeast two-hybrid assays to investigate the interactions between RIN2/RIN3 and RPM1. We generated seven RPM1 bait vectors containing different domains and combinations of domains (CC, NB and LRR; Figure 2a). We used only the C-terminal half of RIN2 (amino acids 329–578), including the RING-finger and CUE domains, to avoid complications in nuclear localization that might be caused by the probable transmembrane domains (Figure 2a). The C4 fragment of RIN2 (amino acids 419–578) is the fragment identified originally from the yeast two-hybrid screen with RPM1. The C9 fragment represents an alternatively spliced *RIN2* cDNA that deletes only D561L562Q563, a tripeptide conserved in all AMFR orthologs. C9 was truncated *in vitro* at the N-terminus to match fragment C4 (Figure 1a). We also generated analogous bait and prey vectors from RIN3 termed RING-CUE (amino acids 329–577) and C4 (amino acids 419–577).

An RPM1 N-terminal fragment (amino acids 1–176; pEG7) exhibited the strongest interaction with the RIN2 RING-CUE and C4 domains, indicating that the C4 region is sufficient for interaction with RPM1 (Figure 2b). RPM1 fragments contained in pEG5, pEG8 and pEG10 exhibited weaker interactions than pEG7. Thus, the CC region of RPM1 contributes to, but is not sufficient for, efficient interaction with RIN2. The interactions of RIN3 with RPM1 were essentially the same as those of RIN2. We also defined the regions of RIN2 required for interaction with RPM1 (pEG7) (Figure 2c). The RING-CUE domain and the C4 truncation gave the strongest interactions, while RING or CUE domains alone did not. These results suggest a requirement for the region between the RING and CUE domains in the RIN2 interaction with RPM1. In addition, deletion of the D561L562Q563 sequence conserved in the CUE domain (construct C9) resulted in loss of interaction with RPM1.

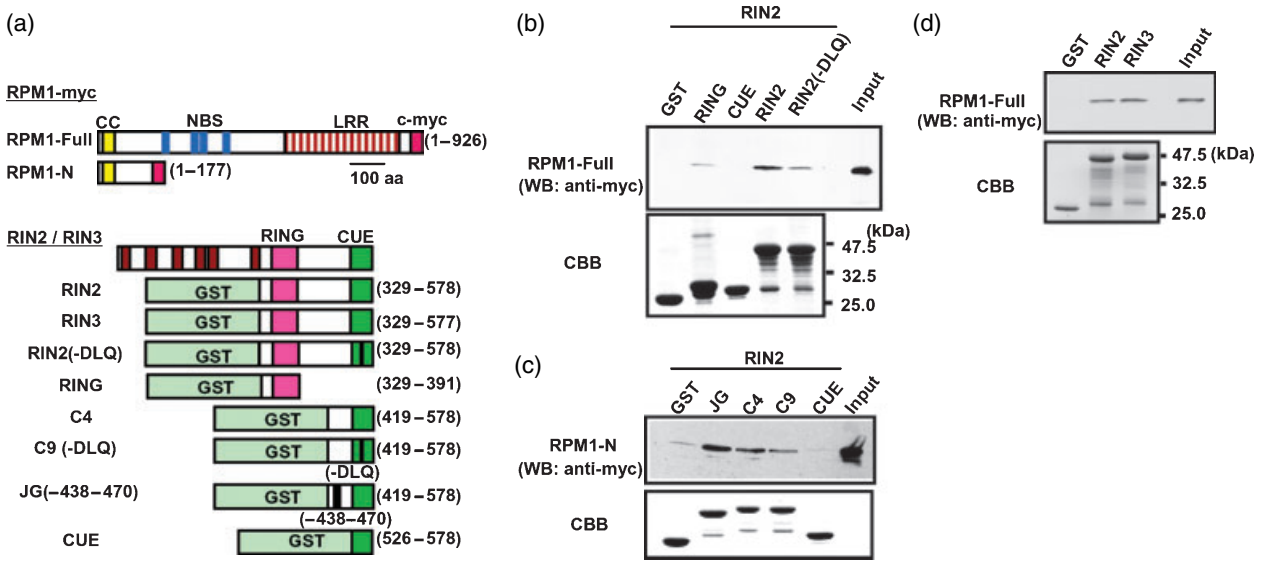

Figure 3. RIN2/RIN3 and RPM1 interact *in vitro*.
(a) Constructs used for *in vitro* interaction. The indicated fragments of RPM1 with a C-terminal c-myc epitope tag were introduced into pET14b (Novagen). The RPM1 constructs were composed of the following amino acids: RPM1-Full, amino acids 1–926; RPM1-N, amino acids 1–177. Each protein was produced by *in vitro* transcription/translation system (Promega). The indicated fragments of RIN2 and RIN3 were inserted into pGEX4T-1(Amersham Pharmacia) to produce GST fusions. The RIN2 constructs were composed of the following amino acids: RIN2, amino acids 329–578; RIN2(-DLQ), amino acids 329–578(-DLQ, amino acids 561–563); RING, amino acids 329–391; C4, amino acids 419–578; C9(-DLQ), amino acids 419–578 (deleted for amino acids DLQ at 561–563); JG, amino acids 419–578 (deleted for amino acids 438–470); CUE, amino acids 526–578. These proteins were expressed in *E. coli* and extracted.
(b) Pull-down assay between RIN2 and RPM1 reveals *in vitro* interaction mediated by the RIN2 cytoplasmic domain. RPM1 was detected by protein blot analysis using anti-myc antibody (top), and the GST-fused RIN2 proteins were detected by Coomassie brilliant blue (CBB) staining of the gel (bottom).
(c) The RIN2 CUE domain is sufficient for interaction with RPM1-myc; organized as in (b).
(d) RIN3 also interacts with RPM1 *in vitro*; organized as in (b).

