

RPG1-B-Derived Resistance to *AvrB*-Expressing *Pseudomonas syringae* Requires RIN4-Like Proteins in Soybean^{1[W][OA]}

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Soybean (*Glycine max*) RPG1-B (for resistance to *Pseudomonas syringae* pv *glycinea*) mediates species-specific resistance to *P. syringae* expressing the avirulence protein AvrB, similar to the nonorthologous RPM1 in Arabidopsis (*Arabidopsis thaliana*). RPM1-derived signaling is presumably induced upon AvrB-derived modification of the RPM1-interacting protein, RIN4 (for RPM1-interacting 4). We show that, similar to RPM1, RPG1-B does not directly interact with AvrB but associates with RIN4-like proteins from soybean. Unlike Arabidopsis, soybean contains at least four RIN4-like proteins (GmRIN4a to GmRIN4d). GmRIN4b, but not GmRIN4a, complements the Arabidopsis *rin4* mutation. Both GmRIN4a and GmRIN4b bind AvrB, but only GmRIN4b binds RPG1-B. Silencing either GmRIN4a or GmRIN4b abrogates RPG1-B-derived resistance to *P. syringae* expressing AvrB. Binding studies show that GmRIN4b interacts with GmRIN4a as well as with two other AvrB/RPG1-B-interacting isoforms, GmRIN4c and GmRIN4d. The lack of functional redundancy among GmRIN4a and GmRIN4b and their abilities to interact with each other suggest that the two proteins might function as a heteromeric complex in mediating RPG1-B-derived resistance. Silencing *GmRIN4a* or *GmRIN4b* in *rpg1-b* plants enhances basal resistance to virulent strains of *P. syringae* and the oomycete *Phytophthora sojae*. Interestingly, *GmRIN4a*- or *GmRIN4b*-silenced *rpg1-b* plants respond differently to AvrB-expressing bacteria. Although both GmRIN4a and GmRIN4b function to monitor AvrB in the presence of RPG1-B, GmRIN4a, but not GmRIN4b, negatively regulates AvrB virulence activity in the absence of RPG1-B.

One of the myriad plant defense responses activated upon pathogen invasion is signaling induced via the activation of resistance (R) proteins. R gene-mediated resistance is generally activated in response to race-specific pathogen effectors, termed avirulence proteins (Avr), and often results in the development of a hypersensitive reaction at the site of pathogen entry (Dangl et al., 1996). The hypersensitive reaction is a form of programmed cell death that results in the formation of necrotic lesions around the site of pathogen entry and is thought to help prevent pathogen spread by confining it to the dead cells.

A majority of the known R proteins contain conserved structural domains, including N-terminal coiled coil (CC) or Toll-interleukin 1 receptor (TIR)-like domains, central nucleotide-binding site (NBS),

and C-terminal Leu-rich repeat (LRR) domains (Martin et al., 2003). While some R proteins “perceive” pathogen presence via direct physical interactions with the cognate Avr proteins (Scofield et al., 1996; Jia et al., 2000; Leister and Katagiri, 2000; Deslandes et al., 2003), several others likely do so indirectly. This led to the suggestion that R proteins monitor the presence of Avr proteins by “guarding” other host proteins targeted by the pathogen effector (Van der Biezen and Jones, 1998; Innes, 2004; Jones and Dangl, 2006). Avr proteins enhance pathogen virulence in genetic backgrounds lacking cognate R proteins by targeting components of the host basal defense machinery, including “guardee” proteins (Chang et al., 2000; Guttman and Greenberg, 2001; Chen et al., 2004; Kim et al., 2005b; Ong and Innes, 2006; van Esse et al., 2007; Shan et al., 2008; Xiang et al., 2008). However, some Avr proteins were found to also target host proteins that do not contribute to the virulence function of the effector (Shang et al., 2006; Shabab et al., 2008; Zhou and Chai, 2008; Zipfel and Rathjen, 2008). This led to the proposition that plants express “decoy” proteins that mimic Avr-guardee recognition in the presence of the R protein. This decoy model suggests that, unlike guardees, decoy proteins do not directly contribute to host basal immunity, such that Avr-derived alterations of decoys do not enhance pathogen virulence in plants lacking the R protein (van der Hoorn and Kamoun, 2008).

A well-studied example of an indirect mode of effector recognition is that of the Arabidopsis (*Arabidopsis thaliana*) R protein, RPM1 (for resistance to

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Pseudomonas syringae pv *maculicola* 1). RPM1 mediates resistance against bacteria expressing two different Avr proteins, AvrRpm1 (AvrRpm1_{PmaM6}) and AvrB (AvrB1_{Pgyrace4}). Although RPM1 does not directly interact with either AvrRpm1 or AvrB, it does associate with RIN4 (for RPM1-interacting 4), which interacts with AvrRpm1 and AvrB. RIN4 is required for RPM1-induced resistance to AvrRpm1/AvrB-expressing *P. syringae* (Mackey et al., 2002). Both AvrRpm1 and AvrB induce the phosphorylation of RIN4, which is thought to induce RPM1-mediated resistance signaling. RIN4 also associates with a second Arabidopsis R protein, RPS2 (for resistance to *P. syringae*), which mediates resistance against *P. syringae* expressing AvrRpt2. RPS2-mediated signaling is activated when AvrRpt2 (AvrRpt2_{PtoJL1065}), a Cys protease, cleaves RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2003; Kim et al., 2005a). The AvrRpt2-triggered loss of RIN4 compromises RPM1-mediated resistance, because RIN4 is not available for phosphorylation (Ritter and Dangl, 1996; Axtell and Staskawicz, 2003; Mackey et al., 2003).

The avirulence effector AvrB was first isolated from a *P. syringae* strain colonizing soybean (*Glycine max*) and used to identify the cognate resistance locus *RPG1* in soybean (Staskawicz et al., 1987; Keen and Buzzell, 1991). This locus contains the *RPG1-B* (for resistance to *P. syringae* pv *glycinea*) gene, which encodes a CC-NBS-LRR protein conferring resistance to AvrB-expressing *P. syringae* in soybean (Bisgrove et al., 1994; Ashfield et al., 2004). Unlike *RPM1*, *RPG1-B* does not confer specificity to AvrRpm1 (Ashfield et al., 1995). However, as in Arabidopsis, the soybean *RPG1-B*-derived hypersensitive reaction to AvrB-expressing bacteria is inhibited by the presence of AvrRpt2-expressing bacteria (Axtell and Staskawicz, 2003; Mackey et al., 2003; Ashfield et al., 2004). This suggests that *RPG1-B* and *RPM1* might utilize common signaling components even though they share very limited sequence identity. Therefore, we investigated the possible involvement of RIN4-like proteins in *RPG1-B*-mediated resistance signaling. In addition to Arabidopsis, RIN4-like proteins have also been identified in tomato (*Solanum lycopersicum*) and lettuce (*Lactuca sativa*; Jeuken et al., 2009; Luo et al., 2009). In tomato, the NBS-LRR protein, Prf (for *Pseudomonas* resistance and fenthion sensitivity), and its interacting protein kinase, Pto, mediate resistance to the AvrPto (AvrPto1_{PtoJL1065})-expressing strain of *P. syringae* (Scofield et al., 1996; Tang et al., 1996; Kim et al., 2002; Mucyn et al., 2006). AvrPto binds RIN4 proteins from both Arabidopsis (AtRIN4) and tomato (SIRIN4). Similar to AvrRpt2, AvrPto induces the proteolysis of RIN4, albeit only in the presence of Pto and Prf (Luo et al., 2009). However, in the case of AvrPto, degradation of RIN4 is the result of induced proteolytic activity in the plant, rather than that of AvrPto itself. In *Lactuca* (lettuce) species, the *L. saligna* RIN4 allele was recently shown to be essential for resistance to an avirulent strain of the downy mildew pathogen, *Bremia lactucae* (Jeuken et al., 2009).

