Structure-Function Analysis of the Coiled-Coil and Leucine-Rich Repeat Domains of the RPS5 Disease Resistance Protein\textsuperscript{1[W][OA]}

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The Arabidopsis (Arabidopsis thaliana) RESISTANCE TO PSEUDOMONAS SYRINGAE5 (RPS5) disease resistance protein mediates recognition of the Pseudomonas syringae effector protein AvrPphB. RPS5 belongs to the coiled-coil-nucleotide-binding site-leucine-rich repeat (CC-NBS-LRR) family and is activated by AvrPphB-mediated cleavage of the protein kinase PBS1. Here, we present a structure-function analysis of the CC and LRR domains of RPS5 using transient expression assays in Nicotiana benthamiana. We found that substituting the CC domain of RPS2 for the RPS5 CC domain did not alter RPS5 specificity and only moderately reduced its ability to activate programmed cell death, suggesting that the CC domain does not play a direct role in the recognition of PBS1 cleavage. Analysis of an RPS5-super Yellow Fluorescent Protein fusion revealed that RPS5 localizes to the plasma membrane (PM). Alanine substitutions of predicted myristoylation (glycine-2) and palmitoylation (cysteine-4) residues affected RPS5 PM localization, protein stability, and function in an additive manner, indicating that PM localization is essential to RPS5 function. The first 20 amino acids of RPS5 were sufficient for directing super Yellow Fluorescent Protein to the PM. C-terminal truncations of RPS5 revealed that the first four LRR repeats are sufficient for inhibiting RPS5 autoactivation; however, the complete LRR domain was required for the recognition of PBS1 cleavage. Substitution of the RPS2 LRR domain resulted in the autoactivation of RPS5, indicating that the LRR domain must coevolve with the NBS domain. We conclude that the RPS5 LRR domain functions to suppress RPS5 activation in the absence of PBS1 cleavage and promotes RPS5 activation in its presence.

To defend themselves against pathogens, plants have evolved a two-tiered innate immune system. The first tier uses transmembrane pattern recognition receptors to detect the presence of pathogen-associated molecular patterns (PAMPs), which are broadly conserved molecules produced by the majority of a given class of microorganism (e.g. flagellin for bacteria and chitin for fungi; Jones and Dangl, 2006). Upon detection of PAMPs, these receptors activate multiple signaling pathways, including a mitogen-activated protein kinase cascade. This is called PAMP-triggered immunity. Successful pathogens can suppress PAMP-triggered immunity by delivering effector proteins into host cells to block signaling pathways at various steps (Desveaux et al., 2006; Kamoun, 2006; Dodds et al., 2009). This has led to the evolution of a second tier in the plant immune system consisting of intracellular receptors capable of recognizing the presence of effectors (Jones and Dangl, 2006). This effector-triggered immunity (ETI) typically leads to a localized activation of programmed cell death called the hypersensitive response (HR).

The immune receptors of the effector-triggered immunity system are encoded by resistance (R) genes. Most R proteins are intracellular and are composed of a central nucleotide-binding site (NBS) and a C-terminal leucine-rich repeat (LRR) domain (McHale et al., 2006; Caplan et al., 2008; Takken and Tameling, 2009). The NBS domain of plant R proteins shares structural and functional similarities with the metazoan apoptosis factors Apaf-1 and CED-4 (van der Biezen and Jones, 1998a; Albrecht and Takken, 2006; Takken et al., 2006) and appears to function as a molecular switch regulated by nucleotide-dependent conformational changes (Tameling et al., 2002, 2006; Collier and Moffett, 2009). Indeed, the NBS domain of a plant R protein has been shown to bind and hydrolyze ATP (Tameling et al., 2002), and mutations in the NBS domain that block ATP-binding also compromise function (Tameling et al., 2006).

Plant NBS-LRR proteins are divided into two classes based on their N-terminal domains. The TIR-NBS-LRR class contains an N-terminal domain with similarity to Toll and IL-1 receptors (TIR), while the non-TIR class most often contains a predicted coiled-coil (CC) domain (Meyers et al., 1999; Cannon et al., 2002). Over-expression of the TIR or CC domain of some NBS-LRR
proteins can induce HR independent of the presence of their corresponding effectors (Frost et al., 2004; Zhang et al., 2004; Swiderski et al., 2009; Krasileva et al., 2010; Bernoux et al., 2011; Collier et al., 2011; Maekawa et al., 2011). HR induction appears to require homodimerization, as amino acid substitutions that reduced dimerization reduced HR strength (Bernoux et al., 2011; Maekawa et al., 2011). However, overexpression of the Arabidopsis (Arabidopsis thaliana) RESISTANCE TO PSEUDOMONAS SYRINGAE5 (RPS5) CC domain by itself in Nicotiana benthamiana does not induce HR, whereas overexpression of the CC-NBS region does (Ade et al., 2007). In addition, overexpression of the NB subdomain of the potato (Solanum tuberosum) CC-NBS-LRR protein Rx can induce HR by itself when fused to GFP (Rairdan et al., 2008), suggesting that the NBS domain of at least some CC-NBS-LRR proteins plays a role in downstream signaling. The molecular mechanism regulating downstream signaling by CC-NBS-LRR proteins thus remains unclear.

The N termini of plant NBS-LRR proteins are also proposed to participate in effector recognition. Many plant NBS-LRR proteins appear to recognize their corresponding effectors by sensing effector-induced modifications of other host proteins (Van der Biezen and Jones, 1998b; Dangl and Jones, 2001). Several R proteins have been shown to interact with their corresponding effector targets via their N-terminal domains (Mackey et al., 2002; Mucyn et al., 2006; Ade et al., 2007; Burch-Smith et al., 2007), indicating that N-terminal domains contribute to the specificity of effector recognition. This is supported by the observation that recognition specificities of specific alleles of the flax (Linum usitatissimum) L locus can be swapped by swapping only the N-terminal TIR domains (Luck et al., 2000).

