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Rpp2 Encodes a TIR-NBS-WH-LRR Protein that Confers Resistance to *Phakopsora pachyrhizi* in Soybean

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The obligate biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd., the causal agent of soybean rust, is among the most formidable pathogens of soybean (*Glycine max* [L.] Merr.). The pathogen is now established in all major soybean-growing areas of the world and presents a significant impediment to global soybean production. Most soybean germplasm is susceptible, enabling the fungus to penetrate and colonize the leaf tissue, causing tan-colored necrotic lesions to form at the site of infection. Severe infection reduces photosynthesis and causes premature defoliation, which ultimately decreases crop yield and seed quality. Eight genetic loci, *Rpp1/Rpp1b* to *Rpp7* and *Rpp6907*, that confer race-specific resistance to *P. pachyrhizi* (*Rpp*) have

been identified. *Rpp2* was identified and characterized in the soybean accession PI 230970 and fine-mapped to a 188.1-kb interval on chromosome 16, a region predicted to contain several toll/interleukin-1 receptor nucleotide-binding leucine-rich repeat (TIR-NLR) genes. To identify *Rpp2*, we constructed a bacterial artificial chromosome (BAC) library from the resistant soybean accession PI 230970. Sequencing BACs that span the *Rpp2* locus identified 14 candidate genes with homology to the TIR-NLR family of resistance genes. Of these, seven are predicted to encode full-length R proteins. Co-silencing the *Rpp2* candidate genes compromised resistance in soybean accession PI 230970. Gene expression analysis suggests that a single gene, *Rpp2C7_PI*, which shares the greatest homology to *Rpp2C6_Wms82* (Glyma.16G136600) in the Williams 82 reference genome, is responsible for *Rpp2*-mediated resistance.

Keywords: disease resistance, soybean, soybean rust, VIGS

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Soybean rust (SBR), caused by the fungal pathogen *Phakopsora pachyrhizi* Syd. & P. Syd., is one of the most economically impactful diseases affecting global soybean (*Glycine max* (L.) Merrill) production. *P. pachyrhizi* is an aggressive pathogen capable of causing yield losses of up to 90% under conditions that favor disease development (Hartman et al. 2015). The fungus thrives in the tropical and subtropical regions of Asia, Africa, and South America, and it became a cause for concern for growers in the United States upon its arrival in 2004 (Schneider et al. 2005). Unlike other rusts, which typically have narrow host ranges, *P. pachyrhizi* has been found to infect at least 31 legume species in the field. These include kudzu (*Pueraria montana*), a fast-growing invasive legume species prevalent across the southeastern United States, which enables to *P. pachyrhizi* to survive annually in the absence of soybean (Koenning et al. 2007; Ono et al. 1992). Infection of kudzu and other wild hosts also serves as a potential source of inoculum that can be transferred via wind dispersal of spores to northern states during the soybean growing season.

Because nearly all commercially grown cultivars of soybean are susceptible to *P. pachyrhizi*, effective control measures are required to combat SBR in high-risk areas. Although fungicides can be effective, they significantly add to production costs and

typically need to be applied multiple times per growing season for adequate control, depending on the timing of disease onset and disease pressure (Godoy 2012). Heavy use of some classes of fungicides has also led to the development of fungicide-resistant *P. pachyrhizi* strains (Aguiar et al. 2016; Dalla Lana et al. 2018; Godoy et al. 2016; Klosowski et al. 2016). Genetic resistance offers a more sustainable means of management by reducing grower costs and possibly extending the efficacy of fungicides.

P. pachyrhizi is primarily a foliar pathogen, and susceptible plants develop a “TAN” infection phenotype, characterized by the development of tan-colored lesions at the point of infection as the fungus colonizes the leaf tissue (Bromfield 1984). TAN lesions yield several fungal reproductive structures, called uredinia, that produce numerous asexual urediniospores. Germplasm screening efforts have identified two SBR resistance phenotypes (Bromfield 1984; Bromfield and Hartwig 1980). The most common type of SBR resistance is an incomplete resistance that results in fewer uredinia and less sporulation than fully susceptible plants. A defining characteristic of this partial resistance is the development of red-brown lesions (Bonde et al. 2006). The other, less common, form of SBR resistance is the immune response (IR), in which the host is able to completely restrict *P. pachyrhizi* growth (Bonde et al. 2006). The IR typically lacks macroscopic symptoms, but occasionally, light tan flecks can be detected at the point of attempted infection.

Both the red-brown phenotype and IR are types of hypersensitive responses governed by the products of soybean *resistance* (*R*) genes that presumably recognize, either directly or indirectly, *P. pachyrhizi* effector proteins secreted into the host during infection. Breeding for SBR resistance typically focuses on nucleotide-binding leucine-rich repeat genes (NLRs) that confer *Resistance to P. pachyrhizi* (*Rpp*). However, the use of single dominant *R* genes in soybean cultivars results in high genetic pressure on *P. pachyrhizi* populations. Coupled with the extreme numbers of urediniospores that can be produced on a single plant, virulent pathogen populations are common even on soybean lines carrying *R* genes. As a result, *Rpp* genes are only effective against specific populations of *P. pachyrhizi*, which may change year over year even in a given location (Kato 2017). Recent efforts to breed for more durable resistance have included gene pyramiding, in which multiple *Rpp* genes are bred into a given line. Combining multiple sources of resistance is a promising strategy for introducing more effective and more durable resistance (Panho et al. 2022; Yamanaka et al. 2013, 2015).