Here, we report that two functionally nonredundant isoforms of soybean *RIN4* (*GmRIN4*) function in *RPG1-B*-derived resistance as well as in the virulence activity of AvrB in the absence of *RPG1-B*.

RESULTS

RIN4 Orthologous Sequences in Soybean

The soybean genome contains at least four genes that encode proteins with high similarities to Arabidopsis *RIN4* (AtRIN4, At3g25070). These were designated *GmRIN4a* (Glyma03g19920), *GmRIN4b* (Glyma16g12160), *GmRIN4c* (Glyma18g36000), and *GmRIN4d* (Glyma08g46400). Amino acid alignments (Fig. 1A) showed that *GmRIN4a* and *GmRIN4b* were approximately 94% identical to each other, approximately 50% identical to AtRIN4, approximately 48% identical to two *RIN4* isoforms from lettuce (LsRIN4_774/780-GQ497774/GQ497780; Jeuken et al., 2009), and approximately 45% identical to a tomato *RIN4* (SIRIN4-TC174419; Luo et al., 2009). *GmRIN4c* and *GmRIN4d* were approximately 91% identical to each other, approximately 46%/48% identical to AtRIN4, respectively, approximately 49% identical to the two LsRIN4 proteins, and approximately 47% identical to SIRIN4. All four *GmRIN4* proteins contained several conserved domains present in AtRIN4, including those involved in binding to AvrB, an amino acid sequence required for AvrRpt2-mediated cleavage, and a putative palmitoylation site for plasma membrane localization (Chisholm et al., 2005; Kim et al., 2005a; Takemoto and Jones, 2005; Desveaux et al., 2007; Fig. 1A). Phylogenetic analysis showed that all four *GmRIN4* proteins clustered together and were more closely related to the Ls/SIRIN4 proteins than to AtRIN4. AtRIN4 was placed in a separate clade from all the other proteins analyzed (Fig. 1B).

GmRIN4 Proteins Interact with AvrB

Since AtRIN4 associates with AvrB, we first tested binding between AvrB and the four *GmRIN4* proteins using the yeast two-hybrid (Y2H) assay. AvrB was expressed as a GAL4 binding domain (BD) fusion protein, while *GmRIN4a*, *GmRIN4b*, *GmRIN4c*, and *GmRIN4d* were expressed as GAL4 activating domain (AD) fusion proteins. Yeast cells coexpressing AvrB-BD and *GmRIN4a/b/c/d*-AD were tested for His autotrophy or β -galactosidase activity (selectable markers for positive interactions). Y2H assay detected interactions between AvrB and all four *GmRIN4* proteins but not the empty AD vector (Fig. 2A). *GmRIN4a/b/c/d*-AD also did not interact with the empty BD vector (data not shown). In vitro "pull-down" affinity assays confirmed these interactions. AvrB and *GmRIN4a* were expressed as His-tagged, *GmRIN4b* as Myc-His-tagged, and *GmRIN4c* and *GmRIN4d* as glutathione S-transferase (GST)-tagged proteins in bacteria. Affinity-purified *GmRIN4a* and *GmRIN4b* were immobilized on nickel-nitrilotriacetic acid agarose (Ni-NTA),

GmRIN4 Proteins Interact with RPG1-B

We next tested interactions between GmRIN4a, GmRIN4b, GmRIN4c, or GmRIN4d and RPG1-B. RPG1-B (CC domain, N-terminal 187 amino acids) was expressed as a GAL4 BD fusion protein, and its interaction with the GmRIN4a, GmRIN4b, GmRIN4c, or GmRIN4d AD protein was assayed in Y2H assays. Interestingly, RPG1-B interacted with GmRIN4b, GmRIN4c, and GmRIN4d but not GmRIN4a or AvrB (Fig. 3A; data not shown). Similar to RPM1, the CC domain of RPG1-B was sufficient for its interactions with GmRIN4b, GmRIN4c, or GmRIN4d. However, truncating 16 amino acid from the C terminus of the RPG1-B CC domain abolished the RPG1-B-GmRIN4b/c/d interactions (Fig. 3A). Likewise, *in vitro* pull-down assays detected binding between the CC domain of RPG1-B and GmRIN4b, GmRIN4c, or GmRIN4d but not AvrB. RPG1-B (CC domain) was expressed as a His-tagged protein in bacteria, immobilized on Ni-NTA, and incubated with increasing amounts of AvrB (0.05–0.5 μ g) or GmRIN4b, GmRIN4c, or GmRIN4d (0.1–1 μ g). The His tag present on AvrB and GmRIN4b was removed prior to incubation with the RPG1-B affinity column. Bound proteins were visualized by immunoblot analysis using AvrB-specific, Myc (GmRIN4b)-specific, or GST (GmRIN4c or GmRIN4d)-specific antibodies. Saturable binding was detected between RPG1-B and GmRIN4b, GmRIN4c, or GmRIN4d (Fig. 3B). AvrB (0.5 μ g) and GmRIN4b (1 μ g) lacking the His tag or GST-tagged GmRIN4c (1 μ g) and GmRIN4d (1 μ g) were not retained on empty Ni-NTA or Ni-NTA preincubated with protein extracts from cells expressing the empty pET30A vector (used for expressing RPG1-B) when applied at maximum concentrations used in the assay (Fig. 3B, – lanes; Supplemental Fig. S3A).

These interactions were further confirmed by *in planta* BiFC assays. Consistent with the Y2H and pull-down assays, fluorescence was detected only in *N. benthamiana* leaves coexpressing RPG1-B with GmRIN4b, GmRIN4c, or GmRIN4d but not AvrB, GST, or GmRIN4a (Fig. 3C). Western-blot analysis of total protein extracts from AvrB/GST/GmRIN4a and RPG1-B coexpressing leaves showed that RPG1-B was expressed at similar levels as in the GmRIN4b coexpressing leaves, indicating that lack of fluorescence was not due to inadequate protein expression (Supplemental Fig. S3B). These leaves also expressed detectable amounts of AvrB, GST, or GmRIN4a. Furthermore, GmRIN4a was detected at comparable levels as GmRIN4b, GmRIN4c, or GmRIN4d in the membrane fractions of *N. benthamiana* leaves coexpressing the respective proteins with RPG1-B (Fig. 3D). This indicated that the lack of interaction between GmRIN4a and RPG1-B was not due to the improper localization or insufficient expression of this protein in *N. benthamiana*. These data showed that three (GmRIN4b, GmRIN4c, and GmRIN4d) of the four GmRIN4 proteins directly interact with RPG1-B.