Generally, the LRR domain is presumed to play a central role in the recognition of pathogen effectors. In support of this, the LRR domains of many plant R proteins have been shown to be under diversifying selection (McDowell et al., 1998; Meyers et al., 1998; Ellis et al., 2000), implying that this domain in particular has been coevolving with pathogen effectors. Indeed, the LRR domains of some R proteins are known to bind pathogen effectors directly (Deslandes et al., 2003; Dodds et al., 2006; Krasileva et al., 2010), and in the case of the L and P loci from flax, recognition specificity can be swapped by swapping the LRR domains between alleles (Ellis et al., 1999; Dodds et al., 2001). In the case of the RPS5 NBS-LRR protein, however, the LRR domain appears to be required to keep RPS5 in the “off” state, as overexpression of the CC-NBS region of RPS5 induces HR but the full-length wild-type RPS5 does not (Ade et al., 2007). Consistent with an autoinhibitory role, the LRR domains of several NBS-LRR proteins have been shown to coimmunoprecipitate with the NBS domain when expressed separately (Moffett et al., 2002; Rairdan and Moffett, 2006; Ade et al., 2007; van Ooijen et al., 2008). Significantly, swapping of LRR domains between closely related paralogues often results in constitutive activation of NBS-LRR proteins (Hwang and Williamson, 2003; Rairdan and Moffett, 2006; van Ooijen et al., 2008). These observations suggest that the interaction surface between the NBS and LRR domains must have coevolved to maintain the inhibition of NBS-LRR protein autoactivation.

To reconcile the presumed positive role of the LRR domain in effector recognition and NBS-LRR activation with its apparent negative role in keeping NBS-LRR proteins in the off state in the absence of recognition, current models assume that the presence of effectors alters LRR conformation and/or interdomain interactions to enable nucleotide exchange (Collier and Moffett, 2009; Takken and Tameling, 2009). R protein activation must involve more than simply the release of LRR-mediated inhibition, however, as physical removal of LRR domains does not always result in the activation of plant NBS-LRR proteins, and some autoactivating mutations in the NBS domain require the presence of the LRR domain (Moffett et al., 2002; Rairdan and Moffett, 2006; van Ooijen et al., 2008).

To obtain a more detailed understanding of how the CC and LRR domains regulate NBS-LRR protein activation, we have focused on the Arabidopsis CC-NBS-LRR protein RPS5. RPS5 confers resistance to Pseudomonas syringae strains carrying the type III effector protein AvrPphB, which is a Cys protease. Recognition of AvrPphB by RPS5 requires the presence of PBS1, a protein kinase, which is a substrate of AvrPphB (Shao et al., 2003). Coimmunoprecipitation (Co-IP) analyses indicate that PBS1 associates with the RPS5 CC domain prior to cleavage by AvrPphB, and mutational analysis of PBS1 and AvrPphB indicates that PBS1 cleavage is required for RPS5 activation (Ade et al., 2007). How PBS1 cleavage activates RPS5 has not been determined, however, nor has the subcellular localization of PBS1 and RPS5. Here, we present a structure-function analysis of the RPS5 CC and LRR domains that revealed that both play positive roles in the recognition of PBS1 cleavage and the activation of downstream signaling. These analyses also revealed that RPS5 localizes to the plasma membrane (PM) and that the N-terminal myristoylation and palmitoylation motifs of RPS5 contribute additively to PM localization. PM localization appears to be required for RPS5 function.

RESULTS

The RPS2 CC Domain Can Functionally Substitute for the RPS5 CC Domain in Transient Expression Assays

To evaluate the role of the RPS5 CC domain in the recognition of PBS1 cleavage and induction of the HR, we swapped the CC domain of RPS5 with the CC domain of RPS2, which mediates the recognition of a different P. syringae effector, AvrRpt2 (Bent et al., 1994). Based on BLASTP analysis (Altschul et al., 1997), the RPS5 CC domain shows the highest amino acid sim-
ilarity (45%) to RPS2 among the Arabidopsis R proteins of known function. This is consistent with a previous phylogenetic analysis that assigned RPS2 and RPS5 to the same clade distinguished by the lack of an EDVID motif found in the CC domain of most other CC-NBS-LRR proteins (Meyers et al., 1999; Rairdan et al., 2008). Therefore, we hypothesized that RPS2 and RPS5 might share a similar signaling mechanism and that the RPS2 and RPS5 CC domains might be functionally interchangeable. We substituted the RPS2 CC domain (amino acids 1–153) for the RPS5 CC domain (amino acids 1–154) to create a chimeric protein, which we refer to as 2-5-5 (for RPS2 CC and RPS5 NBS-LRR). It has been previously shown that overexpression of the RPS2 protein by itself in N. benthamiana causes a cell death response similar to the HR response induced by RPS2 in Arabidopsis (Day et al., 2005). Therefore, we first tested whether the 2-5-5 chimera could induce HR by itself. Unlike RPS2, the 2-5-5 chimera was not autoactive (Supplemental Fig. S1A). The 2-5-5 chimera also did not induce HR when coexpressed with only PBS1 or only AvrPphB. However, when the 2-5-5 protein was coexpressed with PBS1 and AvrPphB, a typical HR was observed (Supplemental Fig. S1A). HR induction required the cleavage of PBS1 by AvrPphB, as coexpression with PBS1 and C98S, a protease-inactive mutant of AvrPphB, failed to initiate HR (Fig. 1A). This indicates that the RPS2 CC can functionally substitute for the RPS5 CC in N. benthamiana transient assays. However, when we tested the reciprocal construct (5-2-2), no autoactive HR-inducing activity was observed (data not shown), indicating that these two CC domains are not fully interchangeable. We were unable to test the activation of the 5-2-2 construct by AvrRpt2 and RIN4 in N. benthamiana, as we found that AvrRpt2 by itself induced HR. Significantly, overexpression of either the RPS5 CC or the RPS2 CC domain alone was not sufficient to induce cell death, even when fused to eGFP to enhance protein accumulation (Supplemental Fig. S1 D and F), indicating that the CC domains by themselves are not sufficient for HR activation, unlike the CC domains of barley (Hordeum vulgare) Mla10, tobacco (Nicotiana tabacum) NRG1, and Arabidopsis ADR1, which have recently been reported to induce cell death in N. benthamiana when overexpressed (Collier et al., 2011; Maekawa et al., 2011).