Currently, eight *Rpp* loci have been described: *Rpp1/Rpp1b*, *Rpp4*, *Rpp6*, and *Rpp6907*, all on chromosome Gm18; *Rpp2* on chromosome Gm16; *Rpp3* on chromosome Gm06; *Rpp5* on chromosome Gm03; and *Rpp7* on chromosome Gm19 (Chen et al. 2015; Chicowski et al. 2024; Hao et al. 2024). Of these, only *Rpp1*, *Rpp3*, *Rpp4*, and *Rpp6907* have had their causal *NLR* genes identified (Bish et al. 2024; Hao et al. 2024; Meyer et al. 2009; Pedley et al. 2019). *Rpp2* is of particular interest in the United States, as this gene is still an effective mode of resistance. Originating from plant introduction (PI) 230970, *Rpp2* was fine-mapped to a 188.1-kb interval on chromosome 16, which was predicted to contain 10 genes encoding toll/interleukin-1 receptor (TIR) NLR proteins in the Williams 82 (Glyma.Wm82.a2.v1) genome assembly (Hartwig and Bromfield 1983; Silva et al. 2008; Yu et al. 2015). Williams 82 carries no *Rpp* genes. Further, two KASP markers associated with the *Rpp2* locus were identified to assist in marker-selection breeding programs utilizing this source of resistance (Meira et al. 2023).

Virus-induced gene silencing (VIGS) has proven to be a viable method for identification of causal *R* genes in soybean (Liu et al. 2015), particularly for genes in complex loci with several duplications or transposable element insertions, as is common for *R* genes. For example, the causal gene for *Rpp4* was narrowed

down to three coiled-coil (CC) NLRs via VIGS, and subsequent quantitative reverse-transcription PCR (qRT-PCR) assays highlighted a single candidate gene (Meyer et al. 2009). Another study found that at least two separate genes are responsible for the *Resistance to brown stem rot 1* (*Rbs1*) resistance phenotype in soybeans through the use of VIGS constructs targeting clusters of putative *R* genes (McCabe et al. 2024). Narrowing candidates or identification of causal *R* genes can make breeding for resistance easier and more efficient, particularly when pyramiding multiple sources of resistance into single soybean lines.

The objective of our work was to identify the source of resistance at the *Rpp2* locus. We screened a bacterial artificial chromosome (BAC) library developed from the *Rpp2* accession PI 230970 with markers targeting the *Rpp2* locus fine-mapped by Yu et al. (2015). Sequencing the BAC contig generated a 318-kb contig, spanning the entire *Rpp2* locus. We identified 14 TIR-NLR candidate *R* genes, seven of which are predicted to be full-length. Three bean pod mottle virus (BPMV) VIGS constructs were used to target the *Rpp2* candidate genes, with all three constructs compromising resistance to *P. pachyrhizi* in the resistant line. RNA sequencing (RNA-seq) of VIGS plants confirmed suppression of defense responses. Gene expression analyses and sequence comparisons between PI 230970 and the SBR-susceptible line Williams 82 enabled us to identify a single candidate for *Rpp2*.

Results

Identification and characterization of *Rpp2* candidate genes

The sequences of the markers used to fine-map *Rpp2* (Yu et al. 2015), BARCSOYSSR_16_0902 and BARCSOYSSR_16_0908, were used to screen the Williams 82 reference genome sequence version Wm82.a4 (Schmutz et al. 2010) using BLASTN (Camacho et al. 2009). The markers defined a 104-kb region on chromosome 16 (Gm16:29,448,565 to 29,553,131, Wm82.a4). However, Yu et al. (2015) reported that the markers spanned 188 kb (Gm16:29,253,139 to 29,441,257, Wm82.a2) in the previous version of the genome. To ensure we identified all the *R* genes in this region that could correspond to *Rpp2*, we used BLASTN to compare the 188-kb region identified by Yu et al. (2015) with the Wm82.a4 genome. A region spanning 188,256 bp (Gm16:29,448,705 to 29,636,961) was identified that included 10 potential *R* genes. Scanning this region with the SoyBase genome browser identified one additional *R* gene candidate located 31.7 kb downstream. To include all possible *R* genes in our analyses, the region was expanded to 220 kb (Gm16:29,448,705 to 29,668,593, Wm82.a4) and included 11 *R* gene homologs. This region was used to develop seven primer pairs (Supplementary Table S1) distributed across the region for BAC contig development in PI 230970 (*Rpp2*). Using this approach, we identified four BACs spanning the *Rpp2* locus: BACs PI_230970_103N04, PI_230970_53A08, PI_230970_41E24, and PI_230970_70O04. Primers developed from PI_230970_53A08 BAC-end sequences were used to confirm overlapping BACs and their orientation.