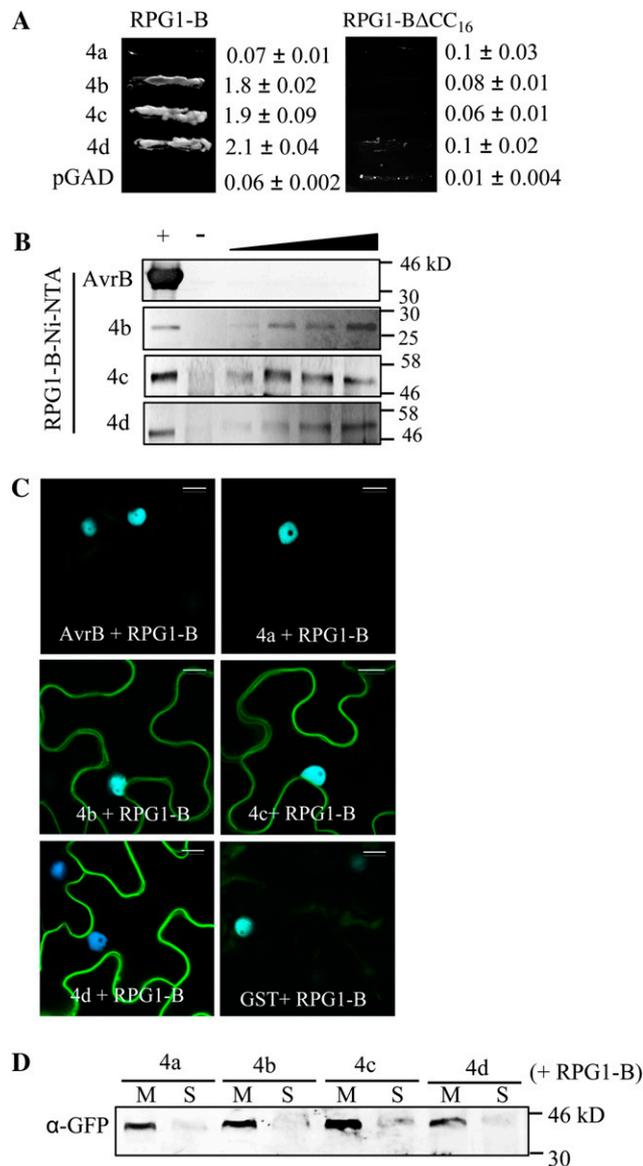


Figure 3. GmRIN4 (GmRIN4a to GmRIN4d = 4a to 4d) proteins interact with RPG1-B. **A**, Y2H assay between 4a, 4b, 4c, or 4d and RPG1-B (N-terminal 187 amino acids) or RPG1-B lacking 16 amino acids of the CC domain (N-terminal 171 amino acids; RPG1- Δ ACC₁₆). Growth on His⁻Trp⁻Leu⁻ plates is shown. Numbers indicate β -galactosidase activity with *sd* ($n = 3$). **B**, Pull-down assays showing *in vitro* protein binding. RPG1-B was immobilized on Ni-NTA incubated with increasing amounts of AvrB (0.05–0.5 μ g) or 4b, 4c, or 4d (0.1–1 μ g), and bound proteins were visualized using anti-AvrB, anti-Myc (4b), or anti-GST (4c/4d) antibodies. Purified AvrB, 4b, 4c, or 4d was loaded as a positive control (+) or incubated with empty Ni-NTA as a negative control (–) on respective gels. **C**, BiFC assay showing *in planta* interactions. CFP and YFP overlay images (40 \times magnification) of micrographs at 48 h post infiltration from leaves coexpressing the indicated proteins are shown. Images are representative of three separate infiltrations from two independent experiments for each interaction. Bars = 10 μ m. **D**, Western-blot analysis showing expression and localization of 4a/4b/4c/4d in 10 μ g each of membrane (M) or soluble (S) fractions of *N. benthamiana* leaves coexpressing the four nEYFP-fused GmRIN4 isoforms and cEYFP-fused RPG1-B. Proteins were detected using anti-GFP antibodies that cross-react with nEYFP alone.

GmRIN4b Complements the Arabidopsis *rin4* Mutation

To determine if their differing affinities for RPG1-B reflected functional differences in the GmRIN4a and GmRIN4b proteins, we overexpressed the *GmRIN4a* and *GmRIN4b* isoforms in the Arabidopsis *rin4 rps2* mutant. T2 plants containing *GmRIN4a* (*35S-GmRIN4a*) or *GmRIN4b* (*35S-GmRIN4b*) expressed the respective transgenes constitutively (Fig. 4A). Western-blot analysis of total protein extracts from the *35S-GmRIN4a* and *35S-GmRIN4b* lines showed

that these plants also expressed the respective GmRIN4 proteins constitutively (Fig. 4B). The *rin4 rps2* mutant exhibits constitutive *PR-1* accumulation due to residual RPM1 activity (Belkhadir et al., 2004). Therefore, we assessed *PR-1* expression in the transgenic plants as a measure of complementation of the *rin4* mutation. Interestingly, increased *PR-1* expression associated with the *rin4* mutation was suppressed in *35S-GmRIN4b rin4 rps2* but not *35S-GmRIN4a rin4 rps2* transgenics (Fig. 4A). To assess