We next asked whether the RPS2 CC domain could substitute for the RPS5 CC domain in the context of the autoactive RPS5 mutant D266E (Ade et al., 2007). The resulting chimeric protein 2-5(D266E)-5 lost the autoactivation phenotype, indicating that the RPS2 CC domain is not fully functional in this context (Fig. 1B). Interestingly, when the 2-5(D266E)-5 chimera was coexpressed with PBS1 and AvrPphB, a strong HR was observed (Fig. 1B). This HR depended on PBS1 cleavage, as coexpression with PBS1 and C98S did not trigger HR (Supplemental Fig. S1B). These observations indicate that substitution of the RPS2 CC domain for the RPS5 CC domain reduces the efficiency of RPS5 activation and/or signaling. They also indicate that the activation of RPS5 caused by the D266E mutation is weaker than that triggered by PBS1 cleavage. We have previously shown that deletion of the LRR domain of RPS5 causes RPS5 to be autoactive in N. benthamiana (Ade et al., 2007). Therefore, we tested whether deletion of the LRR domain from the 2-5-5 chimera (generating a 2-5 chimera) would lead to

Figure 1. The RPS2 CC domain can partially substitute for the RPS5 CC domain. The indicated constructs were transiently coexpressed in N. benthamiana, and leaves were detached for photography 24 h post DEX induction. D266E indicates full-length RPS5 with the D266E substitution. 2-5-5 indicates the chimeric construct with the RPS2 CC domain and the RPS5 NBS-LRR domains. C98S indicates the protease-inactive mutant form of AvrPphB. A, The 2-5-5 chimera can induce HR in response to PBS1 cleavage. B, The 2-5(D266E)-5 chimera is not autoactive but can induce HR in response to PBS1 cleavage. C, Deletion of the LRR domain from the 2-5(D266E)-5 chimera, but not the 2-5-5 chimera, causes autoactivation.
autoactivation. Like the 2-5[D266E]-5 chimera, the 2-5 chimeric protein was not autoactive (Fig. 1C), despite substantial protein accumulation (Supplemental Fig. S2A). Surprisingly, introduction of the D266E mutation into the 2-5 chimera [2-5[D266E]] restored autoactivity (Fig. 1C). Therefore, deletion of the LRR and the D266E substitution have additive effects on autoactivation, and neither one alone is sufficient to overcome the loss of efficiency associated with substitution of the RPS2 CC domain for the RPS5 CC domain.

We have previously reported that PBS1 can be coimmunoprecipitated with the RPS5 CC domain (Ade et al., 2007), suggesting that the CC domain may play a role in the recognition of PBS1 cleavage. We thus tested whether the RPS2 CC domain can also be coprecipitated with PBS1. Consistent with the observed function of the 2-5-5 chimera in N. benthamiana, the RPS2 CC domain did indeed coimmunoprecipitate with PBS1 (Supplemental Fig. S3A). The ability of PBS1 to coimmunoprecipitate with the RPS2 CC domain suggests that the interaction between PBS1 and the RPS5 and RPS2 CC domains may be indirect, or alternatively that there is sufficient similarity between RPS5 and RPS2 CC domains for PBS1 to bind to both when transiently overexpressed. Under native expression levels, however, the RPS2 CC domain cannot substitute for the RPS5 CC domain, as transgenic Arabidopsis plants expressing the 2-5-5 chimera driven by the native RPS5 promoter failed to display an HR when challenged with P. syringae strain DC3000 (AvrPphB; data not shown). This result is consistent with the observed reduction in HR observed for the 2-5[D266E]-5 chimera in the transient system (Fig. 1B) and suggests that the 2-5-5 chimera falls below a threshold required for HR induction when expressed under native protein levels.

The Myristoylation and Palmitoylation Motifs of RPS5 Contribute in an Additive Fashion to RPS5 Function

RPS5 contains both a predicted myristoylation motif at Gly-2 and a palmitoylation motif at Cys-4. It also contains a Gly at position 3 that could be potentially myristoylated if Gly-2 were removed. To investigate the importance of acylation in RPS5 function, we mutated the Gly residues at positions 2 and 3 to Ala (G2/3A) and the Cys at position 4 to Ala (C4A), both as separate mutations and combined. G2/3A-RPS5 was still able to activate HR in response to PBS1 cleavage by AvrPphB when transiently overexpressed in N. benthamiana (Fig. 2A). However, the G2/3A substitution nearly eliminated the autoactivity of the full-length RPS5 mutant D266E without affecting protein accumulation at 4 h post induction (Supplemental Fig. S2B). Like the 2-5[D266E]-5 chimera, the G2/3A-D266E construct could be activated by coexpression with PBS1 and AvrPphB (Fig. 2A). These data indicate that the D266E substitution, which is believed to reduce ATP hydrolysis and increase the percentage of RPS5 in the ATP-bound state (Ade et al., 2007), is not equivalent to activation by PBS1 cleavage. C4A-RPS5