Paired-end sequencing of 970, 704, 460, and 423 subclones from BACs PI_230970_103N04, PI_230970_53A08, PI_230970_41E24, and PI_230970_70O04, respectively, was used to generate individual contig assemblies for each BAC. BAC coverage ranged from 5.8× for PI_230970_70O04 to 13.6× for BAC PI_230970_103N04. Once completely assembled, individual BAC contigs were combined to generate the *Rpp2* contig, spanning 318,346 bp (Fig. 1). Using sequencing reads of the subclones for all four BACs resulted in 12.3× coverage across the sequenced region. The sequence of markers BARCSOYSSR_16_0902 and BARCSOYSSR_16_0908, used to map *Rpp2* by Yu et al. (2015), confirmed that the contig

extended past the *Rpp2* locus in both directions (Fig. 1; Supplementary Table S2). Further, five single-nucleotide polymorphism markers that defined the unique PI 230970 haplotype (Yu et al. 2015), relative to 11 SBR-resistant lines and 32 ancestral lines, were also located within the sequenced region (Supplementary Table S2).

Using BLASTN (Camacho et al. 2009), we compared the sequence of the *Rpp2* PI 230970 locus with predicted primary transcripts from the same region in the Wm82.a4 genome. We identified 20 orthologous genes, of which 14 had homology to the NLR family of *R* genes. Of the 14 *Rpp2* candidate genes in PI 230970 (*Rpp2C1_PI*, *Rpp2C5_PI*, *Rpp2C6_PI*, *Rpp2C7_PI*, *Rpp2C10_PI*, *Rpp2C12_PI*, and *Rpp2C14_PI*) (Figs. 1 and 2). The full-length *Rpp2* candidate genes encode proteins containing a TIR domain (PFAM01582), NB-ARC domain (PF00931), disease resistance protein Roq-1-like winged helix DNA binding domain (PFAM23282), and L domain-like (SSF52058) containing multiple LRR domains (Fig. 2), making them members of the TIR-NLR-integrated domain (ID) family. Each full-length gene was predicted to have four introns. The remaining candidate *R* genes (*Rpp2C2_PI*, *Rpp2C3_PI*, *Rpp2C4_PI*,

Rpp2C8_PI, *Rpp2C9_PI*, *Rpp2C11_PI*, and *Rpp2C13_PI*) encode likely pseudogenes. *Rpp2C2_PI* contains a premature stop codon within the NB-ARC domain. *Rpp2C3_PI* and *Rpp2C4_PI* likely correspond to a single gene, with *Rpp2C4_PI* containing the TIR and NB-ARC domains and *Rpp2C3_PI* containing the L domain. Both fragments contain in-frame stop codons. Similarly, *Rpp2C8_PI* and *Rpp2C9_PI* likely represent a single gene, with *Rpp2C9_PI* encoding the TIR domain, whereas *Rpp2C8_PI* encodes the NB-ARC, winged-helix, and L domain, though again, both fragments contain in-frame stop codons. *Rpp2C13_PI* has similar disruptions encoding portions of the NB-ARC and L domains. Based on the positions of the five single-nucleotide polymorphisms that define the unique *Rpp2* haplotype (Yu et al. 2015; Supplementary Table S2), which spans the interval from 48,197 to 207,113 in the sequenced PI 230970 contig, only the full-length genes *Rpp2C5_PI*, *Rpp2C6_PI*, *Rpp2C7_PI*, and *Rpp2C10_PI* fall within this region and are candidates for *Rpp2*.

The corresponding region in the Williams 82 genome contains 11 related *R* genes (*Rpp2C_Wms82*; Supplementary Fig. S1). Of these, *Rpp2C1_Wms82*, *Rpp2C2_Wms82*, *Rpp2C4_Wms82*, *Rpp2C5_Wms82*, *Rpp2C6_Wms82*, *Rpp2C10_Wms82*, and *Rpp2C11_Wms82* appear to be full-length. Based on

