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RESEARCH ARTICLE

Proteasome-Dependent Degradation of RPM1 Desensitizes the RPM1-Mediated Hypersensitive Response

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Abstract

The intracellular plant resistance (R) proteins, nucleotide-binding and leucine-rich repeat (NLR) proteins, mediate resistance to pathogens by enabling recognition and rapid response. The response consists of the induction of a defensive suite that typically culminates in the hypersensitive response (HR), death of the plant cells at and around an infection site. The *Arabidopsis* intracellular innate immune receptor protein RESISTANCE TO PSEUDOMONAS MACULICOLA1 (RPM1) is a coiled-coil (CC) type of NLR protein that specifies resistance to strains of the bacterial pathogen *Pseudomonas syringae* expressing the type III effector proteins AvrRpm1 and AvrB. We previously demonstrated that RPM1-myc (an epitope-tagged version of RPM1) disappears coincident with the onset of HR induced by AvrRpm1. Infection with *P. syringae* expressing two other type III effector proteins, AvrRpt2 and AvrRps4, also initiated RPM1-myc disappearance at time points coincident with the HR they initiate through the NLR proteins RESISTANCE TO P. SYRINGAE2 (RPS2) and RESISTANCE TO P. SYRINGAE 4 (RPS4), respectively. Here, we use mutants impaired in *NLR* gene dependent signaling to demonstrate that disappearance of RPM1-myc requires normal *NLR* gene dependent signaling steps, but does not require HR. Inhibitors of the 26S proteasome block the disappearance of RPM1-myc and enhance RPM1-myc-dependent cell death. Our data are consistent with a model in which RPM1 is degraded by the 26S proteasome to limit the extent of RPM1-dependent signaling and/or cell death. Furthermore, AvrRpt2 induces disappearance of RPM1-myc in *rps2* mutant plants without HR, suggesting that RPM1 is part of the host target of the virulence activity of AvrRpt2.

Keywords Nucleotide-binding and leucine-rich repeat (NLR) proteins · R protein desensitization · *Pseudomonas syringae* · Type III effectors · 26S proteasome inhibitors

Introduction

Plants resist many pathogens based on recognition of a specific avirulence (*avr*) gene. This so-called gene-for-gene recognition of pathogens does not function if either the plant or the pathogen lacks the corresponding *R* or *avr* gene. Upon recognizing a pathogen, the plant induces a suite of defensive measures that include cell wall deposition, production of anti-microbial compounds, transcription of defense genes, and ultimately localized cell death termed the HR. Molecular mechanism remains unclear on how this complex response actually halts pathogen growth (reviewed in Dangl and Jones 2001; Jones and Dangl 2006). Plants have evolved sophisticated regulation systems to control the NLR steady-state levels for efficient activation of the immune system as well as for inhibition of autoimmune response.

The avirulence proteins of phytopathogenic enterobacteria like *P. syringae* are type III effector proteins delivered

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into the plant cell via the evolutionarily conserved type III pilus. On a resistant host, these effectors induce defenses and render the pathogen “avirulent”. On susceptible hosts, these effectors can enhance the virulence of pathogens (reviewed in Staskawicz et al. 2001). AvrRpm1 and AvrRpt2 can enhance the growth of *P. syringae* on *rpm1* or *rps2 Arabidopsis* plants, respectively (Ritter and Dangl 1995; Chen et al. 2000). AvrRpt2 can also inhibit RPM1 function in an RPS2-independent manner, and thus enhance the growth of bacteria even when they also express *AvrRpm1* (Ritter and Dangl 1996). Thus, the Avr proteins actually function to enhance the virulence of the pathogen. R proteins of the plant counter the virulence activity of these effectors by detecting their presence and inducing defense responses.

The largest class of R proteins function as intracellular immune receptors and is comprised of a central nucleotide binding (NB) and carboxy-terminal leucine-rich repeat (LRR) domains. The sequences of the LRRs from numerous R proteins have evolved under diversifying selection. The LRRs of various proteins have been shown to mediate protein–protein interactions (reviewed in Mchale et al. 2006). Together, these facts have led to models in which the LRRs of R proteins serve as the specificity determinants for recognition of Avr proteins. The LRRs of a given R protein could specify recognition of the cognate Avr protein itself or to molecular perturbations induced by the Avr protein. The amino-terminal end of these so-called NB-LRR proteins contains either a coiled-coil domain (CC-NB-LRR) or a domain with homology to the cytoplasmic domain of Toll and interleukin receptors (TIR-NB-LRR). *Arabidopsis* encodes for approximately 100 and 50 genes with the domain structure TIR-NB-LRR and CC-NB-LRR, respectively (Meyers et al. 2003).