Figure 2. The RPS5 N-terminal acylation motif is required for RPS5-mediated HR. The indicated constructs were transiently coexpressed in N. benthamiana leaves as described in Figure 1. A, Modification of the N-terminal myristoylation motif (G2/3A) disables D266E autoactivation but not the recognition of PBS1 cleavage. B, Modification of the N-terminal palmitoylation residue (C4A) produces the same phenotype as the G2/3A substitution. C, Mutations of both myristoylation and palmitoylation residues (G2/3AC4A) completely disable RPS5-mediated HR in the presence of PBS1 and AvrPphB. D, Quantification of RPS5-mediated cell death in N. benthamiana by measurement of electrolyte leakage. sYFP was included as the negative control.
and C4A-D266E behaved the same as the G2/3A derivatives, with both retaining the ability to be activated by coexpression with PBS1 and AvrPphB and C4A-D266E losing autoactivity (Fig. 2B; Supplemental Fig. S2C). Significantly, the triple substitution derivative G2/3AC4A-RPS5 was completely disabled for HR initiation even in the presence of PBS1 and AvrPphB (Fig. 2C). For a more quantitative assay of the HR mediated by RPS5 acylation mutants, we expressed each RPS5 derivative with PBS1 and AvrPphB and measured electrolyte leakage at 8 or 24 h post dexamethasone (DEX) induction. Compared with the negative control of super Yellow Fluorescent Protein (sYFP), wild-type RPS5 induced an obvious conductivity increase as early as 8 h post DEX induction (Fig. 2D). However, the G2/3AC4A mutation completely abolished RPS5-induced electrolyte leakage. Interestingly, the G2/3A-RPS5 mutant induced only a moderate conductivity increase at 8 h post DEX induction but an electrolyte leakage comparable to wild-type RPS5 at 24 h post DEX induction (Fig. 2D). This is consistent with the macroscopic HR assay. Taken together, these results suggest that N-terminal myristoylation and palmitoylation play additive and partially redundant roles in RPS5 function.

A possible explanation for the functional defect of RPS5 acylation mutants is that these modifications compromise interactions of the CC domain with PBS1 and/or with the NBS domain. We tested this possibility using Co-IP assays, but we observed no significant reduction in the ability of the RPS5 CC domain containing these substitutions to communoprecipitate either PBS1 or the RPS5 NBS domain (Fig. 3). Likewise, the G2/3AC4A-RPS5 full-length protein retained the ability to interact with PBS1 (Supplemental Fig. S3B). Note, however, that these interactions may be occurring in solution following cell lysis and do not necessarily indicate that the G2/3AC4A derivatives colocalize with PBS1 inside the cell.

**N-Terminal Acylation Is Essential for PM Localization of RPS5 and Its Stabilization**

The requirement for myristoylation and palmitoylation motifs in RPS5 suggests that RPS5 must localize to a membrane to be functional. Therefore, we investigated the subcellular localization of RPS5. A C-terminal sYFP fusion of RPS5 was transiently expressed in *N. benthamiana* and visualized by laser scanning confocal microscopy. The RPS5:sYFP fusion induced HR when coexpressed with PBS1 and AvrPphB (Supplemental Fig. S1C), demonstrating that the addition of a C-terminal fluorescent tag did not impair RPS5 function. RPS5:sYFP displayed a peripheral localization when expressed in *N. benthamiana* epidermal cells (Fig. 4A), indicating localization to the PM. A three-dimensional (3D) projection of RPS5 from a Z-stack showed an even distribution of RPS5 on the PM, with no localization to cytoplasmic strands (visualized by coexpressing with free mCherry; Fig. 4A). In contrast, G2/3A-RPS5 displayed an accumulation of fluorescence in cytoplasmic strands but not in the nucleus (Fig. 4A), suggesting that RPS5 lacks a functional nuclear localization signal. Cytoplasmic localization was also observed for the C4A-RPS5: sYFP fusion (Supplemental Fig. S4A) and G2/3AC4A-RPS5:sYFP (Fig. 4A), indicating that both myristoylation and palmitoylation contribute to the PM localization.

The above imaging was performed at 6 to 8 h post DEX induction. To gain more insight into the dynamics of RPS5 localization, we also analyzed samples at 24 h post DEX induction. Surprisingly, at this time point, G2/3A-RPS5 displayed a wild-type-like localization pattern in the majority of cells analyzed. This was manifested as both a peripheral localization in a single confocal section and PM distribution in the 3D projection (Fig. 4A). The same pattern was also observed for C4A-RPS5 at 24 h post DEX induction (data not shown). These observations indicate that modification of just one acylation site significantly reduces, but does not completely abolish, RPS5 localization to the PM. This partial PM localization likely accounts for the HR-inducing ability of G2/3A-RPS5 or C4A-RPS5 in the presence of PBS1 cleavage by AvrPphB (Fig. 2).

Although both G2/3A-RPS5:sYFP and C4A-RPS5: sYFP were easily detected using laser scanning confo-

**Figure 3.** The N-terminal acylation motifs of the RPS5 CC domain are not required for Co-IP with PBS1 or with the NBS domain. G2/3AC4A substitutions in the predicted RPS5 N-terminal acylation residues do not abolish Co-IP between the RPS5 CC domain and PBS1 (left panels) or the RPS5 CC domain and the NBS domain (right panels). All constructs were transiently expressed in *N. benthamiana*. Empty vector (EV) was employed as the negative control. IB, Immunoblot; IP, immunoprecipitation.
cal microscopy at 24 h post DEX induction, G2/3AC4A-RPS5:sYFP was not visible (data not shown). This observation suggested that when not membrane associated, RPS5 protein may be destabilized and degraded. To test this, we compared the relative levels of G2/3A-RPS5:myc and G2/3AC4A-RPS5:myc proteins with wild-type RPS5:myc protein at 6 or 24 h post DEX induction. At 6 h post induction, both G2/3A-RPS5:myc and G2/3AC4A-RPS5:myc proteins accumulated to levels similar to wild-type RPS5:myc (Fig. 4B). By 24 h post DEX induction, however, G2/3A-RPS5:myc levels were significantly reduced compared with wild-type RPS5:myc, and G2/3AC4A-RPS5:myc was nearly undetectable (Fig. 4B); only a very weak band showed up after overexposure (data not shown). Thus, the stability of RPS5 protein during transient overexpression correlates with PM localization. Note that the initial signs of HR are usually apparent by 8 h post DEX treatment, at a time when total protein accumulation is similar between wild-type and mutant constructs. We thus infer that the reduction in HR caused by the acylation mutations is due to the reduction in PM localization rather than a reduction in protein levels.