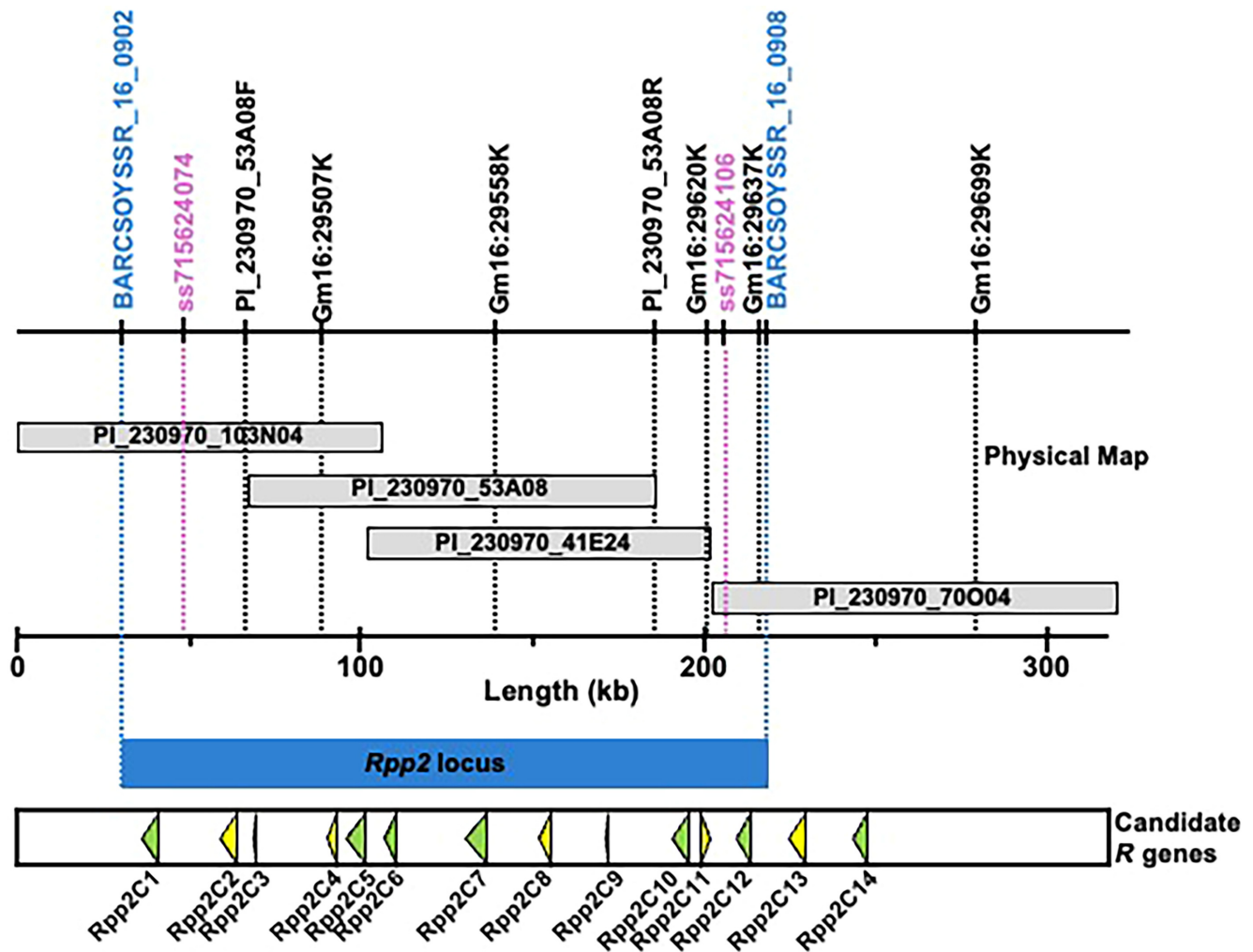


Fig. 1. Sequence of the *Rpp2* locus in PI 230970. The Williams 82 reference genome was used to develop primers to screen a PI 230970 (*Rpp2*) bacterial artificial chromosome (BAC) library (Supplementary Table S1, black font). Primer names and positions are depicted at the top of the figure in black font. Gray boxes indicate the position of sequenced PI 230970 BACs. The assembled sequence was screened for the target sequences of single-nucleotide polymorphism (SNP) markers (blue font) used to fine-map *Rpp2* (Yu et al. 2015). These markers define the *Rpp2* locus in PI 230970 (blue box, Supplementary Table S2). The position of the outside flanking SNP markers (magenta font) that defined the unique PI 230970 haplotype (Yu et al. 2015), relative to 11 SBR-resistant lines and 32 ancestral lines, are also indicated. The location and orientation of 14 candidate resistance genes within the assembled sequence is depicted at the bottom of the figure. Genes encoding full-length NLR proteins are green, while pseudogenes are yellow.

sequence identity, structure, and position and orientation within the cluster, we were able to identify corresponding genes across genotypes: *Rpp2C1_PI* corresponds to *Rpp2C1_Wms82* (Glyma.16G135200), *Rpp2C2_PI* corresponds to *Rpp2C2_Wms82* (Glyma.16G135500), *Rpp2C4_PI* corresponds to *Rpp2C3_Wms82* (Glyma.16G135900), *Rpp2C5_PI* corresponds to *Rpp2C4_Wms82* (Glyma.16G136000), *Rpp2C6_PI* corresponds to *Rpp2C5_Wms82* (Glyma.16G136200), *Rpp2C7_PI* corresponds to *Rpp2C6_Wms82* (Glyma.16G136600), *Rpp2C8_PI* and *Rpp2C9_PI* correspond to *Rpp2C7_Wms82* (Glyma.16G136900), *Rpp2C11_PI* corresponds to *Rpp2C9_*