We tested whether the interaction of RIN2 with RPM1 was specific or could also be observed using similar domains from related NB-LRR proteins (Figure 2d). We tested RPS2 (Bent *et al.*, 1994; Mindrinos *et al.*, 1994) and RPP5 (resistance to *Peronospora parasitica* isolate Noco2; van der Biezen *et al.*, 2000; Parker *et al.*, 1997). RPS2 is a CC-NBS-LRR protein, as is RPM1. RPP5, by contrast, contains an N-terminal TIR domain (Dangl and Jones, 2001). RIN2 and RIN3 weakly interacted with RPS2, but not at all with RPP5, consistent with a requirement for the CC domain in this interaction.

RIN2 and RIN3 can homo- and heterodimerize, and this was strongest when using the C4 domain from each (Figure 2e). The CUE domains alone could weakly bind C4, but not the RING-CUE domain. This indicates that sequences upstream of CUE in C4 are required for homo- and/or hetero-oligomerization.

RIN2 and RIN3 interact with RPM1 in vitro

We used *in vitro* binding experiments to confirm the interaction between RIN2/RIN3 and RPM1. We produced epitope-tagged RPM1-c-myc protein using an *in vitro* transcription/translation system and a series of RIN2/RIN3 proteins fused to glutathione-S-transferase (GST) (Figure 3a; see Experimental procedures). The *RIN2* cDNA clone designated JG was also isolated during the initial two-hybrid screen as a splice variant that eliminates RIN2 residues 438–470 (data not shown). The GST-fused RIN2 proteins were bound to glutathione Sepharose 4B, washed and then incubated with RPM1-myc proteins (see Experimental procedures). The RING-CUE fragment of RIN2 interacted with full-length RPM1. Deletion of D561L562Q563 in this context resulted in weaker interaction (Figure 3b). The same results were obtained using a shorter RIN2 truncation (compare C4 with C9; Figure 3c). As expected from the two-hybrid results, the CUE domain of RIN2 alone did not interact with RPM1 (Figure 3c). Interestingly, the RIN2 splice variant (JG) exhibited enhanced binding to RPM1. RIN3 also bound to RPM1 *in vitro* (Figure 3d).

RIN2 and RIN3 express ubiquitin ligase activity

RING-finger domains typically act as E3 ubiquitin ligases (Jackson *et al.*, 2000; Joazeiro and Weissman, 2000). To address whether RIN2 and RIN3 have this activity, we constructed, expressed and incubated a series of GST–RIN2 fusions with wheat E1 enzyme, the *Arabidopsis* E2 enzyme AtUBC1 and His-tagged ubiquitin (Figure 4a; see Experimental procedures). These reactions were separated by SDS-PAGE and transferred to nitrocellulose

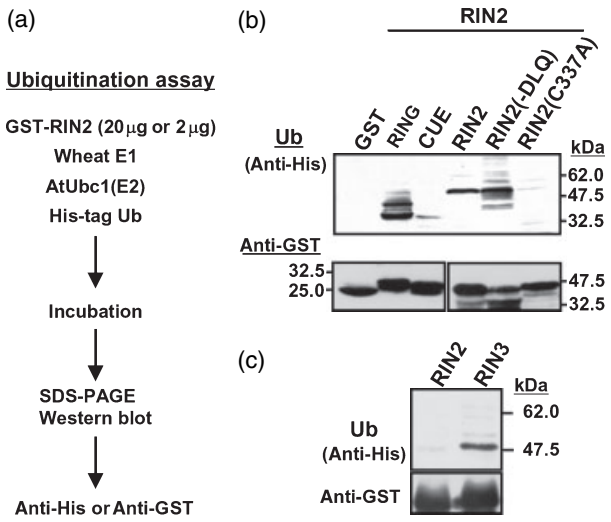

Figure 4. RIN2 and RIN3 express ubiquitin ligase activity.
(a) Outline of the procedure for ubiquitination assay. The GST-fused RIN2 and RIN3 proteins (RING-CUE) were purified using glutathione Sepharose 4B beads. AtUBC1 was purified according to Sullivan and Vierstra (1991).
(b) RIN2 autoubiquitination mediated by the RING domain. The GST-fused RIN2 proteins described as in Figure 3(a). RIN2 (C337A) is a mutant of a crucial Cys within the RING finger (see Figure 1c). Twenty micrograms of RIN2 proteins were used for each ubiquitin assay, and ubiquitinated proteins were detected by protein blot with anti-His antibody.
(c) RIN3 has ubiquitin ligase activity. Note that only 2 μg of RIN2 and RIN3 proteins were used in this assay, revealing that RIN3 has higher E3 ligase activity than RIN2.