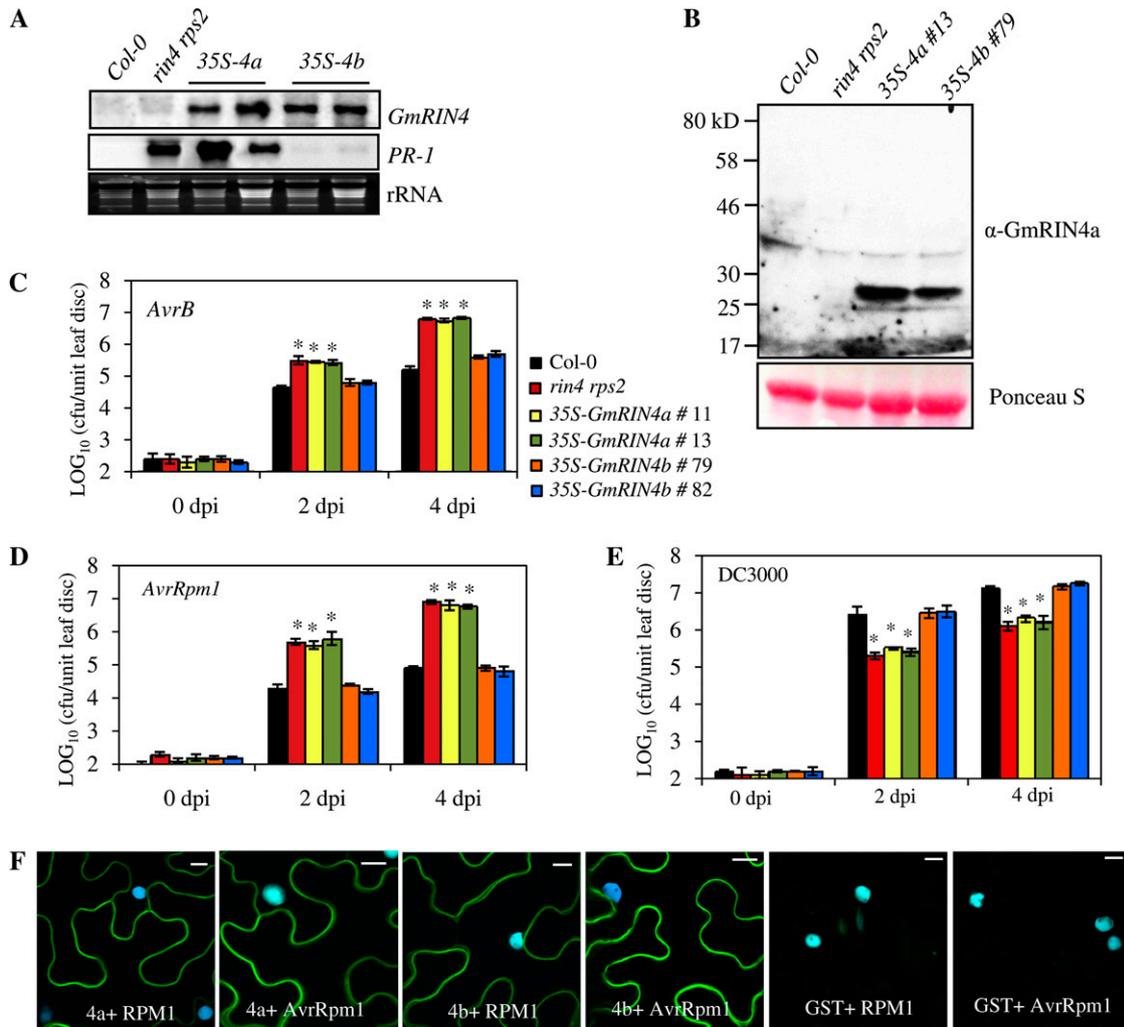


Figure 4. *GmRIN4b* rescues the *rin4* mutation in Arabidopsis. A, Northern-blot analysis showing expression of *GmRIN4a* or *GmRIN4b* and *PR-1* in wild-type (Col-0), *rin4 rps2*, *35S-GmRIN4a* (*35S-4a*), and *35S-GmRIN4b* (*35S-4b*) plants. Ethidium bromide staining of rRNA was used as a loading control. B, Western-blot analysis of protein extracts from the indicated genotypes. GmRIN4a and GmRIN4b proteins in the respective transgenic lines were visualized using GmRIN4a-specific antibodies. Ponceau-S staining of the membrane was used as a loading control. C to E, Response to *AvrB* (C), *AvrRpm1* (D), or DC3000 (E) strains of *P. syringae* in the wild type (Col-0; black bars), a mutant (*rin4 rps2*; red bars), and two transgenic lines each expressing *GmRIN4a* (green and yellow bars) or *GmRIN4b* (orange and blue bars). Bacterial numbers at 0, 2, or 4 d post inoculation (dpi) are presented as log₁₀ values of colony-forming units (cfu) per unit of leaf area. Error bars indicate SD (n = 5). Statistical significance was determined using Student's *t* test. Asterisks denote data significantly different from the wild type (Col-0), where *P* < 0.001 (n = 5). F, BiFC assay showing in planta interactions. CFP and YFP overlay images (40× magnification) of micrographs at 48 h post infiltration from leaves coexpressing the indicated proteins are shown. Images are representative of three separate infiltrations from two independent experiments for each interaction. Bars = 10 μm.

RPM1-mediated defenses, we inoculated wild-type, mutant, and transgenic plants with *AvrB*- or *AvrRpm1*-expressing strains of *P. syringae*. The *rin4 rps2* mutant and 35S-*GmRIN4a rin4 rps2* transgenic plants accumulated approximately 40-fold more *AvrB* bacteria ($P < 0.001$) and approximately 100-fold more *AvrRpm1* bacteria ($P < 0.001$) than the wild-type plants. In contrast, 35S-*GmRIN4b rin4 rps2* plants contained wild-type-like levels of *AvrB* and *AvrRpm1* bacteria (Fig. 4, C and D). We tested several (five to seven each) transgenic lines expressing the *GmRIN4a* or *GmRIN4b* transgene at various levels (high or low). For all lines tested, 35S-*GmRIN4a* lines always continued to show phenotypes similar to the *rin4 rps2* mutant, while the 35S-*GmRIN4b* lines behaved like wild-type plants (data not shown). This suggested that complementation of the Arabidopsis *rin4* mutant was not associated with levels of the *GmRIN4b* transcript.

The *rin4 rps2* mutant also exhibits enhanced resistance to virulent bacteria (Belkhadir et al., 2004; Kim et al., 2005b). Therefore, we next challenged the 35S-*GmRIN4b rin4 rps2* and 35S-*GmRIN4a rin4 rps2* transgenic plants with a virulent strain (DC3000) of *P. syringae*. Consistent with their response to avirulent *P. syringae*, the 35S-*GmRIN4a rin4 rps2* plants behaved similar to *rin4 rps2* mutant plants and exhibited enhanced resistance to virulent *P. syringae*. Both *rin4 rps2* and 35S-*GmRIN4a rin4 rps2* plants accumulated approximately 30-fold fewer bacteria than wild-type plants ($P < 0.001$). In comparison, the 35S-*GmRIN4b rin4 rps2* transgenic plants showed a wild-type-like response (Fig. 4E). Together, these results suggest that *GmRIN4b*, but not *GmRIN4a*, was able to compensate for the loss of *AtRIN4*.

We next tested if the ability of *GmRIN4b* or the inability of *GmRIN4a* to complement the Arabidopsis *rin4* mutation was associated with their respective abilities to bind the corresponding R protein, RPM1. In planta binding between *GmRIN4a* or *GmRIN4b* and full-length RPM1 as well as *AvrRpm1* was tested using EYFP-fused proteins in BiFC assays. Fluorescence was detected when RPM1 was coexpressed with either *GmRIN4a* or *GmRIN4b* (Fig. 4E). This indicated that both *GmRIN4a* and *GmRIN4b* interacted with RPM1 in planta. Similarly, *GmRIN4a* and *GmRIN4b* also interacted with *AvrRpm1* in planta (Fig. 4F). These data suggest that the inability of *GmRIN4a* to complement *AtRIN4* function is not associated with its ability to interact with RPM1 or *AvrRPM1*.

GmRIN4a and GmRIN4b Are Required for RPG1-B-Mediated Resistance in Soybean

We next tested the functional requirements for *GmRIN4a* and *GmRIN4b* in RPG1-B-derived resistance signaling in soybean. Silencing *GmRIN4a* or *GmRIN4b* in plants (cv Harosoy) containing *RPG1-B* was achieved using the bean pod mottle virus (BPMV)-based vector (Zhang and Ghabrial, 2006; Kachroo et al., 2008; Fu et al., 2009). Plants were inoculated

with recombinant vectors targeting *GmRIN4a* (S_{4a}) or *GmRIN4b* (S_{4b}). Similar expression of all four *GmRIN4* isoforms observed in vector (V)- or mock (M)-inoculated plants suggested that BPMV infection did not alter *GmRIN4* expression (Fig. 5A). Plants inoculated with S_{4a} or S_{4b} were silenced for the *GmRIN4a* or *GmRIN4b* isoform, respectively, but continued to express other *GmRIN4* isoforms at basal levels (Fig. 5A). Unlike the Arabidopsis *rin4* mutant, the S_{4a} or S_{4b} plants did not exhibit ectopic *PR1a* expression (Fig. 5B). Inoculation with an avirulent (*AvrB*) strain of *P. syringae* pv *glycinea* race 4 showed that S_{4a} and S_{4b} plants containing *RPG1-B* were significantly more susceptible than V plants, supporting 15- and 10-fold more growth

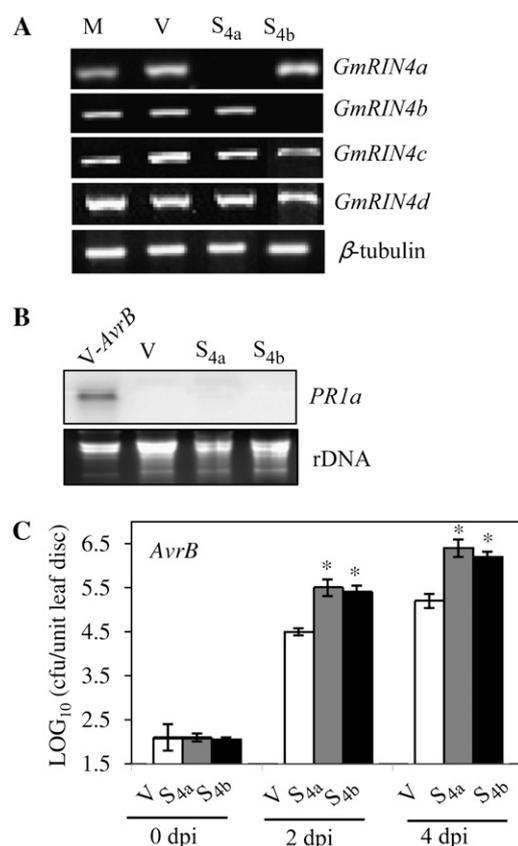


Figure 5. *GmRIN4a* and *GmRIN4b* are required for *RPG1-B*-mediated resistance signaling. A, RT-PCR analysis showing expression of the *GmRIN4* isoforms in mock/vector (M/V)-inoculated and *GmRIN4a* (S_{4a})- or *GmRIN4b* (S_{4b})-silenced plants. β -Tubulin levels were used as an internal control for cDNA amounts. B, Northern-blot analysis showing expression of *PR1a* in V, S_{4a} , or S_{4b} plants or V plants inoculated with an avirulent (*AvrB*) strain of *P. syringae* pv *glycinea* (V-*AvrB*; 24 h). Ethidium bromide staining of rRNA was used as a loading control. C, Bacterial counts in *RPG1-B* (cv Harosoy) S_{4a} (gray bars) or S_{4b} (black bars) plants as compared with V (white bars) plants. Plants were infiltrated with *AvrB* bacteria. Log_{10} values of colony-forming units (cfu) per unit of leaf area from infected leaves at 0, 2, or 4 d post inoculation (dpi) are presented. Error bars indicate sd ($n = 5$). Statistical significance was determined using Student's *t* test. Asterisks denote data significantly different from control (V), where $P < 0.001$.