RPM1 is a CC-NB-LRR type R protein of *Arabidopsis* (Grant et al. 1995). Though predicted to be cytoplasmic, RPM1-myc associates peripherally with the plasma membrane (Boyes et al. 1998). RIN4, which is required for RPM1 function, binds to RPM1-myc and is also membrane-associated (Mackey et al. 2002). Following delivery into the plant cell via the type III pilus, AvrRpm1 and AvrB are myristoylated by host proteins and thus localized to the plasma membrane (Nimchuk et al. 2000). Both AvrRpm1 and AvrB physically interact with RIN4 and induce phosphorylation of it via receptor-like cytoplasmic kinases, RIPKs (Liu et al. 2011). AvrRpm1 directly induces ADP-ribosylation of RIN4 proteins from both *Arabidopsis* and soybean within two highly conserved nitrate-induced (NOI) domains and promotes phosphorylation of RIN4 (Redditt et al. 2019). RPM1 perceives these events and activates defense responses, including the HR.

Upon activation of the HR by RPM1, RPM1-myc itself disappears. Interestingly, HR induced through activation of RPS2 (CC-NB-LRR) or RPS4 (TIR-NB-LRR) also induces disappearance of RPM1-myc. Disappearance of RPM1-myc does not appear to be an inadvertent consequence of the HR, since most proteins, including another associated with the plasma membrane, do not similarly disappear. Thus we inferred that the disappearance of RPM1-myc is a regulated process (Boyes et al. 1998). Here we show that the disappearance of RPM1-myc correlates with the appearance of the HR, and is controlled by genes that are required for R function. We demonstrate that inhibitors of the 26S proteasome also inhibit the disappearance of RPM1-myc. These inhibitors enhance the magnitude of RPM1-dependent cell-death. We suggest that RPM1 activity is controlled by proteasome-mediated degradation. Interestingly, in *rps2* plants, AvrRpt2 still induces disappearance of RPM1-myc in the absence of HR. We suggest that this AvrRpt2 activity is intimately associated with its virulence function and that RPM1 is part of the host target of the virulence activity of AvrRpt2.

Materials and Methods

DNA Manipulation and Generation of Transgenic Plants

The vector used to construct plants expressing RPM1-myc has been previously described (Boyes et al. 1998). In short, the plasmid allows *Agrobacterium tumefaciens* mediated delivery of an *RPM1* genomic clone with five full and one partial copy of the myc epitope at its C-terminus. A gene conferring hygromycin resistance is also contained on the transferred DNA. The various ecotypes and mutants were transformed by vacuum-infiltrated (Clough and Bent 1998) and plants with insertion at only one locus were propagated to homozygosity.

Pseudomonas Bacterial Strains and Infection

Isolates of *Pseudomonas. syringae* pv. *tomato* (*Pst*) DC3000 carrying pVSP61 or derivatives of pVSP61 containing *avrRpm1*, *avrRpt2*, and *avrRps4* have been described (Grant et al. 1995). Bacteria were resuspended at 5×10^7 cfu/ml in 10 mM $MgCl_2$. Leaves of five-week-old plants were infiltrated and three leaves were pooled per time-point.

Treatments of Proteasome Inhibitors and β-estradiol

MG-132 and Lactacystin (LAC; Sigma-Aldrich) were suspended in 100% DMSO at 10 and 2 mM, respectively. These stocks, or just DMSO, were added at 1 part in 100 into solutions for experiments. β-estradiol (Sigma-Aldrich) was resuspended at 10 mM in 100% EtOH. This stock, or just EtOH, was added at one part in 1000 into solutions for experiments. Solutions were infiltrated into leaves of five-week-old plants and three leaves were pooled per time point. Alternatively, solutions were spotted onto the surface of leaves of five-week-old plants.

Ion Leakage Measurement

Ion leakage measurements were made as previously described (Mackeyet al. 2002). Briefly, leaf discs were collected and washed in water. Discs were then transferred into test solutions and conductance of these solutions was measured over time.