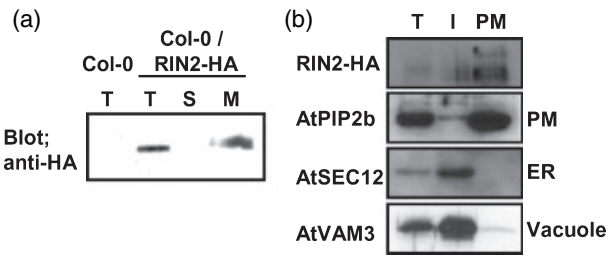

Figure 5. RIN2 is predominantly localized in the plasma membrane.
(a) Protein blot analysis of total (T), soluble (S), and microsomal membrane (M) fractions from untransformed (Col-0) and from transgenic Col-0 carrying *RIN2p-RIN2:HA*. The RIN2 protein was detected by anti-HA antibody.
(b) Protein blot analysis of membrane fractions obtained by aqueous two-phase partitioning. Total extract (T), intracellular membrane (I) and plasma membrane (PM) enriched vesicle fractions were subjected to protein blot with antibodies against plasma membrane intrinsic protein (AtPIP2b; Kammerloher *et al.*, 1994), ER-localized GTP-exchange protein (AtSEC12 (ER); (Bar-Peled and Raikhel, 1997), vacuolar syntaxin (AtVAM3; Sato *et al.*, 1997) (see Experimental procedures).

membranes. Ubiquitinated proteins were detected by protein blot analysis with anti-His antibody. As displayed in Figure 4(b), the RING domain of RIN2 indeed exhibited ubiquitin ligase activity. This activity was not inhibited by deletion of the D561L562Q563 sequence. Mutation of the highly conserved cysteine 337 (Figure 1c) to alanine resulted in loss of ubiquitin ligase activity (Figure 4b), as did removal of E1 or E2 from the ubiquitination reaction (data not shown). The CUE domain always gave a faint signal in repeated experiments, perhaps reflecting the reported ubiquitin-binding activity of this motif (Prag *et al.*, 2003; Shih *et al.*, 2003). RIN3 also had ubiquitin ligase activity, which was in fact much stronger than that of RIN2. We incubated RPM1-myc with the ubiquitination reaction mixtures containing RIN2 and/or RIN3. However, we failed to detect ubiquitination of RPM1 by either RIN2 or RIN3 (data not shown; see Discussion).

RIN2 localizes to the plant cell plasma membrane

To analyze RIN2 localization, we produced transgenic plants expressing C-terminal hemagglutinin (HA)-tagged RIN2 driven by a 1.9 kb native *RIN2* promoter. Total extract, and either crude soluble or microsomal membrane fractions derived from it, were prepared from wild-type Col-0 and transgenic RIN2-HA plants. Protein analysis with anti-HA antibody showed that RIN2 was localized in a membrane fraction (Figure 5a). A RIN2-HA doublet was consistently detected in the microsomal fraction, although we could see only one band in total extracts. This may be due to the different extraction conditions used for the total extract and the membrane fraction. RIN3 was similarly localized in the microsomal membrane fraction (data not shown).

RPM1 and RPS2 were previously localized as peripheral plasma membrane proteins (Axtell and Staskawicz, 2003; Boyes *et al.*, 1998). To analyze the subcellular localization of RIN2, microsomal membrane fractions were subjected to two-phase partitioning to enrich for plasma membrane vesicles (Daniels *et al.*, 1994). Protein extracts from total microsomes, intracellular membrane vesicles and plasma membrane vesicles were subjected to protein blot analysis with antibodies for the HA epitope and various subcellular makers. As shown in Figure 5(b), AtPIP2 was enriched in the plasma membrane fraction and AtSEC12 and AtVAM3 were enriched in intracellular membrane fractions. RIN2-HA was enriched in the plasma membrane fraction, although it was detectable at low levels in intracellular membrane fractions. Thus, RIN2-HA is predominantly localized in the plasma membrane.

RIN2 modification occurs at the time of HR

RPM1 disappears at a time coincident with the onset of HR induced by avirulent *Pto* strains that trigger various NB-LRR proteins (Figure 6a; Boyes *et al.*, 1998). We addressed whether RIN2 was altered after infection using total protein extracts prepared from leaves inoculated with *Pto* DC3000 carrying either *avrRpm1* or *avrRpt2*.