($P < 0.001$) of *AvrB* bacteria, respectively (Fig. 5C). Thus, *RPG1-B*-mediated resistance to *AvrB*-expressing *P. syringae* requires both *GmRIN4a* and *GmRIN4b*, even though *RPG1-B* does not interact with *GmRIN4a*.

GmRIN4b Interacts with Itself as Well as with GmRIN4a, GmRIN4c, and GmRIN4d

The fact that *GmRIN4a* was required for *RPG1-B*-mediated resistance to *AvrB*-expressing bacteria even though it did not interact with *RPG1-B* raised the possibility that the *GmRIN4* proteins might function as a heteromeric complex. Indeed, Y2H analysis showed interactions between the *GmRIN4a* and *GmRIN4b* and between the *GmRIN4b* and *GmRIN4c* proteins (Fig. 6A). However, no interactions were detected for *GmRIN4a* with *GmRIN4c* or *GmRIN4d* or for *GmRIN4b* with *GmRIN4d*. Furthermore, only *GmRIN4b* was able to self-interact in Y2H assays (Fig. 6A). In vitro pull-down assays and in planta BiFC assays were used to confirm the Y2H interactions. For pull-down assays between *GmRIN4a* and *GmRIN4b*, *GmRIN4c*, or *GmRIN4d*, *GmRIN4a*-His was immobilized on Ni-NTA and incubated with *GmRIN4b*-Myc, *GmRIN4c*-GST, or *GmRIN4d*-GST. For pull-down assays between *GmRIN4b* and *GmRIN4c* or *GmRIN4d*, *GmRIN4b*-Myc-His was immobilized on Ni-NTA and incubated with the GST-tagged *GmRIN4c* or *GmRIN4d*. Bound proteins were visualized by immunoblot analysis using Myc- or GST-specific antibodies, as appropriate. *GmRIN4b* lacking a His tag, *GmRIN4c*-GST, or *GmRIN4d*-GST did not bind nonspecifically to Ni-NTA lacking bait proteins (Fig. 6B, - lanes) or Ni-NTA preincubated with protein extracts from cells expressing the empty pET28A (vector used for expressing *GmRIN4a*) or pET30A (vector used for expressing *GmRIN4b*) plasmids (Supplemental Fig. S3A). Pull-down assays showed that *GmRIN4b* interacted with itself and with *GmRIN4c* or *GmRIN4d* (Fig. 6B). Consistent with Y2H and pull-down assays, BiFC showed binding between *GmRIN4b* and *GmRIN4a* or *GmRIN4c* but not between *GmRIN4a* and *GmRIN4c* or *GmRIN4d* (Fig. 6C). In addition, except for *GmRIN4b*, other *GmRIN4* isoforms did not self-interact even though all proteins were expressed at comparable levels (Fig. 6C; Supplemental Fig. S4, A and B). However, in contrast to Y2H assay, both pull-down and BiFC assays showed binding between *GmRIN4b* and *GmRIN4d*. As before, all four *GmRIN4* proteins were localized to the membrane fraction (data not shown). Together, these data showed that the *GmRIN4b* protein interacts with itself and the other three *GmRIN4* isoforms.

GmRIN4a and GmRIN4b Mediate Basal Resistance in Soybean

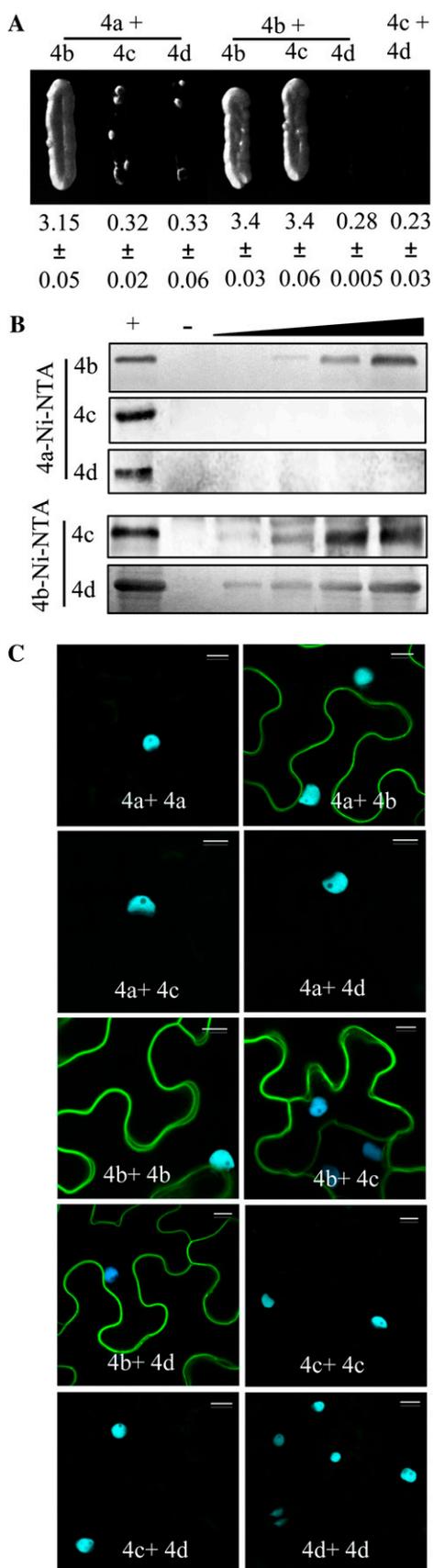
The *RIN4* protein also mediates basal defense in Arabidopsis (Kim et al., 2005b). Therefore, we next tested basal resistance in *S_{4a}* and *S_{4b}* plants to virulent

(vir) strains of *P. syringae* pv *glycinea* race 4 and the oomycete *P. sojae*. *GmRIN4a* (*S_{4a}*) or *GmRIN4b* (*S_{4b}*) was silenced in the Essex cultivar (lacking *RPG1-B*). Silencing of the respective genes was confirmed using reverse transcription (RT)-PCR analysis as described above (data not shown). All four *GmRIN4* isoforms were expressed to similar levels in V and M plants. Thus, BPMV infection did not alter *GmRIN4* expression in Essex plants, similar to that observed in Harosoy plants (data not shown). Essex (*rpg1-b*) V, *rpg1-b S_{4a}*, and *rpg1-b S_{4b}* plants were inoculated with vir bacteria. Both *rpg1-b S_{4a}* and *rpg1-b S_{4b}* plants consistently accumulated approximately 2-fold reduced levels of vir bacteria in comparison with V plants (Fig. 7A). Likewise, *rpg1-b S_{4a}* and *rpg1-b S_{4b}* plants also showed significantly enhanced basal resistance ($P < 0.001$) to virulent *P. sojae* than V plants; at 9 d, approximately 60% of the *S_{4a}* or *S_{4b}* plants survived infection versus none of the control plants (Fig. 7B). Together, these results suggest that *GmRIN4a* and *GmRIN4b* contribute to basal resistance to virulent pathogens in soybean.