Since the 2-5(D266E)-5 chimera only triggered HR when coexpressed with PBS1 and AvrPphB, but not by itself, and the RPS2 CC domain contains a predicted palmitoylation residue, but no myristoylation residue, we hypothesized that the 2-5-5 chimera might be only partially associated with the PM, thus reducing signaling activity. To test this, we analyzed the subcellular localization of 2-5-5:sYFP; however, 2-5-5:sYFP was exclusively localized to the PM (Supplemental Fig. S4B), indicating that the absence of autoactivity in the 2-5(D266E)-5 background cannot be attributed to disassociation from the PM.

Figure 4. The N-terminal acylation motifs are required for the localization of RPS5 to the PM and for protein stabilization. A, RPS5 is localized to the PM. Mutating the RPS5 myristoylation and palmitoylation motifs individually slows the accumulation of RPS5-sYFP at the PM, while modification of both together eliminates PM accumulation. The indicated constructs were transiently coexpressed in N. benthamiana leaves and imaged using confocal laser scanning microscopy at the indicated times following DEX induction. Free mCherry was included as a reference for cytoplasmic localization (C) and nuclear localization (N). B, Modification of the myristoylation and palmitoylation motifs of RPS5 causes reduced protein accumulation. Coomassie Brilliant Blue staining was included as a control to show equal loading. Samples were prepared at 6 or 24 h post DEX induction and loaded as indicated.
The First 20 Amino Acids of RPS5 Are Sufficient for Directing It to the PM

To further define the PM localization signal of RPS5, we generated C-terminal sYFP fusions to the CC domain, NBS domain, and NBS-LRR region of RPS5. As expected, only the CC:sYFP fusion localized to the PM (Fig. 5; data not shown). A 3D projection of a CC:sYFP Z-stack revealed the presence of punctate structures in addition to a PM localization profile. Similarly, a RPS2 CC:eGFP fusion was also found to localize to the PM (Supplemental Fig. S4C), which is consistent with previous findings that wild-type RPS2 is associated with the membrane fraction in Arabidopsis cell extracts (Axtell and Staskawicz, 2003). We next fused only the first 20 amino acids of RPS5 to sYFP (N20AA:sYFP) to test whether the predicted N-terminal acylation motifs were sufficient to direct PM localization. Similar to RPS5 CC:sYFP, the N20AA:sYFP fusion displayed a typical PM localization pattern along with the presence of numerous punctate structures in the 3D projection view (Fig. 5). Time-lapse imaging showed that the punctate structures observed for RPS5 CC:sYFP and N20AA:sYFP were highly mobile, suggesting that they might be vesicle-like structures (data not shown). The formation of physiologically inert vesicles as a consequence of overexpression has been reported for other membrane-associated GFP fusions, particularly those with an acylation motif (Vilas et al., 2006; Joensuu et al., 2010). Unlike free mCherry, no intranuclear fluorescence was observed for the N20AA:sYFP fusion (Fig. 5). PM localization of the N20AA:sYFP fusion depends on the acylation motifs, as the triple substitution G2/3AC4A abolished the PM localization of the sYFP fusion (Fig. 5).

The association of the N20AA:sYFP fusion with the PM provided us with an ideal negative control for Co-IP experiments involving membrane-associated proteins. Therefore, we performed Co-IP analysis between an N20AA:sYFP:myc fusion and PBS1:HA (for hemagglutinin), RPS5-CC:HA, and RPS5-NBS:HA protein fusions. No Co-IPs were detected for any of these combinations, although a co-IP was observed for the positive control of RPS5-CC:myc and PBS1:HA (Supplemental Fig. S3C), demonstrating the utility of this N20AA:sYFP fusion as a negative control for Co-IP assays.

The RPS5 LRR Domain Is Required for the Recognition of PBS1 Cleavage

A positive role for the LRR in NBS-LRR protein activation has been reported for the R proteins Rx and Mi1.2 (Moffett et al., 2002; van Ooijen et al., 2008). Determining whether the RPS5 LRR domain contributes positively to RPS5 activation is complicated by the finding that deletion of the entire LRR domain leads to autoactivation of RPS5 (Ade et al., 2007). To circumvent this problem, we introduced the G2/3A mutation into the RPS5 CC-NBS and CC-NBS(D266E) derivatives, hoping that this would suppress their autoactivation phenotype. Indeed, both G2/3A-CC-NBS and G2/3A-CC-NBS(D266E) lost the autoactivation phenotype (Fig. 6A), while protein accumulation was unaffected (Supplemental Fig. S2D). Unlike the full-length G2/3-RPS5 and G2/3-D266E (Fig. 2), coexpression of G2/3A-CC-NBS or G2/3A-CC-NBS(D266E) with PBS1 and AvrPphB failed to induce an HR (Fig. 6A). These observations indicate that the RPS5 LRR domain plays a positive regulatory role in RPS5 activity in addition to its known negative regulatory role in preventing RPS5 CC-NBS autoactivation.