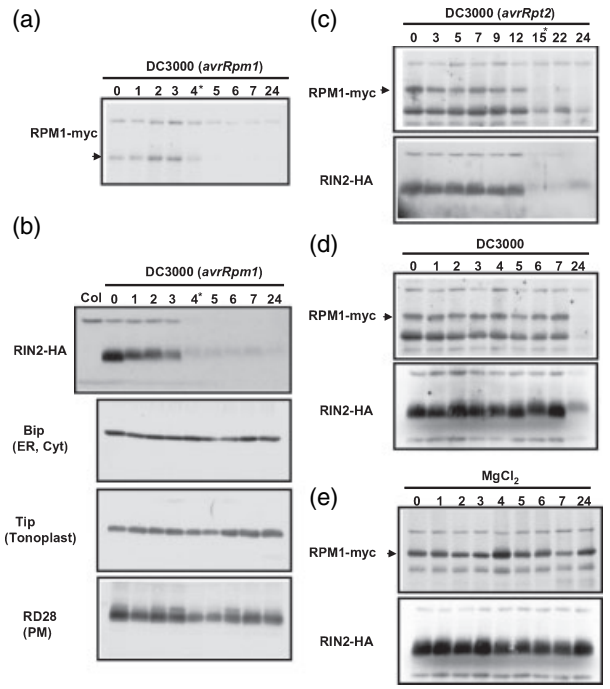

Figure 6. Modification of RIN2 occurs specifically after triggering of RPM1- and RPS2-mediated responses.
(a) RPM1 degradation occurs at the timing of HR. Transgenic plants expressing RPM1-myc were inoculated with *Pto* DC3000(*avrRpm1*) at 5×10^7 cfu ml^{-1} (for all panels). RPM1-myc was detected by protein blot with anti-myc antibody. An asterisk indicates the earliest time point of visible RPM1-dependent HR.
(b) Bi-phasic modification of RIN2 following inoculation with *Pto* DC3000(*avrRpm1*). An asterisk indicates the earliest time point of visible RPM1-dependent HR. RIN2-HA was detected by protein blot with anti-HA antibody. Subsequent panels are protein blots to detect marker proteins for the noted subcellular compartments.
(c) Disappearance of RPM1 (upper panel) and modification of RIN2 (lower panel) were induced by inoculation of DC3000(*avrRpt2*), respectively. An asterisk indicates the time point where visible RPS2-dependent HR was first noted.
(d) Disappearance of RPM1 (upper panel) and modification of RIN2 (lower panel) following inoculation with *Pto* (empty vector) control. An asterisk indicates a time point where *Pto* induced the necrotic phenotype.
(e) Control experiment using $MgCl_2$ buffer.

Inoculation with *Pto* DC3000(*avrRpm1*) caused RPM1-mediated HR at 4 hours post-inoculation (hpi). RIN2-HA exhibited reduced mobility in SDS-PAGE beginning at 1 hpi (Figure 6b). RIN2-HA levels declined at the onset of HR, but a further mobility reduction of the remaining RIN2-HA continued to 24 hpi. These data indicate that (i) the initial modification of RIN2-HA is rapid and precedes the disappearance of RPM1-myc, (ii) RPM1, and most of the RIN2, disappear coincident with HR and (iii) a small fraction of modified RIN2 remains post-HR. RPS2-dependent HR triggered by *Pto* DC3000(*avrRpt2*) was induced around 15 hpi in these experiments. RIN2-HA also disappeared at times coincident with RPS2-dependent HR, and the remaining RIN2-HA exhibited a slight mobility shift. The very early mobility shift observed during the RPM1-dependent response was not observed (Figure 5c). Eventual tissue collapse induced by virulent *Pto* DC3000 at 24 hpi also led to the disappearance of both RPM1 and RIN2 (Boyes *et al.*, 1998). Neither modification nor disappearance of RIN2 were observed in control extracts from leaves inoculated with $MgCl_2$, or with *Pto* DC3000(*hrcC*), a mutant incapable of type III effector delivery (data not shown). Identical RIN2 modification was also observed in extracts from transgenic plants expressing C-terminal c-myc-tagged RIN2 (data not shown), indicating that the modification and disappearance of RIN2 was not an artifact associated with either epitope tag. Extracts from transgenic plants expressing RIN3-HA also exhibited similar modifications after infection of *Pto* DC3000(*avrRpm1*) (data not shown).

We noted a slight upregulation of accumulation of *RIN2* mRNA following inoculation of *Pto* DC3000(*avrRpm1*) into either resistant or susceptible plants on RNA blots (not shown). Analysis of publicly available expression arrays (AtGenExpress) confirmed this—accumulation of *RIN2* mRNA is relatively upregulated by *Pto* DC3000(*avrRpm1*), but not by *Pto* DC3000, *Pto* DC3000(*hrcC*) or any other pathogen tested (not shown). Further, *RIN2* mRNA is relatively upregulated in seedlings and pollen (not shown). We did not observe differentially sized mRNAs that would correlate with the cDNA defined by clone JG in Figure 3(a), which lacks 32 codons (data not shown). Collectively, these observations suggest that the modification of RIN2 we observed is post-translational. Treatments with either calf alkaline intestinal phosphatase or endoglycosidase H did not alter the modification of RIN2, suggesting that the mobility changes are not due to phosphorylation or glycosylation (data not shown). Immunoprecipitated RIN2 proteins from leaves inoculated with *Pto* DC3000(*avrRpm1*) were subjected to protein blot analysis using anti-ubiquitin antibody. However, we failed to identify ubiquitinated RIN2 (data not shown).

RIN2 and RIN3 are positive regulators of RPM1- and RPS2-dependent HR

To assign a function to RIN2 and RIN3 in RPM1-dependent responses, we isolated two T-DNA insertion mutants in *RIN2* (*rin2-1*, SAIL 392 C08; *rin2-2*, SALK_141408) and one in *RIN3* (*rin3-1*, SALK_064875) (Figure 7a). The T-DNAs of *rin2-1* and *rin3-1* were located in the eighth intron of *RIN2* and the tenth intron of *RIN3*, respectively, both of which resulted in loss of functional RING-finger domains. Expression of *RIN2* and *RIN3* was undetectable in *rin2-1* and *rin3-1*, respectively, suggesting that each is a null allele. We produced a *rin2-1 rin3-1* double mutant. Both single mutants and the double mutant were morphologically wild type (data not shown), suggesting that