Since *AvrB* enhances virulence on plants lacking *RPG1-B* (Ashfield et al., 1995; Ong and Innes, 2006), we next tested the response of the *rpg1-b S_{4a}* and *rpg1-b S_{4b}* plants to bacteria expressing *AvrB*. The *AvrB*-expressing strain of *P. syringae* was consistently 2-fold more virulent in *rpg1-b* V plants than the vir strain ($P < 0.0001$; Fig. 7A). *AvrB* bacteria also exhibited enhanced virulence in the *rpg1-b S_{4b}* plants, consistently accumulating to 2-fold greater levels than the vir bacteria ($P < 0.0001$; Fig. 7A). In contrast, *AvrB* bacteria showed more pronounced virulence in the *rpg1-b S_{4a}* plants, accumulating to levels approximately 4-fold higher than vir bacteria in *rpg1-b* V plants ($P < 0.001$). These levels were also 2-fold higher than the *AvrB* bacteria in *rpg1-b* V plants ($P < 0.05$). Together, these results suggest that *GmRIN4a* negatively regulates the virulence of *AvrB* bacteria on plants lacking *RPG1-B*.

DISCUSSION

We demonstrate that the soybean R protein *RPG1-B* recruits *RIN4*-like proteins for resistance signaling against *AvrB*-expressing *P. syringae*, similar to its non-orthologous counterpart, *RPM1* in Arabidopsis. The Arabidopsis *RIN4* protein is thought to serve as a guard for *RPM1*, which presumably monitors the *AvrB*-mediated phosphorylation of *RIN4* to induce resistance signaling. The lack of sequence conservation between *RPM1* and *RPG1-B* indicates independent evolution of the two R proteins, in spite of their shared specificity for the same avirulence effector (Bisgrove et al., 1994; Grant et al., 1995; Ashfield et al., 2004). This is supported by the differences in their requirements for signaling components. For example, *RPM1* but not *RPG1-B* requires *HSP90*, whereas *RPG1-B* but not *RPM1* requires *SGT1* for resistance signaling (Austin et al., 2002; Hubert et al., 2003; Fu et al.,



2009). However, as we show here, RPM1 and RPG1-B do share their requirements for RIN4. Unlike in Arabidopsis, four AvrB-interacting GmRIN4 proteins are present in soybean, and three of these also interact with RPG1-B. Interestingly, in spite of their low sequence conservation, both RPM1 and RPG1-B interact with the respective Arabidopsis and soybean RIN4 proteins via their N-terminal CC domains (Mackey et al., 2002; this work). In soybean, more than one RIN4-like protein mediates effector-triggered immunity to *AvrB*-expressing *P. syringae*, since the absence of either GmRIN4a or GmRIN4b abrogates RPG1-B-derived resistance. The presence of multiple RIN4 proteins in soybean and the fact that more than one of them is required for RPG1-B-mediated resistance raises the possibility that multiple RIN4-like proteins may also be present in other genomes. Indeed, multiple RIN4-like proteins are reported in both tomato and lettuce species (Jeuken et al., 2009; Luo et al., 2009). Notably, the Arabidopsis genome also encodes several other as yet uninvestigated proteins with significant similarities to AtRIN4 (At3g25070), raising the possibility that some of these too might function in defense signaling.

Of particular interest is the fact that GmRIN4a is required for RPG1-B-mediated resistance even though this protein does not directly interact with RPG1-B. Moreover, GmRIN4a is unable to complement the Arabidopsis *rin4* mutation even though it interacts with RPM1. Thus, the functioning of RIN4-like proteins in R-derived resistance does not rely on direct interactions with the R protein. However, GmRIN4a does interact with GmRIN4b, which in turn interacts with RPG1-B. Thus, the requirement for GmRIN4a in RPG1-B-derived resistance signaling could be because GmRIN4 proteins function as a heteromeric complex. GmRIN4b also interacts with two other RIN4 isoforms, GmRIN4c and GmRIN4d. It is likely that GmRIN4c and GmRIN4d also function in RPG1-B-derived resistance, since both interact with AvrB,

Figure 6. GmRIN4 isoforms (GmRIN4a to GmRIN4d = 4a to 4d) interact with each other. A, Y2H assay showing interactions between the various GmRIN4 proteins. Growth on His⁻Trp⁻Leu⁻ plates is shown. Numbers indicate β -galactosidase activity \pm SD ($n = 3$). B, Pull-down assays showing in vitro binding between the GmRIN4 proteins. For pull-down assay between 4a and 4b, 4c, or 4d, 4a was immobilized on Ni-NTA and incubated with increasing amounts (0.1–1 μ g) of 4b, 4c, or 4d. The His tag was cleaved from 4b before application to a 4a affinity column. For interactions between 4b and 4c or 4d, 4b was immobilized on Ni-NTA and incubated with increasing amounts (0.1–1 μ g) of 4c or 4d. Purified 4b, 4c, or 4d was loaded as a positive control (+) and incubated with empty Ni-NTA/glutathione agarose as a negative control (–) on respective gels. Proteins were visualized by immunoblot analysis using anti-Myc (4b) or anti-GST (4c and 4d) antibodies. C, BiFC assay showing in planta interactions. CFP and YFP overlay images (40 \times magnification) of micrographs at 48 h post infiltration from leaves coexpressing the indicated proteins are shown. Images are representative of three separate infiltrations from two independent experiments for each interaction. Bars = 10 μ m.

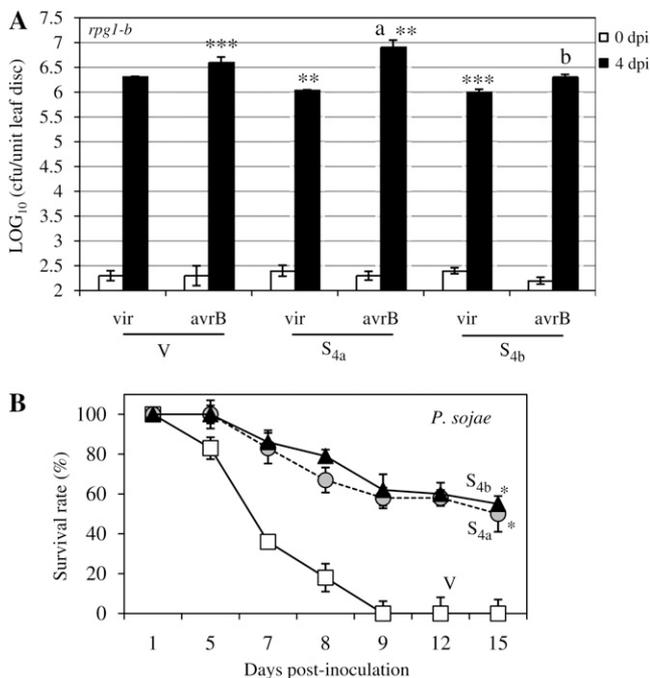


Figure 7. GmRIN4a and GmRIN4b mediate basal immunity in soybean. **A**, Bacterial counts in *rpg1-b* (cv Essex) plants silenced for GmRIN4a (S_{4a}) or GmRIN4b (S_{4b}) and those inoculated with the empty silencing vector (V). Plants were infiltrated with virulent (vir) or avirulent (avrB) strains of *P. syringae* pv *glycinea*. Log₁₀ values of colony-forming units (cfu) per unit of leaf area from infected leaves at 0 d post inoculation (dpi; white bars) or 4 dpi (black bars) are presented. Error bars indicate SD ($n = 5$). Student's *t* test was used to determine statistical significance. Asterisks denote significant differences from V plants inoculated with vir bacteria as follows: ** $P < 0.001$, *** $P < 0.0001$. Significant difference between avrB-inoculated V and S_{4a} plants is denoted by a, where $P < 0.05$. Significant difference between vir and avrB-inoculated S_{4b} plants is denoted by b, where $P < 0.0001$. **B**, Percentage survival of V (white squares), S_{4a} (gray circles), or S_{4b} (black triangles) plants at 1, 5, 7, 8, 9, 12, and 15 d post inoculation with *P. sojae*. Error bars indicate SD ($n = 3$). Asterisks denote significant differences from V, where $P < 0.001$, as determined using Student's *t* test. Results are representative of three independent experiments.