To test whether the positive regulatory role of the RPS5 LRR is sequence specific, we swapped the RPS2 LRR (amino acids 508–919) for the RPS5 LRR (amino acids 513–889), creating a 5-5-2 chimera. Interestingly,
this 5-5-2 chimera proved to be autoactive (Fig. 6B). This observation is consistent with findings from other NBS-LRR proteins (Rairdan and Moffett, 2006; van Ooijen et al., 2008), which suggests that the NBS and LRR domains coevolve and that negative regulation requires precise interactions between the NBS and LRR domains.

Negative Regulation of RPS5 Activity by the LRR Domain Requires Only the First Four LRR Repeats

To identify the minimum RPS5 LRR region required to inhibit RPS5 autoactivation, five C-terminal truncation mutants of different LRR lengths were created (Fig. 7A). Transient expression of RPS5 CC-NBS-3LRR (amino acids 1–577), incorporating the CC-NBS domain plus the first three LRRs, induced a strong HR similar to the CC-NBS construct lacking the entire LRR domain (Fig. 7, B and C). However, with the addition of the fourth LRR (CC-NBS-4LRR; amino acids 1–605), the HR was dramatically suppressed, although protein accumulation was similar (Supplemental Fig. S2E). There was no obvious tissue collapse in the CC-NBS-4LRR injected area, and only some mild isolated cell death was observed (Fig. 7C). Constructs with longer LRR fragments displayed similar phenotypes (Fig. 7C). Consistent with the macroscopic observations, electrolyte leakage measurements demonstrated a strong conductivity increase for the CC-NBS-3LRR construct but only moderate or mild conductivity increases for other RPS5 C-terminal truncation mutants (Fig. 7B). Hence, the first four LRRs appear to be the minimum region required to suppress RPS5 autoactivation.

Activation of RPS5 in Response to PBS1 Cleavage Requires the Entire RPS5 LRR Region

Because the C-terminal truncation constructs with four or more LRR repeats were not autoactive, we were able to test whether they could be activated by cleavage of PBS1. None of these constructs was capable of inducing HR in the presence of PBS1 and AvrPphB (Fig. 7D), indicating that they are unable to recognize PBS1 cleavage.

To further assess the role of different LRRs in the recognition of PBS1 cleavage, we constructed a second series of mutants by introducing internal deletions of three LRRs at different sites (RPS5D1-3LRR, deletion of amino acids 513–579; RPS5D4-6LRR, deletion of amino acids 583–650; RPS5D7-9LRR, deletion of amino acids 651–734; RPS5D10-12LRR, deletion of amino acids 735–819; and RPS5D13LRR, deletion of amino acids 820–889). These mutants did not display an autoactive phenotype (data not shown). Next, we tested whether these mutants were still functional in recognizing cleavage of PBS1 by AvrPphB. None of these mutants induced HR in response to PBS1 cleavage (Fig. 8), although protein accumulated to levels similar to wild-type RPS5 (Supplemental Fig. S2F). To exclude the possibility that these mutants were actually defective in signaling rather than in PBS1 cleavage recognition, the same mutations were introduced into the autoactive RPS5 mutant D266E (i.e. D266ED1-3LRR, D266ED4-6LRR, D266ED7-9LRR, D266ED10-12LRR, and D266ED13LRR). All of these D266E derivatives retained their autoactivity (Fig. 8), indicating that these internal LRR deletions compromised the recognition of PBS1 cleavage but not RPS5 signaling ability. Collectively, these results indicate that an intact RPS5 LRR domain is required for the recognition of PBS1 cleavage. Conversely, suppression of autoactivation is dependent on a minimum length of the RPS5 LRR region.

DISCUSSION

The Role of the RPS5 CC Domain in Downstream Signaling

The role of the CC domain in the function of CC-NBS-LRR proteins is poorly understood and contro-
versial. For example, the CC domain of the barley MLA protein interacts with WRKY transcription factors in the nucleus, apparently inhibiting their ability to repress defense genes (Shen et al., 2007), and overexpression of this CC domain alone in *N. benthamiana* can induce cell death (Maekawa et al., 2011). Unlike MLA, however, exclusion of the potato Rx CC-NBS-LRR protein from the nucleus appears to enhance its HR-inducing ability (Tameling et al., 2010), suggesting that signaling is occurring in the cytoplasm. Furthermore, the CC domain of Rx is not required for activation of the HR, as overexpression of the NB subdomain alone, when fused to eGFP, induces HR in *N. benthamiana* but overexpression of the Rx CC domain alone does not (Rairdan et al., 2008). This is unlikely to be the case for the RPS5 NB subdomain, however, as we demonstrated that the RPS5-mediated HR requires PM localization mediated by the N terminus (Figs. 2 and 4). In addition, overexpression of the RPS5 CC-NB fragment (without the ARC subdomain) failed to cause HR as well, even when fused to GFP to enhance stability (data not shown). We also expressed the full NBS domain of RPS5 fused to the first 20 amino acids of RPS5 to direct it to the PM, but this was also unable to induce HR (Supplemental Fig. S1E). Thus, the minimal region of RPS5 required to induce HR in *N. benthamiana* is the CC plus full NBS domain.

One of the more surprising results presented in our study is that the RPS2 CC domain can partially substitute for the RPS5 CC domain in transient overexpression assays (Fig. 1A). However, this finding is consistent with a phylogenetic analysis of CC-NBS-LRR genes, which places *RPS2* and *RPS5* in the same clade, separate from well-characterized *R* genes such as *RPM1*, *Rx*, *Prf*, *Mla*, and *I2* (Meyers et al., 1999; Rairdan et al., 2008). The *RPS5/RPS2* clade is characterized by CC domains that lack an EDVID motif conserved in most CC-NBS-LRR proteins outside this clade (Rairdan et al., 2008). The EDVID motif is essential for Rx function and for interaction of the Rx CC domain with its NBS-LRR domain, but it is not required for interaction with RanGAP (Rairdan et al., 2008). The MLA CC domain forms an antiparallel homodimer, and the side chains of the EDVID motif are exposed on the surface (Maekawa et al., 2011), available for intramolecular and intermolecular interactions. Taken together, these observations suggest that RPS5 and RPS2 CC domains may function differently from the EDVID-containing CC domains.