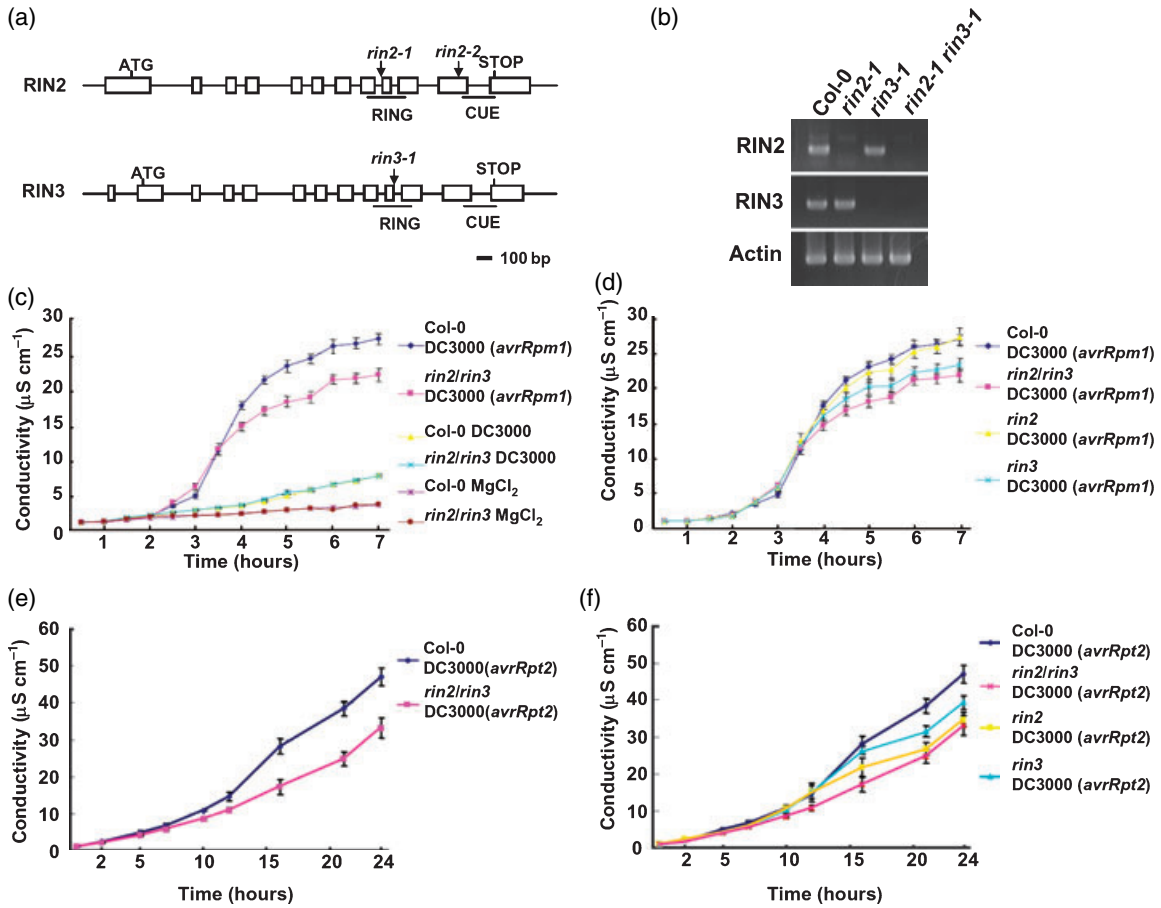

Figure 7. RIN2 and RIN3 contribute to RPM1- and RPS2-dependent HR.
(a) Schematic map of T-DNA insertion mutations in RIN2 and RIN3. Blocks indicate exons; key domains are underlined and labeled under each gene. Arrows indicate the position of T-DNA insertions in *rin2-1*(SAIL 392), *rin2-2* (SALK_141408), and *rin3-1* (SALK_064875).
(b) RT-PCR analysis of the *RIN2* and *RIN3* mRNAs in the T-DNA insertion mutants. Total RNAs extracted from wild-type Col-0, *rin2-1*, *rin3-1* and *rin2-1 rin3-1* double mutants (top) were used as templates. Actin was used as a control.
(c) Conductivity (μS cm^{-1}) of a solution containing four leaf disks from either Col-0 or the *rin2 rin3* mutant inoculated with either *Pto* DC3000(*avrRpm1*), *Pto* DC3000(vector) (both at 10^7 cfu ml^{-1}), or 10 mM $MgCl_2$.
(d) Conductivity (μS cm^{-1}) of a solution containing four leaf disks from either Col-0 or the *rin2* and *rin3* single mutants after inoculation of *Pto* DC3000(*avrRpm1*) at 10^7 cfu ml^{-1}.
(e) Conductivity (μS cm^{-1}) of a solution containing four leaf disks from either Col-0 or the *rin2 rin3* mutant inoculated with either *Pto* DC3000(*avrRpt2*) (at 10^7 cfu ml^{-1}).
(f) Conductivity (μS cm^{-1}) of a solution containing four leaf discs from either Col-0, the *rin2 rin3* mutant or the *rin2* and *rin3* single mutants after inoculation of *Pto* DC3000(*avrRpt2*) at 10^7 cfu ml^{-1}. Each value (c–f) represents the mean and SD of five replicates per experiment. Experiments were repeated.

RIN2 and RIN3 are dispensable for normal plant development under our growth conditions.

We quantified RPM1-dependent HR by monitoring electrolyte leakage(Dellagi *et al.*, 1998). Wild-type Col-0 leaves inoculated with *Pto* DC3000(*avrRpm1*) displayed dramatically increased ion leakage 3–6 hpi, which was not seen in Col-0 inoculated with *Pto* DC3000. The *rin2-1 rin3-1* double mutant displayed significantly less ion leakage than Col-0 in repeated experiments (Figure 7c). The *rin3-1* single mutant had more effect on RPM1-dependent ion leakage than the *rin2-1* single mutant (Figure 7d), suggesting that RIN2 and RIN3 act additively as positive regulators of RPM1-dependent ion leakage. Similarly, RIN2 and RIN3 act additively to generate maximal RPS2-dependent ion leakage (Figure 7e), but the roles of each are apparently reversed: *rin2-1* has a larger effect on RPS2-dependent ion leakage than *rin3-1* (Figure 7f). However, measurement of *in planta* growth of *Pto* DC3000(*avrRpm1*) and DC3000 indicated no significant differences between Col-0 and *rin2-1 rin3-1* (data not shown). To address whether RIN2 and RIN3 are required for the disappearance of RPM1 during HR, we generated *rin2-1 rin3-1* plants with the *RPM1-myc* transgene (Boyes *et al.*, 1998). However, the timing of the disappearance of RPM1 induced by Pto DC3000(*avrRpm1*) was essentially the same in Col-0 and *rin2-1 rin3-1* (data not shown), suggesting that RIN2 and RIN3 are not required for degradation of RPM1.