RPG1-B, and GmRIN4b. However, silencing the GmRIN4c and GmRIN4d isoforms separately (without altering the expression of the other three isoforms) to test their individual functions in RPG1-B-mediated resistance has proven challenging.

A knockout mutation in RIN4 is lethal, while anti-sense reduction of RIN4 expression constitutively activates defense responses due to the ectopic induction of a second R protein, RPS2, in Arabidopsis (Mackey et al., 2002, 2003). Arabidopsis RIN4 also prevents the inappropriate activation of RPM1, because the absence of RIN4 enhances basal defense to virulent *P. syringae* (Belkhadir et al., 2004). This enhanced basal resistance and increased PR-1 expression in the *rin4 rps2* double mutant plants were attributed to residual RPM1 activity, since both phenotypes are absent in the *rin4 rps2 rpm1* triple mutant (Belkhadir et al., 2004). The soybean cv Harosoy (RPG1-B) did not exhibit constitutive

induction of defense responses in response to silencing of GmRIN4a or GmRIN4b, suggesting that silencing GmRIN4a or GmRIN4b does not affect the activities of other R proteins in this cultivar. Furthermore, unlike RPM1 in Arabidopsis, silencing GmRIN4a or GmRIN4b does not inappropriately induce RPG1-B activity; the RPG1-B GmRIN4a- or GmRIN4b-silenced plants do not exhibit enhanced resistance to virulent bacteria (data not shown) and express basal levels of PR genes. However, silencing GmRIN4a or GmRIN4b in *rpg1-b* plants (cv Essex) does confer increased resistance to virulent bacteria and *P. sojae*. Strikingly, the GmRIN4a- or GmRIN4b-silenced *rpg1-b* plants showed much better resistance to virulent *P. sojae* than to *P. syringae*. The nominal increase in resistance to virulent *P. syringae* might result from derepression of basal immunity, albeit only in cv Essex. Alternatively, the various GmRIN4 proteins might function additively in basal defense to *P. syringae*, such that absence of all or multiple isoforms is required for the induction of a more robust response. Analyzing basal defense in plants simultaneously silenced for multiple GmRIN4 isoforms would help address this. The more pronounced resistance to *P. sojae* might be associated with the ectopic activation of other unidentified R/R-like protein(s) in cv Essex that are specifically active against *P. sojae*.

Unlike their increased resistance to DC3000, *rin4 rps2* plants do not exhibit enhanced resistance to *P. syringae* expressing AvrB or AvrRPM1 (Belkhadir et al., 2004). Likewise, *rin4 rps2 rpm1* triple mutant plants accumulate similar levels of bacteria or induce chlorosis like the *rps2 rpm1* double mutant plants in response to AvrRPM1- or AvrB-expressing strains of the weak pathogen *P. syringae* pv *maculicola* M6CAE, respectively (Nimchuk et al., 2000; Rohmer et al., 2003; Belkhadir et al., 2004). Thus, although RIN4 is a negative regulator of immunity, it is not required for the virulence activities of either AvrRPM1 or AvrB in Arabidopsis (Nimchuk et al., 2000; Belkhadir et al., 2004). This is also true for the two GmRIN4 proteins tested here; both GmRIN4a- and GmRIN4b-silenced *rpg1-b* plants support more growth of AvrB-expressing *P. syringae* than the virulent strain. Interestingly, however, GmRIN4a-silenced *rpg1-b* plants consistently accumulate higher levels of AvrB bacteria than the control plants, suggesting that GmRIN4a negatively regulates the virulence function of AvrB. Thus, the virulence activity of AvrB is enhanced in plants lacking both the R protein and GmRIN4a. A likely possibility is that GmRIN4a serves as a decoy to mimic AvrB interaction with its target and thereby limit pathogen fitness. This scenario is in agreement with the proposed function for decoy proteins that might serve to both perceive and compete for binding with the effector (van der Hoorn and Kamoun, 2008). A second possibility is that the Essex background contains another R protein with specificity for AvrB and that GmRIN4a but not GmRIN4b is required for the function of this unknown R protein. Indeed, besides

RPM1, the Arabidopsis TIR-NBS-LRR protein TAO1 also exhibits AvrB specificity but does not require RIN4 for its function (Eitas et al., 2008). Comparing pathogen fitness in *RPG1-b* and *rpm1-b* plants silenced for either *GmRIN4a* or *GmRIN4b* versus those silenced for both *GmRIN4a* and *b* simultaneously could help resolve the functions of the encoded proteins and their relative contributions to the perception of AvrB and the virulence activity of AvrB.

MATERIALS AND METHODS

Plant Growth Conditions

Soybean (*Glycine max* 'Harosoy' [*RPG1-B*] and 'Essex' [*rpm1-b*]) were grown in the greenhouse with day and night temperatures of 25°C and 20°C, respectively. For silencing experiments, inoculation of recombinant BPMV vectors and confirmation of silencing were carried out as described before (Kachroo et al., 2008). Arabidopsis (*Arabidopsis thaliana*) plants (wild type or *rln4 rps2* mutant in the Columbia [Col-0] ecotype) were grown in MTPS 144 Conviron walk-in chambers at 22°C, 65% relative humidity, and 14-h photoperiod.

Y2H Assays

Y2H assays were carried out using standard protocols for the GAL4 system (Clontech). Interactions were tested in the yeast strain Y190, with AvrB or RPG1-B (N-terminal 187 amino acids) expressed from the GAL4 bait (pGADT7) plasmid and GmRIN4 proteins expressed from the prey (pGBKT7) plasmid.

In Vitro Binding Assays

The AvrB, GmRIN4a, GmRIN4b, and RPG1-B (CC domain, N-terminal 187 amino acids) proteins were expressed as His-tagged fusions in *Escherichia coli* (AvrB-pET28A, GmRIN4a-, and RPG1-B-pET30). For GmRIN4b, a Myc tag was also added at the C terminus (pBAD). GmRIN4c and GmRIN4d were expressed as GST fusion proteins in *E. coli* (pGEX-5X). Proteins were purified using Ni-NTA (His) and glutathione Sepharose (GST) affinity chromatography. For binding between AvrB and GmRIN4a, -b, -c, -d, or RPG1-B, the His tag was removed from AvrB using thrombin. To ensure complete cleavage of the His tag, 0.5 µg of the cleaved AvrB was applied to empty Ni-NTA. Lack of nonspecific retention was assessed by immunoblot analysis using AvrB-specific antibodies. One microgram each of GmRIN4a-His, GmRIN4b-His, and RPG1-B-His was immobilized on Ni-NTA and GmRIN4c-GST or GmRIN4d-GST immobilized on glutathione agarose. The immobilized proteins were incubated with increasing amounts (50, 100, 250, and 500 ng) of AvrB. For pull down between GmRIN4b, GmRIN4c, or GmRIN4d and RPG1-B, the His tag was cleaved from GmRIN4b using thrombin, and nonretention of the cleaved protein on empty Ni-NTA or Ni-NTA bound to protein extracts from cells expressing the empty pET28A/pET30A vectors was assessed using Myc-specific antibodies. One microgram of RPG1-B-His was immobilized on Ni-NTA and incubated with 100, 250, and 500 ng and 1 µg of GmRIN4b-Myc (His tag removed), GmRIN4c-GST, or GmRIN4d-GST. For pull-down assay between GmRIN4a and GmRIN4b, GmRIN4c, or GmRIN4d, GmRIN4a-His was immobilized on Ni-NTA incubated with increasing amounts (0.1–1 µg) of GmRIN4b-Myc (His tag removed), GmRIN4c-GST, or GmRIN4d-GST. For pull-down between GmRIN4b and GmRIN4c or GmRIN4d, GmRIN4b-Myc-His was immobilized on Ni-NTA and incubated with increasing amounts (0.1–1 µg) of GmRIN4c-GST or GmRIN4d-GST. Purified proteins were loaded as positive controls (+) and incubated with empty Ni-NTA/glutathione Sepharose as negative controls (–) on the respective gels. Purified proteins were also incubated with Ni-NTA preincubated with extracts from cells expressing the empty pET28A/pET30A vectors or with glutathione Sepharose (GE Healthcare) preincubated with extract from cells expressing the empty pGEX-5X vector. Affinity columns were washed extensively with sodium phosphate buffer (0.01 M, pH 7.0, 150 mM NaCl, 10 mM imidazole) to remove unbound proteins, and immunoblot analysis of bound proteins was carried out using anti-AvrB, anti-Myc (GmRIN4b), and anti-GST (GmRIN4c and GmRIN4d) antibodies. Results presented are representative of two to three