Protein Blot Analysis

Total protein extracts were prepared by grinding approximately three-square centimeters of leaf tissue in 100 µl of grinding buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM DTT, and plant protease inhibitor cocktail (Sigma-Aldrich) and insoluble debris was pelleted by centrifugation at 20,000×*g* at 4 ℃ for 10 min. Concentration of protein in the supernatant was measured with the Bio-Rad protein assay (Bio-Rad). Samples containing 30 µg of protein were separated on 7.5% SDS-PAGE gels (mini protean, Bio-Rad) and transferred to nitrocellulose. Standard western blot procedure with the mouse monoclonal antibody 9E10 was used to detect RPM1-myc.

Results

RPM1-myc Disappearance in Various Defense Signaling Mutant Backgrounds

A genomic *RPM1-myc* clone, under the control of the *RPM1* promoter, was introduced into various mutants and ecotypes of *Arabidopsis*. In these transgenic lines, we tested the disappearance of RPM1-myc triggered by RPM1 (induced by AvrRpm1), RPS2 (AvrRpt2), and RPS4 (AvrRps4). The *Non-Race-Specific Disease Resistance 1* (*npr1*) and *Enhanced Disease Susceptibility 1* (*eds1*) mutations are in the Ws-0 accession of *Arabidopsis*. The *npr1* mutation is unable to mount systemic defense responses and is unable to fully transduce signaling from some CC-NB-LRR proteins including RPS2 (Chern et al. 2008). The *eds1* mutation eliminates signaling via TIR-NB-LRR proteins such as RPS4 but does not affect the function of RPM1 or RPS2 (Aarts et al. 1998; Bhttacharjee et al 2011; Heidrich et al. 2011). In Ws-0, *Pst* DC3000 carrying *avrRpm1*, *avrRpt2*, or *avrRps4* causes disappearance of RPM1-myc by 7, 17, and 20 h, respectively (Fig. 1a). These times coincide closely with the onset of macroscopically visible HR idiosyncratic for the action of each R protein assayed (indicated in Fig. 1 by asterisks). When HR occurred, the RPM1-myc protein significantly disappeared compared to intrinsic proteins that non-specifically reacting with the myc antibody (sFig. 1), indicating that the disappearance of RPM1-myc is not due to total protein degradation following HR-mediated cell death. *Pst* DC3000 carrying the empty vector (lacking any *avr* gene) induces neither an HR nor disappearance of RPM1-myc. These results in Ws-0 are consistent with those already described in the ecotype Col-0 (Boyes et al. 1998). In *npr1*, the HR induced by *Pst* DC3000 carrying the *avr* genes is similar to that in Ws-0. The timing of the disappearance of RPM1-myc is unchanged (Fig. 1b), indicating that signals for RPM1-myc disappearance are generated upstream of, or independently from, *NPR1*. In *eds1*, AvrRps4 does not induce an HR or disappearance of RPM1-myc while the effects of AvrRpm1 and AvrRpt2 are unchanged (Fig. 1c). We infer that the induced disappearance of RPM1-myc requires signals downstream of *EDS1* function in RPS4-mediated responses. The *Phytoalexin Deficient4* (*pad4*) mutation (in the Col-0 ecotype) affects the function of the same *R* gene set as *eds1* but has a different function at the infection site since *Pst* DC3000 (*avrRps4*) can induce an HR in *pad4* (Rustérucci et al. 2001). In addition, the disappearance of RPM1-myc is still induced by AvrRps4 (as well as AvrRpm1 and AvrRpt2) in *pad4* (Fig. 1d). Thus, in these mutants, there exists a correlation between the induction of cell death and disappearance of RPM1-myc.

Function of the 26S Proteasome is Required for Disappearance of RPM1-myc

We tried to test inhibitors of the 26S proteasome on the disappearance of RPM1-myc as assayed in Fig. 1. However, the solvents necessary to keep the inhibitors in solution killed the bacteria. Instead, we used transgenic plants that conditionally express *AvrRpm1* following treatment with β-estradiol (β-ED) (Tornero et al. 2002a). By crossing these plants (Col-0 background) to a line in Col-0 that expresses *RPM1-myc*, we established a new line that is homozygous for all these traits. When leaves of these plants were infiltrated with β-ED, RPM1-myc disappeared by 21 h (Fig. 2). When either of two inhibitors of the 26S proteasome (MG-132 or LAC) was included with the β-ED, RPM1-myc no longer disappeared (Fig. 2). While we recognize that these inhibitors can have pleiotropic effects on cell physiology, our results argue for a proteasome-dependent step in the disappearance of RPM1-myc.