Discussion

RIN2 and RIN3 encode new RPM1 partners that are conserved in higher eukaryotes

To date, five proteins, RIN1, RIN4, RAR1, HSP90 and RIN13, have been identified as partners of RPM1 using various assays (Al-Daoude *et al.*, 2005; Belkhadir *et al.*, 2004; Schulze-Lefert, 2004). Here, we identified RIN2 and RIN3, membrane-bound RING-E3 ligases, as new RPM1 partners. A *rin2 rin3* double mutant displayed weakened RPM1- and RPS2-dependent HR, as measured by diminution of electrolyte leakage. Yet this phenotype was not correlated with a loss of RPM1- or RPS2-dependent restriction of bacterial growth, at least at the level of resolution afforded by available assays. We infer that RIN2 and RIN3 act additively as positive regulators of RPM1- and RPS2-dependent HR.

RIN2 and RIN3 encode ubiquitin E3 ligases

In Arabidopsis, there are 469 deduced RING domain-containing proteins (Stone *et al.*, 2005). The RING-finger domain contains an octet of cysteines and histidines that coordinate zinc (Jackson *et al.*, 2000). The RING-finger domains are classified based upon the spacing of the metal-binding amino acids or substitutions at one or more of the metal-bound positions (Stone *et al.*, 2005). Based upon this classification, the RING-finger domains of RIN2 and RIN3 are assigned to the type RING-H2 that contains six cysteines and two histidines (Stone *et al.*, 2005).

The RING-finger domain of RIN2 itself is sufficient for *in vitro* ubiquitin ligase activity that is abolished by a mutation at one of the conserved cysteines, indicating that RIN2 encodes a typical ubiquitin E3 ligase. In addition, the *in vitro* ligase activity of RIN3 is much stronger than that of RIN2, although both RING-finger domains are closely related. We anticipate that this difference is due to different biochemical properties of the C-terminal cytoplasmic domains of RIN2 and RIN3.

RIN2 and RIN3 each possess a CUE domain at their C-terminus. The CUE domain was initially identified as a domain similar to yeast Cue1 protein and was proposed to recruit E2 ubiquitin-conjugating enzyme (Ponting and Birney, 2000). Recent observations indicate that the CUE domain is a monoubiquitin-binding motif that promotes intramolecular monoubiquitination. Monoubiquitination is a key cell regulatory event, involved in endocytosis and regulation of protein activity and localization (Hicke, 2001; Prag *et al.*, 2003). In our *in vitro* ubiquitination experiments of RIN2, the CUE domain always gave a weak signal, suggesting that it has monoubiqutin-binding activity. This interpretation is supported by the fact that ubiquitin was identified as a RIN2 interactor in further two-hybrid screening using the CUE domain as a bait (data not shown).

RIN2 and RIN3 are closely related to the mammalian autocrine motility factor (AMF) receptor. AMF is a cytokine secreted by some tumor cells that stimulate cell migration *in vitro* and, perhaps, metastasis *in vivo* (Watanabe *et al.*, 1996). AMFR functions as a cell surface receptor for AMF. Because the crystal structure of AMF indicates dimerization, an AMF homodimer may interact with an AMFR homodimer to form an AMF/AMFR complex (Tanaka *et al.*, 2002). The interaction between RIN2 and RIN3 via their C-terminal regions, including the CUE domain, supports the idea that RIN2 and RIN3 form homo- or heterodimers.

AMFR is localized to PM caveolae and membranes of the smooth ER at steady state (Benlimame *et al.*, 1998). Endocytosis of AMFR delivers it to the ER (Le *et al.*, 2002). RIN2 is predominantly localized in the plasma membrane consistent with RPM1 localization (Boyes *et al.*, 1998). However, we also found low levels of RIN2 in our intracellular membrane pool, suggesting that RIN2 also might be localized in the ER as found in AMFR. Recently, AMFR has been reported to be involved in ER-associated degradation (ERAD) for protein quality control (Fang *et al.*, 2001). The barley MLO protein is localized to the plasma membrane and plays a critical role in resistance responses to powdery mildew (Devoto *et al.*, 1999). The MLO protein is regulated by ERAD-mediated protein quality control (Muller *et al.*, 2005). RIN2 and RIN3 were tested for, but do not participate in, ERAD of the MLO protein (Muller *et al.*, 2005).