The experiments described in this work, combined with our previous studies, demonstrate that the RPS5 CC domain fulfills multiple roles. First, it promotes the association of RPS5 with its corresponding effector target PBS1 (Ade et al., 2007). A similar interaction
between a CC domain and an effector target has also been shown for the CC-NBS-LRR protein RPM1 and RIN4, which is targeted by the *P. syringae* effectors AvrRpm1 and AvrB (Mackey et al., 2002), suggesting that this may be a common role of CC domains. Second, the N terminus of the RPS5 CC domain contains both myristoylation and palmitoylation motifs, which are necessary for localizing RPS5 to the PM and are necessary for RPS5 function (Figs. 2 and 4). Third, the CC domain interacts with the NBS domain (Ade et al., 2007). The functional significance of this interaction is unknown, but it has also been observed for the CC domain of Rx from potato (Moffett et al., 2002; Rairdan and Moffett, 2006).

**PM Association Is Required for RPS5 Function**

Our results demonstrate that RPS5 localizes to the PM and strongly suggest that this localization is required for RPS5 function. Mutation of N-terminal myristoylation and palmitoylation motifs abrogated both PM localization and RPS5-induced HR in transient expression assays, despite high initial levels of protein accumulation (Fig. 4). Localization of RPS5 to the PM is consistent with previous work showing that AvrPphB becomes myristoylated inside host cells and is targeted to the PM (Dowen et al., 2009). AvrPphB catalyzes the cleavage of several related protein kinases in Arabidopsis in addition to PBS1, including BIK1 (Zhang et al., 2010). BIK1 associates with the flagellin receptor FLS2, and mutation of BIK1 reduces FLS2-mediated defense responses. FLS2 is a PM-localized PAMP receptor (Robatzek et al., 2006); thus, AvrPphB appears to target a family of kinases involved in regulating PAMP receptor signaling at the PM. Localization of RPS5 to the PM, therefore, may in part reflect a requirement to colocalize with the targets of AvrPphB. This cannot be the only reason for PM localization, however, as signaling by autoactive variants of RPS5 is also blocked by mutation of the myristoylation or palmitoylation motifs (Fig. 2). This latter observation suggests that activation of the HR requires that RPS5 be at the PM. Similarly, an autoactive mutant derivative of the CC-NBS-LRR protein RPM1 was found to localize to the PM (Gao et al., 2011), suggesting that PM-based signaling is not unique to RPS5.

Myristoylation and palmitoylation appear to have an additive effect on RPS5 localization and function. Mutation of either motif alone reduced the initial electrolyte leakage (Fig. 2) and the accumulation of RPS5 on the PM (Fig. 4), while the double mutation completely abolished electrolyte leakage/HR induction (Fig. 2) and PM accumulation (Fig. 4). This is consistent with the previously reported dosage effect of RPS5 on HR and resistance, in which the speed of the HR in response to inoculation with *P. syringae* expressing AvrPphB, and the level of resistance, were proportional to protein levels in transgenic Arabidopsis plants expressing RPS5:HA (Holt et al., 2005).
Furthermore, our data indicate that this dosage effect is correlated with RPS5 abundance on the PM. Interestingly, PM localization appears to be essential for stabilization of the RPS5 protein (Fig. 4B). This likely explains why a G2/3A-RPS5 mutant gene driven by the RPS5 promoter in stable transgenic plants fails to complement an rps5 mutation (data not shown), as the amount of G2/3A-RPS5 protein on the PM may not reach the threshold for robust downstream signaling.

As shown in Figure 7, the LRR domain of RPS5 functions in part to keep RPS5 in the off state. The inhibitory function of LRRs is thought to be mediated by physical interactions between the LRR domain and the NBS domain (specifically, the ARC2 subdomain; Rairdan and Moffett, 2006). Consistent with this model, swapping the RPS5 LRR domain with the RPS2 LRR domain resulted in the induction of HR in the absence of PBS1 cleavage (Fig. 6B). The prevailing model for NBS-LRR protein activation is that switching between the off state and the on state is mediated by exchange of ADP for ATP (Takken and Tameling, 2009). Autoinhibition by the LRR domain is thus thought to be a consequence of inhibiting nucleotide exchange. In this study, we found that LRR-mediated inhibition requires as little as the first four LRR repeats (out of 13 total; Fig. 7A). If correct, then the first four LRRs of RPS5 seem to constitute the minimal NBS-interacting surface required to prevent nucleotide exchange.

A Role for the RPS5 LRR Domain in the Recognition of PBS1 Cleavage

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It is also informative that deletion of the LRR domain has an additive effect when combined with the D266E substitution in terms of autoactivation (Fig. 1C). This makes sense if the former promotes the exchange of ADP for ATP and the latter slows ATP hydrolysis. Combined, these mutations should greatly enhance the percentage of RPS5 molecules in the ATP-bound state and, thus, strengthen signaling.

Although the LRR domain clearly plays an autoinhibitory role, our data also establish that it performs a separate positive role in the activation of RPS5 and a...
Plant Growth Conditions

Nicotiana benthamiana and Arabidopsis (Arabidopsis thaliana) plants were grown in Metro-Mix 360 (Sun Gro Horticulture) under a 9-h/15-h photoperiod at 150 μmol m⁻² s⁻¹ and 23°C.