Cell-Death Induced by Activation of RPM1-myc is Enhanced When Its Disappearance is Blocked

We determined what effect inhibition of the 26S proteasome has on the intensity of cell-death induced by RPM1-myc. The same plants used in Fig. 2 were treated with

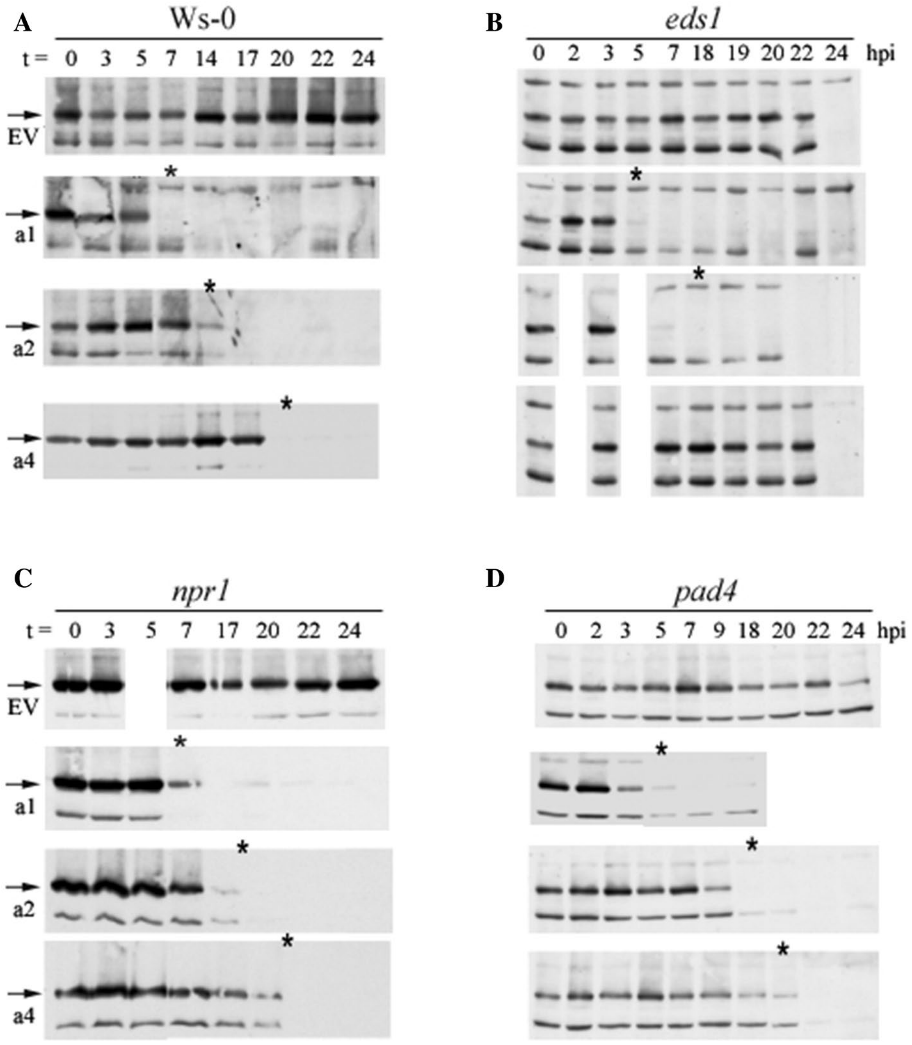

Fig. 1 Disappearance of RPM1-myc correlates with the onset of the HR. Transgenic plants expressing RPM1-myc were inoculated with 5×10^7 cfu/ml of *Pst* DC3000 carrying empty vector (EV), *avrRpm1* (a1), *avrRpt2* (a2), or *avrRps4* (a4). The time of appearance of macroscopic HR is indicated by an asterisk (*). Samples were collected, total protein prepared, and RPM1-myc (position indicated by *arrow*) was detected by anti-myc western blot. (**a**) In the ecotype Ws-0, RPM1-myc is undetectable by 7, 17, or 20 h following treatment with *Pst* DC3000 expressing *avrRpm1*, *avrRpt2*, or *avrRps4*, respectively. Treatment with *Pst* DC3000 carrying the empty vector induces neither an HR nor a diminution in levels of RPM1-myc. (**b**) In *eds1*, no HR is observed in response to *avrRps4*. Here, tissue collapse is indistinguishable between leaves treated with *Pst* DC3000 expressing *avrRps4* or carrying the empty vector (EV). Similarly, the pattern of the disappearance of RPM1-myc following these treatments is identical. In *npr1* (**c**) and *pad4* (**d**) the timing of both the HR and the disappearance of RPM1-myc induced by treatments with *Pst* DC3000 are unaffected. To make all data at the same time points, we separated western data performed at different time points. Note the HR time points at which the protein levels of RPM1-myc are significantly reduced