RIN2 and RIN3 interact with RPM1

RIN2 and RIN3 interact with the N-terminal region of RPM1, including the CC motif. However, the N-terminal fragment (pEG10) that is deleted for the CC motif binds RIN2 or RIN3 very weakly. Thus, the CC region of RPM1 contributes significantly to, but is not sufficient for, full interaction with RIN2. Similarly, RIN2 and RIN3 bind weakly to the CC-NBS-LRR protein RPS2, but not to similar baits derived from the TIR-NBS-LRR protein RPP5. In addition, RIN2 and RIN3 both interact *in vitro* with full-length RPM1 produced by *in vitro* translation, but not in yeast. This might be explained by the fact that full-length RPM1 protein is made in relatively low abundance in yeast (Holt *et al.*, 2002), or by a requirement for conformational changes in RPM1 that might accompany its activation (Belkhadir *et al.*, 2004; Moffett *et al.*, 2002). Examination of *in vivo* interactions between RIN2 and RPM1 by co-immunoprecipitation using plants expressing both RPM1-myc and RIN2-HA were unsuccessful, probably because extraction buffers suitable for solubilization of RIN2 (a multipass integral membrane protein) disrupted RPM1 localized at the periphery of the plasma membrane. Future

experiments using cell biological methods might allow co-localization of RIN2 and RPM1 *in vivo*.

The C-terminal region of RIN2, including the CUE domain, interacts with RPM1. Deletion of the DLQ sequence conserved in the CUE domain results in reduced binding of RPM1. The CUE domain alone does not interact with RPM1, indicating that the CUE domain is required, but not sufficient for, full interaction. This result also suggests that the region between the RING and CUE domains is necessary for interaction with RPM1. Thus, the C-terminal regions of RIN2 and RIN3 are required for both homo- and heterodimerization and for interaction with RPM1. This suggests that RPM1 (or RPS2) may regulate RIN2/RIN3 dimerization.

RIN2 and RIN3 are not required for activation-dependent disappearance of RPM1

The RPM1 protein disappears at the time of HR induced by inoculation with *Pto* DC3000(*avrRpm1*), as well as by other NB-LRR-mediated recognition events (Boyes *et al.*, 1998). The disappearance of RPM1 seems to be mediated by ubiquitination because treatment with a proteasome inhibitor inhibits it (J. Nam, D. Mackey and J. Dangl, unpublished results). Therefore, we considered the possibility that RIN2 and RIN3 are involved in degradation of RPM1. However, we could not detect RIN2- and RIN3-dependent ubiquitination of RPM1 *in vitro* or *in vivo*. Furthermore, the kinetics of the disappearance of RPM1 is not altered in *rin2 rin3* (data not shown). Thus, it is unlikely that disappearance of RPM1 is regulated by a RIN2/RIN3-mediated pathway.

RIN2 and RIN3 are positive regulators for RPM1-mediated HR

Inoculation of *Pto* DC3000(*avrRpm1*) induces various apparent modifications of RIN2 in SDS-PAGE: a rapid mobility shift at 1 hpi, a second and gradual mobility coincident with HR and disappearance also coincident with HR. The very early modification of RIN2 mobility was not detected following inoculation with *Pto* DC3000(*avrRpt2*), and neither modification was seen using *Pto* DC3000, *Pto* DC3000(*hrcC*) or $MgCl_2$. Thus, these molecular events are unlikely to be induced by the other type III effectors delivered into the plant cell from *Pto* DC3000 or by a wound response. The rapid timing of the RPM1-specific RIN2 mobility shift at 1 hpi is essentially coincident with type III effector delivery (Grant *et al.*, 2000), supporting our contention that this first modification of RIN2 may be a very early signature of AvrRpm1-RPM1 signaling. The second modification, and disappearance of RIN2, occur at the same time as disappearance of RPM1. We proposed (Boyes *et al.*, 1998) that disappearance of RPM1 is a desensitization mechanism by which a receptor is eliminated to prevent excessive host responses after activation (Chuang and Ulevitch, 2004). Data presented here are not inconsistent with that idea.

RIN2 and RIN3 function additively as positive regulators of RPM1- and RPS2-mediated HR, but they have no obvious role in regulating pathogen growth restriction. This finding could be interpreted as a separation of HR regulation from the mechanism(s) of disease resistance. Alternatively, this finding could merely suggest that a partial loss of HR is not sufficient to alter the effectiveness of disease resistance. Further definition of the regulators of HR and disease resistance will allow us to differentiate whether HR is the cause or simply a consequence of the mechanisms that ultimately stop proliferation of pathogens.

Experimental procedures

Plant cultivation, Agrobacterium *transformation and herbicide selection*

Plants were cultivated in growth chambers under a 9 h light/15 h dark regime at 22°C and 60% constant relative humidity. The *RIN2p-RIN2:HA* gene, including 1912 bp of promoter sequence upstream of the translation start codon, was subcloned into binary vector pBAR1 (Holt *et al.*, 2002) and transformed into *Agrobacterium* GV3101 by electroporation. *Agrobacterium* containing the construct were used for transformation into plants as described (Bechtold and Pelletier, 1998). Basta (glufosinate-ammonium) selection was performed according to Holt *et al.* (2002).

Yeast two-hybrid constructs and methods

The yeast two-hybrid library was produced as described (Holt *et al.*, 2002). All RPM1, RIN2 and RIN3 yeast two-hybrid baits were cloned into the pEG202 vector. RIN2 was originally identified by two-hybrid screening using pEG10 as bait (Gyuris *et al.*, 1993). The prey vectors containing the fragments of RIN2 and RIN3 were made using the pJG4-5 vector (Gyuris *et al.*, 1993). For two-hybrid assays, bait and prey vectors were co-transformed into yeast EGY48-competent cells prepared by the Frozen-EZ yeast transformation II Kit (Zymo Research, CA, USA). All screening, interaction assays, and plasmid purification from yeast were done according to (Ausubel *et al.*, 1987).