Wms82 (Glyma.16G137200), *Rpp2C12_PI* corresponds to *Rpp2C10_Wms82* (Glyma.16G137300), and *Rpp2C14_PI* corresponds to *Rpp2C11_Wms82* (Glyma.16G137600). *Rpp2C3_PI*, *Rpp2C10_PI*, and *Rpp2C13_PI* could not be clearly assigned to a Williams 82 counterpart. Among the seven full-length *R* genes in PI 230970, *Rpp2C1*, *Rpp2C5*, and *Rpp2C12* shared greater than 99% nucleotide identity within their coding sequences relative to their Williams 82 counterparts, with 3 to 7 amino acid differences per protein. In contrast, *Rpp2C6*, *Rpp2C7*, and *Rpp2C14* were significantly different. *Rpp2C6_PI* shared greater than 98% nucleotide identity with *Rpp2C5_Wms82*, resulting in 20 amino acid differences, with 16 located within the L domain. *Rpp2C7_PI* shared 69% nucleotide identity with *Rpp2C6_Wms82*. Low nucleotide identity was due to a 918-bp duplication in *Rpp2C6_Wms82*, resulting in a duplicated L domain (Supplementary Fig. S1), and a 120-bp insertion in *Rpp2C7_PI* located near the end of the predicted protein. Removing gaps from the alignments resulted in 92% nucleotide identity and 83% amino acid identity. *Rpp2C14_PI* and *Rpp2C11_Wms82* shared 97% nucleotide identity and 94% amino acid identity. Because no clear correspondence could be made for *Rpp2C10_PI* relative to Williams 82, we compared its sequence with the other *R* genes from PI 230970. Removing gaps, *Rpp2C10_PI* was most similar to *Rpp2C14_PI*, sharing 90% nucleotide identity.

Silencing *Rpp2* candidate genes compromises resistance to *P. pachyrhizi*

While developing and sequencing the BAC contig from PI 230970, we took advantage of a library of VIGS constructs that had been developed to target soybean homologs of Arabidopsis genes with demonstrated roles in defense signaling (Pandey et al. 2011; Zhang et al. 2009). We identified three constructs targeting *Rpp2C1_Wms82*, *Rpp2C6_Wms82*, and *Rpp2C11_Wms82* (Supplementary Table S3). Given that our previous research had demonstrated that *R* gene sequences from a susceptible line could silence *P. pachyrhizi* resistance in a resistant line (Meyer et al. 2009), we were interested in testing whether these constructs could silence *Rpp2*-mediated resistance. The three VIGS constructs (BPMV:*Rpp2C1_Wms82*, BPMV:*Rpp2C6_Wms82*, and BPMV:*Rpp2C11_Wms82*) targeted the same region of the L domain of each of these genes but vary in size (309 to 441 bp; Supplementary Fig. S2). Across this region, the three genes shared 78 to 83% nucleotide identity. To conduct the VIGS experiments, 14-day-old PI 230970 plants were subject to one of four pretreatments: inoculation with a BPMV vector with no insert (empty vector), inoculation with BPMV:*Rpp2C1_Wms82*, inoculation with BPMV:*Rpp2C6_Wms82*, or inoculation with BPMV:*Rpp2C11_Wms82*. Four weeks after silencing treatment, all plants were inoculated with *P. pachyrhizi* isolate LA04-1. Two weeks later, plants were phenotyped for resistance to *P. pachyrhizi*. Silencing with all three test constructs resulted in susceptibility to *P. pachyrhizi* isolate LA04-1, with plants exhibiting TAN lesions and sporulating uredinia on both the abaxial and adaxial surface of the leaf (Fig. 3). Empty vector control plants were resistant, developing red-brown lesions with occasional uredinia as expected.

To determine the specificity of the three *Rpp2* VIGS constructs, we used BLASTN (Camacho et al. 2009) to compare the inserted *R* gene fragments (Supplementary Fig. S2) against all predicted transcripts in the Williams 82 reference genome version Wm82.a4 (Schmutz et al. 2010). For each construct, using a default E value of 10, we only identified *R* gene transcripts from the *Rpp2* locus in Williams 82.

To confirm silencing of *Rpp2* candidate genes, mRNA from three independent biological replicates was measured by qRT-PCR (Supplementary Table S4). Primers KP1236 and