Plasmid Construction and Site-Directed Mutagenesis

An adapted multisite Gateway cloning strategy (Invitrogen) was used to generate RPS5 fusions with a 5× Myc tag or sYFP. The wild-type RPS5 open reading frame was PCR amplified from a plasmid template and cloned into the donor vector pBSDONR P1-P4 (an ampicillin-resistant vector derived from pDONR221 P1-P4 from Invitrogen) using the BP cloning Kit (Invitrogen, Grand Island, NY). RPS5 CC derivatives were cloned by amplifying the first 549 nucleotides of the RPS5 open reading frame followed by cloning into pBSDONR P1-P4 using BP clonase. RPS2-RPS5 chimeras were generated using a recombination PCR protocol. All N-terminal acylation mutants of RPS5 were created by PCR amplification with forward primers containing the desired mutations followed by BP cloning into the pBSDONR P1-P4. RPS5 CC derivatives were generated by PCR amplification of nucleotides 1 to 1,536 followed by BP cloning into pBSDONR P1-P4. Similarly, the RPS5 C-terminal truncation mutants were generated by amplifying the desired fragment followed by BP cloning. The LRR internal deletions and substitutions were introduced using an established mutagenesis PCR protocol (Qi and Schotholz, 2008). 5× Myc, 3× HA, and sYFP tags were BP cloned into pBSDONR P4r-P2 (an ampicillin-resistant vector derived from pDONR221 P4r-P3r and pDONR22 P3-P2). To create protein fusions with epitope tags or fluorescent proteins, the desired P1-P4 clone, P4r-P2 clone, and destination vector containing attR1 and attR2 sites were mixed in an approximately 2:2:1 ratio, and recombination was accomplished by using the LR cloning Kit (Invitrogen, Grand Island, NY). The C-terminal HA-tagged RPS5 CC domain, RPS5 NBS domain, and PBS1 were generated by traditional ligation as described previously (Ade et al., 2007). pTA7002-based DEX-inducible constructs were used for transient expression in N. benthamiana (Aoyama and Chua, 1997). All constructs were sequenced for verification. All primers used to generate the above constructs are listed in Supplemental Table S1.

Agrobacterium-Mediated Transient Expression in N. benthamiana

Agrobacterium tumefaciens GV3101 (pMP90) strains carrying pTA7002 DEX-inducible constructs were grown and prepared for transient expression as described previously (Ade et al., 2007). Agrobacterium cultures were resuspended in water to an optical density at 600 nm (OD₆₀₀) of 1.0. For single-construct expression, the suspensions were diluted to OD₆₀₀ of 0.3 before infiltration. For coexpression of multiple constructs, suspensions were mixed in equal ratios before infiltration, such that each strain was present at an OD₆₀₀ of 0.3. Bacterial suspensions were infiltrated into leaves of 4-week-old N. benthamiana plants with a needleless syringe. Transgene expression was induced by spraying the infiltrated leaves with a 50 μg DEX (Sigma) solution at 40 h post injection. Tissues were collected for protein extraction approximately 4 h post DEX induction, unless stated otherwise. Laser scanning confocal microscopy was performed at 6 to 8 h post DEX induction unless stated otherwise. HR was scored at 24 h post DEX induction. For ion leakage measurements, leaf discs were punched at 3 h post DEX induction. For each replicate, 16 discs were briefly washed with distilled water, dried on paper towels, and then floated in 5 mL of distilled water with 0.001% Tween 20. Conductivity was measured at the indicated time points. Data represent averages and 2 of 3 replicates, and the entire experiment was repeated at least twice with similar results.

Immunoprecipitations and Immunoblotting

For total protein extraction, six infiltrated leaves were ground in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and Plant Proteinase Inhibitor Cocktail [Sigma]). Homogenates were centrifuged twice at 13,000 rpm at 4°C for 10 min, and supernatants were transferred to new tubes. For immunoblots, crude extracts were mixed with 4× SDS loading buffer and boiled for 10 min before resolving by SDS-PAGE. Immunoprecipitations were performed as described previously (Ade et al., 2007) using an anti-c-Myc monoclonal antibody matrix (Clontech). The immunocomplexes were resuspended in 30 μL of 1× SDS loading buffer and boiled for 10 min. All protein samples were resolved on 4% to 20% gradient Tris-HEPES-SDS polyacrylamide gels (Thermo Scientific) and transferred to a nitrocellulose membrane for probing with anti-c-Myc- peroxidase (Roche) or anti-HA-peroxidase (Sigma). The Immunostar HR Substrate Kit (Bio-Rad) was used for detecting antibody complexes.
Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was performed on an SP5 AOB5 inverted confocal microscope (Leica Microsystems) equipped with a 63× numerical aperture 1.2 water objective. sYFP fusions were excited with a 514-nm argon laser and detected using a 522- to 545-nm band-pass emission filter. mcCherry was excited using a 561-nm helium-neon laser and detected using a custom 585- to 620-nm band-pass emission filter. To obtain 3D images, a series of Z-stack images were collected and then combined and processed using the 3D image-analysis software IMARIS 7.0 (Bitplane Scientific Software; http://www.bitplane.com).

Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers NC_003070 (RPS5), NC_003076 (PBS1), NC_003074 (RPS2), and AJ879974 (AvrPphB).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. HR tests of various RPS5 derivatives.

Supplemental Figure S2. Immunoblot analysis of RPS5 derivatives transiently expressed in N. benthamiana.

Supplemental Figure S3. Supplemental Co-IP assays.

Supplemental Figure S4. Laser scanning confocal microscopy of RPS5 and RPS2 derivatives.

Supplemental Figure S5. Addition of nuclear exclusion signals does not reduce RPS5 HR-inducing activity in N. benthamiana transient assays.

Supplemental Table S1. Primers used in plasmid construction.

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