droplets of β-ED to induce AvrRpm1-RPM1 dependent cell death (Fig. 3a, second droplet site from top). Similar treatment, including inhibitors of the proteasome (MG-132 and LAC), induced no cell death. When these inhibitors of the proteasome were applied in combination with β-ED, the extent of cell death was clearly enhanced relative to that induced by β-ED alone. We used ion leakage to quantify this difference (Fig. 3b). When leaf discs from these plants were floated on a solution containing β-ED, cell death could be monitored indirectly by measuring the conductance of the solution (see "Materials and Methods" section). When the solution contained proteasome inhibitor (MG-132) and β-ED, the amount of ion leakage was greater than that induced than by β-ED alone (Fig. 3b). We conclude that the persistence of RPM1-myc leads to prolonged and intensified RPM1-mediated signaling.

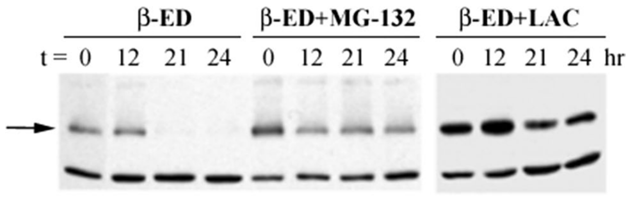

Fig. 2 Disappearance of RPM1-myc is dependent on the 26S proteasome. RPM1-myc disappears in transgenic plants conditionally induced to express *avrRpm1* by treatment with β-ED. When inhibitors of the proteasome (MG-132 and LAC) are introduced along with β-ED, the disappearance of RPM1-myc is no longer observed. Leaves were infiltrated with a solution containing 10 µM β-ED with DMSO carrier (β-ED) or with 100 µM MG-132 (β-ED+MG-132) or 20 µM LAC (β-ED+LAC). Samples were collected, total protein prepared, and RPM1-myc (position indicated by arrow) was detected by anti-myc western blot

A Type-III Effector of *P. syringae* Induces Disappearance of RPM1-myc in the Absence of Its Cognate R Protein

Pst DC3000 expressing *avrRpt2* induces an HR and disappearance of RPM1-myc in Col-0 and Ws-0 (Fig. 1). In *ndr1*, a mutant that impairs signaling through CC-NB-LRR R proteins like RPS2, and in *rps2*, each in the Col-0 background, no HR is induced in response to AvrRpt2 (Aarts et al 1998). Despite the lack of an HR, AvrRpt2 still induces the disappearance of RPM1-myc at the same time point as in wild type plants (Fig. 4). The HR and disappearance of RPM1-myc induced by *Pst* DC3000 expressing *avrRpm1* and *avrRps4* are not altered by these mutations. Thus, HR is not required for disappearance of RPM1-myc induced by AvrRpt2.

Discussion

Disappearance of RPM1-myc Correlates with the Normal Timing of HR Induced via R-Dependent Signaling Pathways

In *eds1*, *Pst* DC3000 expressing *AvrRps4* induced neither an HR nor disappearance of RPM1-myc. In both previous work (Boyes et al. 1998) and in *npr1* and *pad4*, the appearance of the HR correlates with the disappearance of RPM1-myc. Thus, signals leading to cell-death and disappearance of RPM1-myc overlap significantly. PAD4 and EDS1 each encode putative lipases that interact with one another (Feys et al. 2001). Mutations in these two genes affect signaling via the same set of *R* genes. Two notable exceptions are that *EDS1*, but not *PAD4*, is required for both the oxidative burst and HR (Rustérucci et al. 2001). Recent examination of EDS1 structure reveals that the c-terminal α-helical EP-domain (PFAM:PF18117) cavity that is distinct from that of PAD4 is critical is for RPS4-mediated immune responses (Bhandari et al. 2019). Similarly, the disappearance of RPM1-myc induced by AvrRps4 requires *EDS1* but not *PAD4*. Thus, the disappearance of RPM1-myc requires steps occurring subsequent to *EDS1* action. These might include the accumulation of reactive oxygen intermediates and other signals leading to HR. *NPR1* is not required for the HR induced by RPM1, RPS2, or RPS4, but is required for transduction of signals that lead to systemic acquired resistance of the plant. Like the signal(s) that leads to HR, the signal(s) that lead to the disappearance of RPM1-myc do