independent binding assays using proteins from two separate preparations. Approximate protein molecular masses are as follows: AvrB (36 kD), RPG1-B_{CC} (20.68 kD), GmRIN4a (27.12 kD), GmRIN4b (27.08 kD), GmRIN4c-GST (52.66 kD), and GmRIN4d-GST (53.51 kD).

BiFC Assays

BiFC assays were carried out as described before (Martin et al., 2009). Briefly, the various proteins were fused to the N/C-terminal halves of EYFP (nEYFP/cEYFP) using the pSITE-BiFC-C1cec/nec (EYFP) vectors (Martin et al., 2009) and introduced in *Agrobacterium tumefaciens* strain C58C1. *A. tumefaciens* strains were then infiltrated into CFP-H2B-tagged *Nicotiana benthamiana* plants (transgenic plants expressing nucleus-localized CFP). At 48 h later, water-mounted sections of leaf tissue were examined by confocal microscopy using a water-immersion PLAPO60XWLSM (numerical aperture, 1.0) objective on a FV1000 point-scanning/point-detection laser scanning confocal microscope (Olympus) equipped with lasers spanning the spectral range of 405 to 633 nm. CFP and YFP overlay images (40× magnification) were acquired at a scan rate of 10 ms pixel⁻¹. Olympus FLUOVIEW 1.5 was used to control the microscope, image acquisition, and the export of TIFF files. All proteins were expressed as both nEYFP and cEYFP fusions, and all interactions were tested using both combinations of n/cEYFP-fused reciprocal proteins. Results presented are representative of three separate infiltrations for every combination tested. All interactions (positive and negative) were tested at least twice.

Plant Protein Extraction and Immunoblot Analysis

Proteins were extracted in buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 10 mM MgCl₂, 5 mM EDTA, 5 mM dithiothreitol (DTT), and 1× protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured by the Bio-Rad protein assay. For Ponceau-S staining, polyvinylidene difluoride membranes were incubated in Ponceau-S solution (40% methanol [v/v], 15% acetic acid [v/v], and 0.25% Ponceau-S). The membranes were destained using deionized water. Membrane proteins were extracted using buffer containing 50 mM Tris-MES, pH 8.0, 0.5 M Suc, 1 mM MgCl₂, 10 mM EDTA, 10 mM EGTA, 10 mM ascorbic acid, 5 mM DTT, and 1× protease inhibitor cocktail. Total protein extracts were centrifuged at 10,000g followed by a second centrifugation at 125,000g. The microsomal fractions were suspended in a buffer containing 5 mM potassium phosphate, pH 7.8, 2 mM DTT, 1× protease inhibitor cocktail, and 1% Triton X-100. Proteins (30–50 µg) were fractionated on a 7% to 10% SDS-PAGE gel and subjected to immunoblot analysis using anti-TCV-CP or anti-GFP antibody. Approximate protein molecular masses were as follows: AvrB-nEYFP (53 kD), RPG1-B_{CC}-nEYFP (37.67 kD), GmRIN4a-nEYFP (44.12 kD), GmRIN4b-nEYFP (44.08 kD), GmRIN4c-nEYFP (43.66 kD), GmRIN4d-nEYFP (44.5 kD), GST-nEYFP (43 kD), Avr1b-nEYFP (32.7 kD), GST-cEYFP (33 kD), and TCV-CP-cEYFP (45 kD).

Construction of Viral Vectors, In Vitro Transcription, and Plant Inoculation

Generation of silencing vectors, in vitro transcription, and rub inoculation of soybean leaves was carried out as before (Kachroo et al., 2008). A 159-bp fragment (Gly-184 to Gln-236) of GmRIN4a and a 243-bp fragment (Thr-59 to Pro-139) of GmRIN4b were used to generate vectors targeting *GmRIN4a* and *GmRIN4b*, respectively.

RNA Extraction, Northern Blot, and RT-PCR Analysis

RNA from leaf tissues of soybean plants at the V1/V2 growth stage was extracted using the Trizol reagent (Invitrogen) per the manufacturer's instructions. Northern-blot analysis and synthesis of randomly primed probes were as described before (Kachroo et al., 2001). RT and first-strand cDNA synthesis were carried out using SuperScript II (Invitrogen). Two to three independent RNA preparations were analyzed at least twice by RT-PCR using a reduced number of cycles (15–20) for evaluating relative differences in transcript levels.

Trypan Blue Staining and Pathogen Inoculations

Trypan blue staining of *N. benthamiana* leaves was performed as described (Chandra-Shekhara et al., 2006). *Pseudomonas syringae* inoculation of soybean and monitoring of bacterial proliferation were carried out as described before

(Fu et al., 2009). Mock inoculations were carried out with 10 mM MgCl₂ in 0.04% Silwett L-77. Experiments were repeated three to four times. For Arabidopsis inoculations, bacterial suspensions in 10 mM MgCl₂ were infiltrated to the abaxial surface of leaves using a 1-mL needleless syringe. After inoculations, plants were transferred to a Conviron PGV36 walk-in chamber maintained at 22°C with 65% relative humidity and a 10-h photoperiod. For soybean *Phytophthora sojae* inoculations, race 1, strain P6497 was grown on V8 agar at 25°C in the dark. *P. sojae* infections were carried out as described before (Kachroo et al., 2008). Experiments were repeated three times with 15 to 20 plants tested per wild-type or silenced line per experiment.

Plant Transformation

Full-length cDNAs corresponding to *GmRIN4a* or *GmRIN4b* under the control of a double 35S promoter and the 35S terminator were cloned into the binary vector pBAR1. Constructs were introduced into *A. tumefaciens* strain MP90 by electroporation and transformed into Arabidopsis using vacuum infiltration. Transgenic seeds were selected on soil sprayed with the herbicide BASTA.

The sequences described have been submitted to the GenBank database (accession nos. GU132851–GU132854).

Supplemental Data

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

Supplemental Figure S1. AvrB-induced cell death and controls for AvrB pull-down and BiFC assays.

Supplemental Figure S2. GmRIN4 proteins do not interact with GST, TCV-CP, or Avr1b in BiFC assays.

Supplemental Figure S3. Controls for GmRIN4 pull-down and BiFC assays.

Supplemental Figure S4. GmRIN4 isoforms interact with each other.

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