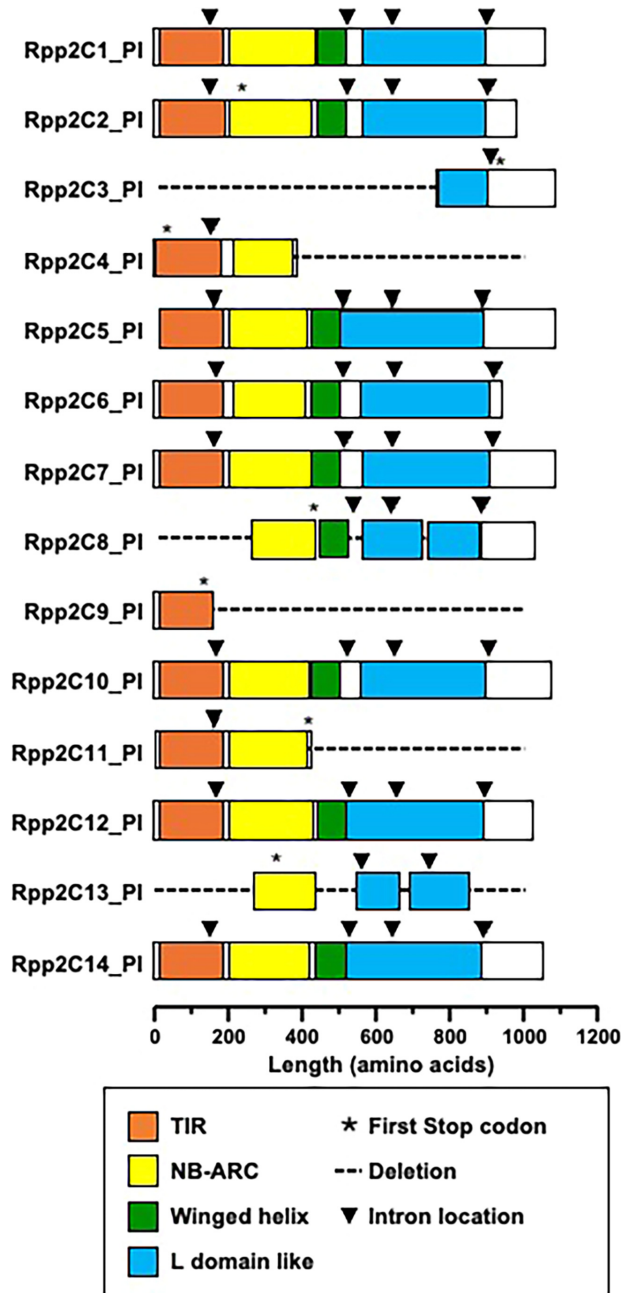


Fig. 2. Schematic representation of the structure of *Rpp2* candidate proteins from PI 230970. FGENESH (Solovyev et al. 2006) and sequence alignments with predicted transcripts from the Williams 82 reference genome (version Wms82.a4.v1) were used to confirm the positions of exons and splice sites within candidate *Rpp2* genes. InterProScan 5 (Jones et al. 2014) was used to identify conserved domains within predicted proteins. TIR, toll/interleukin-1 receptor; NB-ARC, nucleotide-binding, Apaf-1, R proteins, and Ced-4; L domain-like, leucine-rich repeat.

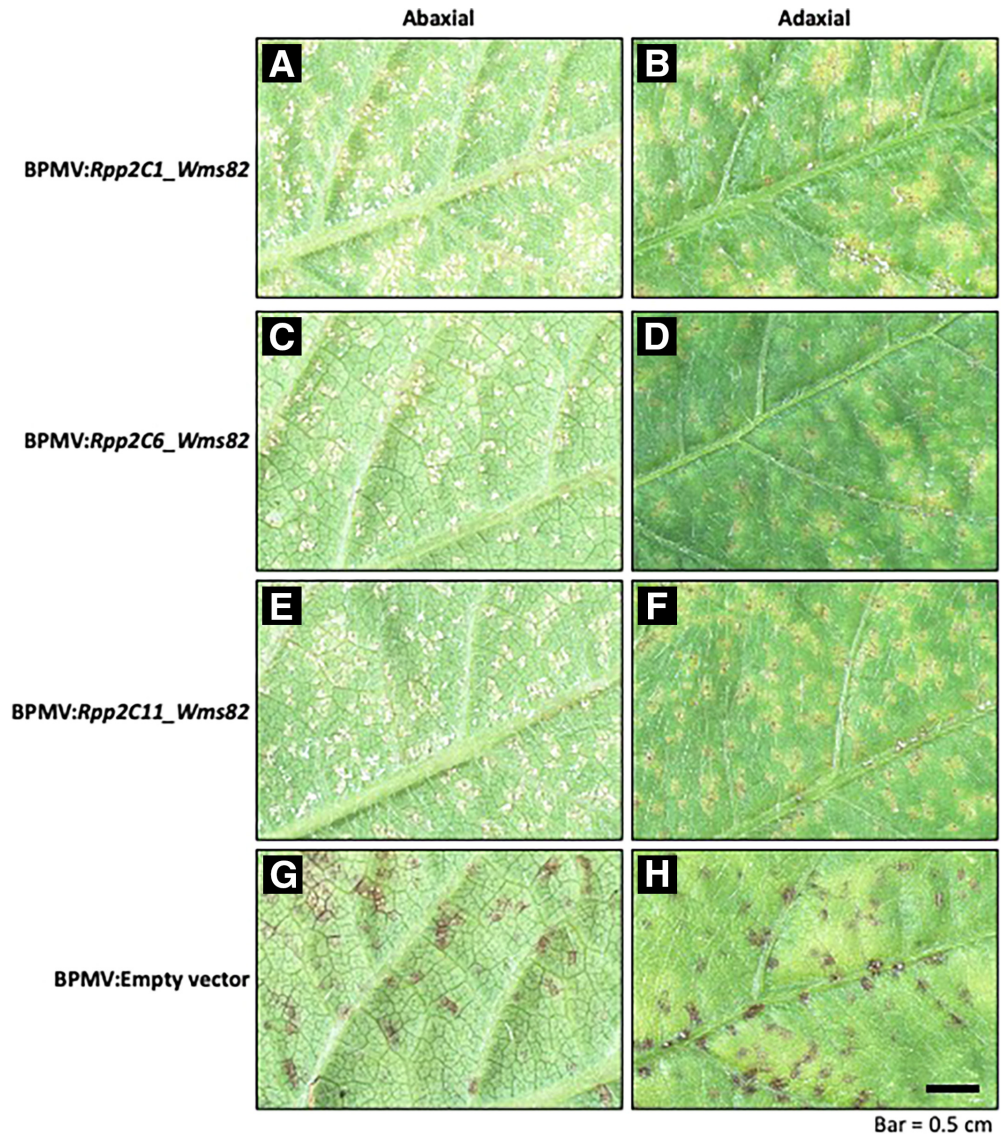
KP1237 were designed to target the TIR domain of the seven full-length PI 2305970 *Rpp2* candidate genes (Supplementary Fig. S3). Primer sites were identical across full-length transcripts. Relative to the empty vector-treated plants, *Rpp2* candidate gene expression was reduced 5.00 ± 0.14 -fold, 4.40 ± 0.15 -fold, and 3.28 ± 0.12 -fold in BPMV:*Rpp2C1_Wms82*-, BPMV:*Rpp2C6_Wms82*-, and BPMV:*Rpp2C11_Wms82*-treated plants, respectively. To confirm that resistance was compromised, we measured the amount of the *P. pachyrhizi* α -tubulin transcript as a proxy for fungal growth. Relative to the empty vector control plants, α -tubulin transcripts were increased 3.00 ± 0.56 -fold, 3.96 ± 0.58 -fold, and 2.58 ± 0.57 -fold in BPMV:*Rpp2C1_Wms82*-, BPMV:*Rpp2C6_Wms82*-, and BPMV:*Rpp2C11_Wms82*-treated plants, respectively. These data suggest that silencing *Rpp2* promoted fungal accumulation and confirm that one of the full-length candidate genes in the *Rpp2* locus from PI 230970 confers resistance.