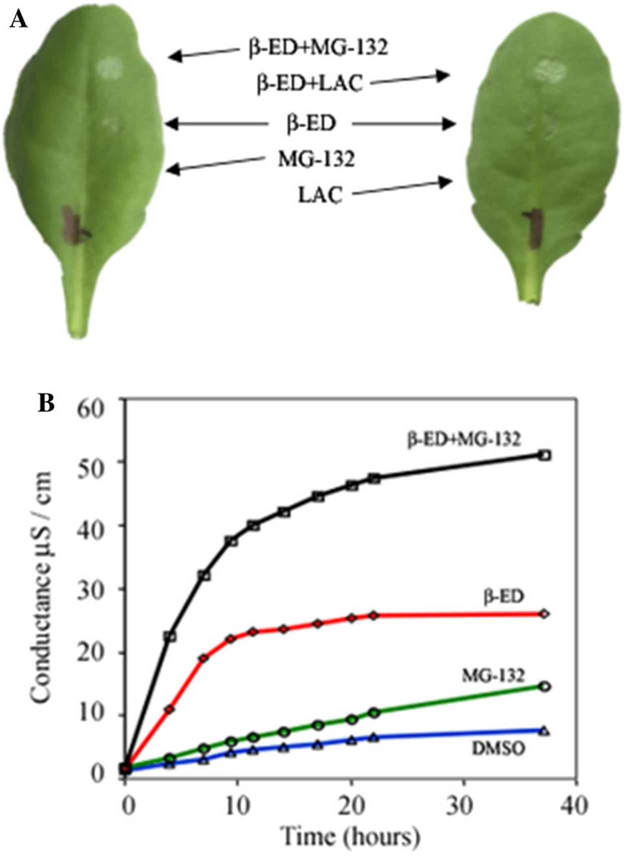

Fig. 3 Inhibition of the 26S proteasome enhances RPM1-dependent cell death. (**a**) Solutions containing the proteasome inhibitors and β-ED alone or in combination were placed in 5 µl droplets onto leaves of the plants that inducibly express *avrRpm1*. β-ED treatment activated macroscopically visible cell death. The proteasome inhibitors in combination with β-ED produced significantly more cell death than did β-ED alone. The proteasome inhibitors alone produced no macroscopically visible cell death. Pictures of leaves were made 24 h after application of the droplets. (**b**) Ion leakage was used to measure quantitatively these differences. Twelve 8 mm leaf discs per sample were punched from the inducible plants and washed in water. These discs were then transferred to the test solutions. Conductance was measured (µSiemens/cm) over time (Mackeyet al. 2002). The solutions used in Fig. 3 are the same as those described in the legend to Fig. 2