In vitro *interaction experiments*

Full-length (amino acids 1–926) and N-terminal region (amino acids 1–177) RPM1 fragments with C-terminal c-myc epitope-tags were cloned into pET14b (Novagen, Madison, WI, USA). RPM1-Myc proteins were produced by the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA). The cDNA fragments of *RIN2* and *RIN3* were cloned into pGEX4T-1 (Amersham Pharmacia, Piscataway, NJ, USA) to produce GST-fused proteins. GST, GST-RIN2 and GST-RIN3 proteins coupled to glutathione Sepharose 4B beads were incubated with the RPM1-Myc proteins in TEDM buffer [80 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 20 mM $MgCl_2$, 4 mM DTT and 1× protease inhibitor cocktail (Sigma, St Louis, MO, USA)] at 4°C for 2 h. After incubation, the beads were washed five times with a

wash buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100, 0.5 mM DTT and 1× protease inhibitor cocktail]. After the final wash, bound proteins were eluted in 3× SDS loading buffer at the same volume as the beads. SDS-PAGE, immunoblotting, and Coomassie staining were performed using standard protocols.

E3 ubiquitin ligase assay

The AtUBC1 protein was purified as previously described (Sullivan and Vierstra, 1991). Either 20 μg or 2 μg of GST-fused RIN2 and RIN3 proteins was incubated with 30 ng of yeast E1 enzyme (Boston Biochem, Cambridge, MA, USA), 2 μg His-tagged ubiquitin (Calbiochem, San Diego, CA, USA), 1 μg of AtUBC1 in a reaction buffer [50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.5 mM DTT and 4 mM ATP] at 30°C for 90 min. The reaction was stopped by heating at 95°C for 2 min. After SDS-PAGE and blotting, the ubiquitinated proteins were detected by anti-His antibody.

Two-phase partitioning

Tissue was homogenized on ice in an extraction buffer [100 mM Tris-HCl (pH 7.5), 12% sucrose, 1 mM EDTA, 1× protease inhibitor cocktail (Sigma)] with a polytron. Total proteins were prepared by filtration through one layer of miracloth, and pelleting of the insoluble debris by centrifugation at 2000 ***g*** for 10 min at 4°C. The soluble and microsomal membrane proteins were separated by centrifugation at 100 000 ***g*** for 1 h. Aqueous two-phase partitioning was done with a polymer concentration of 6.6% (wt/vol) as described previously (Boyes *et al.*, 1998). The anti-AtPIP2b and anti-AtSEC12 antibodies were purchased from Rose Biotechnology (Rose Biotechnology, Hayward, CA, USA). The AtVAM3 antibody was provided by Dr Masa H. Sato (Sato *et al.*, 1997).

Modification of RIN2

Two 8.5 mm diameter leaf disks were homogenized in an extraction buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS], and incubated on ice for 1 h. Total proteins were prepared by pelleting of the insoluble debris by centrifugation at 2000 ***g*** for 10 min at 4°C, and subjected to immunodetection using 10% SDS-PAGE. The anti-RD28 and anti-Tip antibodies were provided by Dr Maarten Chrispeels, and the anti-Bip antibody was provided by Dr Rebecca Boston.

Pathogen culture and inoculation

Pto DC3000 carrying either pVSP61 or derivatives of this plasmid containing *avr* genes have been described (Bisgrove *et al.*, 1994; Grant *et al.*, 1995). For analyzing the modification of RIN2 and RIN3 and degradation of RPM1, *Pto* was resuspended at 5×10^7 colony-forming units (cfu) ml^{-1} in 10 mM $MgCl_2$, and infiltrated into leaves of 4- to 5-week-old plants. For measurements of electrolyte leakage, *Pto* at 10^7 cfu ml^{-1} was infiltrated into leaves of 4- to 5-week-old plants. Four 8.5 mm diameter leaf disks were collected from the infiltrated area and washed with water for 50 min, and then placed in a tube with 15 ml of water. Conductivity was measured from five replicates for each treatment using a Yokogawa (Yokogawa, Musashino, Tokyo, Japan) conductivity meter, model SC82. For assays of bacterial growth, *Pto* at 10^5 cfu ml^{-1} was infiltrated, and numbers of bacteria were measured as described previously (Mackey *et al.*, 2003).

DNA/RNA manipulation

For analysis of RNA, tissue was ground by mortar and pestle in liquid nitrogen. Total RNA was extracted with an RNeasy plant mini kit (Qiagen, Valencia, CA, USA). RT-PCR was done using primers specific to RIN2 (sense, 5′-GCTCTCCATGCAGCCCTCCC-3′; antisense, 5′-CTGGCACCTCAGCAGGAATAA-3′), RIN3 (sense, 5′-GCAACTTCAGAAGAGCTACGGGA-3′; antisense, 5′-AGCCTGCAGAGGAACCCACA-3′) and actin (sense, 5′-GAGAGATTCAGGTGCCCAG-3′; antisense, 5′-AGAGCGAGAGCGGGTTTTCA-3′).

Double-mutant construction

The T-DNA insertion lines for *rin2-2* (SALK_141408), and *rin3-1* (SALK_064875) were identified by searches of the Salk T-DNA insertion mutant collection (http://signal.salk.edu/cgi-bin/tdnaexpress). The seeds were obtained from the ABRC (Columbus, OH, USA). *rin2-1* (SAIL 392 C08) was identified by searching SAIL (the Syngenta Arabidopsis Insertion Library). Genomic DNA was isolated from the mutant lines, and T-DNA insertions were confirmed by PCR reactions using T-DNA left border primers and gene-specific primers, followed by sequencing of the PCR products. The *rin2 rin3* double mutants were produced by a cross of both lines, and identified by PCR.

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