***Rpp2C7_PI* is exclusively expressed in *P. pachyrhizi*-infected leaves of PI 230970**

To narrow the *R* genes conferring *Rpp2*-mediated resistance to *P. pachyrhizi*, the TIR primers, KP1236 and KP1237, were used to amplify genomic DNA and cDNA from mock-inoculated or *P. pachyrhizi*-infected tissue 7 days after treatment in PI 230970 and Williams 82. These primers would amplify

sequence differences that could be used to distinguish candidate genes. Because all candidate genes would be represented in genomic DNA at equal proportions, we could determine the amplification efficiency correction for each gene within a genotype and adjust expressed gene counts accordingly (Supplementary Table S5). For the 223 cDNA clones from mock-inoculated Williams 82, 44, 40, 15, and 1% of clones corresponded to *Rpp2C6_Wms82*, *Rpp2C8_Wms82*, *Rpp2C9_Wms82*, and *Rpp2C11_Wms82*, respectively. For the 123 cDNA clones from *P. pachyrhizi*-infected Williams 82, 48, 31, 19, and 2% of clones corresponded to *Rpp2C6_Wms82*, *Rpp2C8_Wms82*, *Rpp2C9_Wms82*, and *Rpp2C11_Wms82*, respectively. For the 164 cDNA clones from mock-inoculated PI 230970, 98 and 2% of clones corresponded to *Rpp2C7_PI* and *Rpp2C14_PI*, respectively. For the 141 cDNA clones from *P. pachyrhizi*-infected PI 230970, 100% of clones corresponded to *Rpp2C7_PI*. Of the four remaining candidate genes, *Rpp2C7_PI* is exclusively expressed in PI 230970 in response to *P. pachyrhizi*, making it the primary candidate for *Rpp2*. To confirm our silencing results, we compared the predicted transcript of *Rpp2C7_PI* with the sequences of all three VIGS constructs (BPMV:*Rpp2C1_Wms82*, BPMV:*Rpp2C6_Wms82*, and BPMV:*Rpp2C11_Wms82*; Supplementary Fig. S2). As demonstrated by conservation in the alignment, all three constructs would silence *Rpp2C7_PI*.

Fig. 3. Silencing *Rpp2* candidate genes in PI 230970 results in loss of resistance. Fourteen-day-old PI 230970 plants were subjected to one of four virus-induced gene silencing (VIGS) pretreatments: inoculation with BPMV:*Rpp2C1_Wms82*, BPMV:*Rpp2C6_Wms82*, BPMV:*Rpp2C11_Wms82*, or BPMV:Empty-vector. Three weeks after silencing, plants were inoculated with *Phakopsora pachyrhizi* isolate LA04-1, and plants were phenotyped 2 weeks later. **A to F**, Silencing by all three *Rpp2C* constructs resulted in loss of resistance to *P. pachyrhizi*, with plants exhibiting TAN lesions and sporulating uredinia on both the abaxial and adaxial surface of the leaf. **G and H**, Empty-vector control plants were resistant, developing red-brown lesions and occasional uredinia as expected. Scale bar = 0.5 cm.