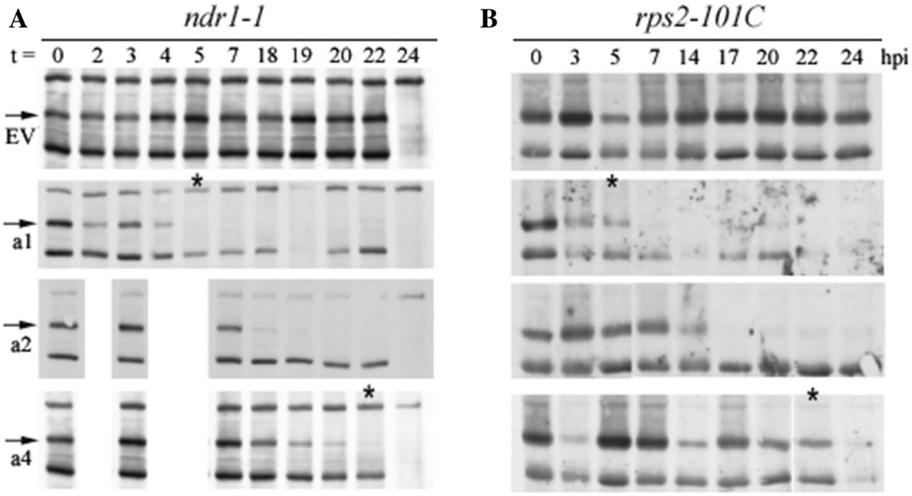

Fig. 4 AvrRpt2 induces disappearance of RPM1-myc in the absence of an HR. In *ndr1* (**a**) and *rps2* (**b**), *Pst* DC3000 expressing *avrRpt2* does not induce an HR but does induce disappearance of RPM1-myc. The HR and disappearance of RPM1-myc induced by *Pst* DC3000 expressing either *avrRpm1* or *avrRps4* were unaffected in these mutants. Experimental details are as described in the legend to Fig. 1. To make all data at the same time points we separated western data performed at different time points. Note the HR time points at which the protein levels of RPM1-myc are significantly reduced

not require *NPR1*. In these mutants, there exists a correlation between the accumulation of reactive oxygen, induction of HR, and the disappearance of RPM1-myc.

Inhibitors of the 26S Proteasome Block the Disappearance of RPM1-myc

In plants that conditionally express *AvrRpm1*, RPM1-myc disappears following induction of AvrRpm1 and disappearance of RPM1-myc is inhibited by MG-132 and LAC (inhibitors of the 26S proteasome) (Fig. 2). A simple interpretation of this result is that RPM1 is directly degraded by the proteasome. However, more complicated models, such as the existence of a proteasome degraded factor that affects the transcription, translation, or stability of RPM1, cannot be ruled out. Recent findings indicate that the regulation of homeostasis of R proteins by the ubiquitin–proteasome system is crucial for effective immunity (Cheng et al. 2011; Wang et al. 2016). RAR1 is required for RPM1 function; and in a *rar1* mutant, RPM1 protein does not accumulate. RAR1 can physically associate with SGT1, which in turn can bind to components of the SCF ubiquitin ligase complex (Azevedo et al. 2002). The trail from an ubiquitin ligase to RPM1 indicates that the 26S proteasome may be the ultimate arbiter of RPM1-myc disappearance.

Inhibitors of the Proteasome Exaggerate Responses Induced by RPM1

Conditional expression of *AvrRpm1* induces RPM1-dependent cell death. The presence of the proteasome inhibitors (MG-132 and LAC) along with the inducing agent (β-ED) enhanced the observed death and blocked RPM1-myc disappearance (Fig. 3). By blocking the disappearance of RPM1-myc, proteasome inhibitors prolong/enhance RPM1-dependent signaling. Thus, the normally observed disappearance of RPM1 desensitizes the plant to signals dependent on RPM1 function.

The relationship between RPM1 protein stability and function is complex. We infer that disappearance of RPM1 concurrent with the HR limits signal output from RPM1 since proteasome inhibitors that block the disappearance augment the signal. How does proteasome-dependent protein degradation regulate R protein function? Mutations in *SGT1b* inhibit the function of numerous R-proteins by reducing homeostasis of R proteins [notably not RPM1; (Austin et al. 2002; Holts et al. 2005; Azevedo et al. 2006)], indicating that SCF-mediated ubiquitination via SGT1/RAR1 is required for the function of these R proteins. Gene silencing of SGT1 eliminates signaling from a variety of R proteins, indicating that there is a universal requirement for SGT1 in R function (Peart et al. 2002). Furthermore, stabilization of R-proteins requires the molecular chaperon HSP90, which makes HSP90-SGT1-RAR1complex that interacts with R proteins (Hubert et al. 2003; Kadota et al. 2008). Thus, the current model postulates that HSP90-SGT1-RAR1chaperon complex stabilizes and maintains a recognition-competent state of R proteins (Kadota et al. 2010). The homeostasis of R proteins also can be tightly regulated by E3 ligases, which determine the specificity of the targets for ubiquitin-mediated protein degradation. The F-box protein CPR1 forming SCF^{CPR1} E3 complex and RING domain E3 ligase MUSE1 are directly involved in the turnover of the R protein SNC1 and its pairing partner R proteins SIKIC family proteins, respectively (Cheng et al. 2011; Dong et al. 2018). Thus, the plant could couple the production of the cell death signal with the control of the magnitude of that signal triggered by the activation of R proteins. Proteasome inhibitors would not block the activation event but would block the subsequent inactivating event, thus leading to increased signal output.

R protein function must be managed carefully since the output of this function is cell death. The fact that most R proteins induce an HR indicates that it is adaptive to sacrifice some tissue to effectively contain a potential pathogen. However, the extent of HR must be limited. R proteins can result in autoimmune response in the absence of invading pathogens (Cheng et al. 2011), and some mutations disrupt the ability of the plant to limit the spread of cell death once it has been initiated (Dietrich et al. 1994). Thus, the plant carefully regulates the control of cell death around the site of R function. By fine-tuning the balance of activation and de-activation of R protein-mediated signaling, the plant may fine-tune the balance between cell-death associated with resistance and preservation of viable tissue around the infection site. Cell death induced by RPM1 is relatively the strongest among the R proteins considered in this work. Thus, its de-sensitization by the plant might be similarly robust. Nevertheless, we cannot rule out the possibility that disappearance of un-activated RPM1-myc in the HR triggered other combinations of gene-for-gene interactions such as AvrRpt2/RPS2 and AvrRps4/RPS4 might result from a subsequential reaction of HR (Fig. 1). Because phospholipase D (PLD) activity and PLD-derived phosphatidic acid (PA) decreased RPM1 protein accumulation level (Yuan et al. 2019), it is speculated that activation of PLD by hydrogen peroxide (H_2O_2) induced during HR might interfere with the plasma-membrane location of RPM1. RPM1 released from the membrane is probably very unstable in the cytosol of plant cells. It is very interesting to examine two or more R proteins simultaneously to confirm the molecular fates of the activated and un-activated R proteins during HR.

R activation is associated with a continuum of responses, from no cell death, to single-cell death, to the death of a patch extending beyond the infected cells. One could imagine that R proteins associated with a weaker HR do not pass a threshold required to activate the desensitization mechanism we describe here for RPM1-myc. A key set of data in this regard comes from Bendahmane et al (1999). The potato Rx protein recognizes the potato virus X coat protein (CP), normally resulting in no HR. Over-expression of CP, however, leads to an Rx-dependent HR. Furthermore, these authors demonstrate that the normal Rx-CP response (no HR) is epistatic to the HR produced by recognition of TMV by the *N* gene. Collectively, these data support a threshold model for activation of HR. Control of stability of R proteins may be one means by which the plant fine-tunes the balance between cell death associated with high amplitude resistance responses and preservation of viable tissue around the infection site.

The HR is not Required for RPM1-myc Disappearance

AvrRpt2 does not induce an HR in the *ndr1* or *rps2* mutants. Despite the lack of HR, the disappearance of RPM1-myc induced by AvrRpt2 is unaffected in these mutants. *NDR1* contributes quantitatively to HR induced by CC-NB-LRR-type R-proteins (Tornero et al. 2002b). It is possible that the disappearance of RPM1-myc induced by AvrRpt2 in *ndr1* results merely from weak signaling through RPS2. This interpretation is inconsistent with the observation in *rps2* (Fig. 4). In the absence of RPS2, AvrRpt2 has a function that leads to the disappearance of RPM1-myc. AvrRpt2 can inhibit RPM1 function, and in so doing enhance the virulence of *P. syringae* carrying *avrRpm1* (Ritter and Dangl 1996). Deletion of RPM1 function and increased susceptibility to virulent pathogen was also observed in AvrRpt2-expressing stable transgenic plants (Chen et al 2000). It is worth noting that although the disappearance of RPM1-myc that parallels HR induction may be mechanistically unrelated to RPM1-myc disappearance induced by AvrRpt2 in the absence of an HR, their time points of RPM1-myc disappearance are similar. We hypothesize that AvrRpt2, acting as a virulence factor, induces disappearance of RPM1. Both RPM1 and RPS2 are localized at the plasma membrane monitor by binding to the plasma membrane-associated defense regulator RIN4 (RPM1 interacting protein 4), which is cleaved by AvrRpt2 and released from membrane and destabilized (Mackey et al. 2002; Axtell and Staskawicz 2003; Kim et al. 2005). Since RIN4 is a positive regulator for RPM1 stability and a negative regulator for RPS2 activation, cleavage of RIN4 by AvrRpt2 might lead to the disappearance of un-activated RPM1 irrespective of RPS-mediated HR. We propose that AvrRpt2 co-opts a plant signaling activity and manipulates it to the advantage of the pathogen.

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Author contributions Nam J. and Mackey D. designed and performed experiments, analyzed data and wrote the manuscript. Yun DJ contributed to the interpretation of the results and the final version of the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The article does not contain any studies with human participants or animals performed by any of the authors.

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