***Rpp2* silencing suppresses defense responses**

Given the analyses above, we focused our RNA-seq analyses of PI 230970 on BPMV:*Rpp2C6_Wms82* plants relative to empty vector control plants, each infected with *P. pachyrhizi* isolate LA04-1, which would allow us to identify downstream components of the *Rpp2* signaling pathway. RNA-seq of three biological replicates of BPMV:*Rpp2C6_Wms82* and empty vector controls resulted in 185 million reads. Using FASTQ Screen (Wingett and Andrews 2018), we confirmed that 35.7 to 90.2% of reads within a sample originated from the BPMV vector. Using a false discovery rate < 0.05, we identified 796 differentially expressed genes (DEGs) repressed by *Rpp2* silencing and 951 DEGs induced by *Rpp2* silencing (Supplementary Table S6). To identify biological processes impacted by *Rpp2* silencing, we examined the Gene Ontology (GO) biological process terms significantly overrepresented (corrected $P < 0.05$) within the data sets (Supplementary Table S7). Among the genes repressed by *Rpp2* silencing, we identified 10 significant GO terms associated with defense (cinnamic acid and phenylpropanoid biosynthesis, salicylic acid and L-phenylalanine catabolism), stress (response to karrikin, UV and wounding), and photosynthesis (photosynthesis, protein-chromophore linkage, and photosynthetic electron transport). Genes induced by *Rpp2* silencing were associated with two significant GO terms: cellular response to hypoxia and response to chitin. These results suggest that defense- and stress-related genes were not induced in response to *P. pachyrhizi* in PI 230970 BPMV:*Rpp2C6_Wms82* plants, which allowed increased fungal accumulation to occur and resulted in the induction of genes responding to chitin.

Gain of TIR-NLR-IDs in the *Rpp2* locus is relatively recent

To better understand the evolution of the *Rpp2* cluster, we used the Legume Information System Genomic Context Viewer (Dash et al. 2016) to identify regions syntenic to the *Rpp2* locus in soybean and other legumes (Fig. 4). Using Glyma.16G136600 (*Rpp2C6_Wms82*) as a query, we focused on the approximately 1.5-Mb region surrounding the cluster (Gm16:28,315,811 to

29,810,481, Wm82.a4). Using this approach, we identified the corresponding region on chromosome Gm16 of multiple *Glycine* species, including *G. max*, *G. sojae*, *G. syndetica*, and *G. stenophita*; the homologous region on chromosome Gm02 of *G. max* and *G. sojae*; and the syntenic region in *Phaseolus vulgaris* (common bean, Pv03), *Vigna unguiculata* (cowpea, Vu03), *Vigna radiata* (mungbean, Vr07), and *Medicago truncatula* (barrel medic, Mt08). Only the regions associated with chromosome 16 in the *Glycine* species contained TIR-NLR genes. Meyer et al. (2009) and Pedley et al. (2019) also found that *R* gene homologs were absent in regions homologous to *Rpp4* and *Rpp1*, respectively. This observation suggests that the presence of *R* genes in *Rpp* loci is relatively recent, occurring after the separation of soybean and *P. vulgaris*, its closest relative.

Long terminal repeat (LTR) retrotransposons have shaped the evolution of the *Rpp2* gene cluster

Because Meyer et al. (2009) and Pedley et al. (2019) both noted the presence of repetitive elements in the *Rpp4* and *Rpp1* loci, we took advantage of the work by Lexa et al. (2020), who identified LTR retrotransposons in the genomes of multiple plant species, including soybean. We used the nucleotide sequence of the LTR transposons identified in the *Rpp2* locus of Williams 82 (version Wm82.a2.v1) to BLAST (Camacho et al. 2009) against the PI 230970 sequence. This revealed that the *Rpp2* candidate genes are located within islands of nested retrotransposons (Fig. 5A). To understand where the *Rpp2* candidate genes might have originated from, we used BLASTN (Camacho et al. 2009) to compare the *Rpp2C_Wms82* transcripts and the *Rpp2C_PI* transcripts with all predicted primary transcripts in the Williams 82 reference genome. Using the default E value of 10, we identified 36 transcripts from Gm03, Gm04, Gm09, Gm13, Gm16, and Gm19, including the 11 TIR-NLRs from the Williams 82 *Rpp2* cluster. The best BLASTN hits ($E = 0$) were associated with a cluster of three *R* genes on Gm19 (Glyma.19G054700, Glyma.19G054900, and Glyma.19G055000), unrelated to the mapped location of *Rpp7* (Childs et al. 2018). As above, we ex-

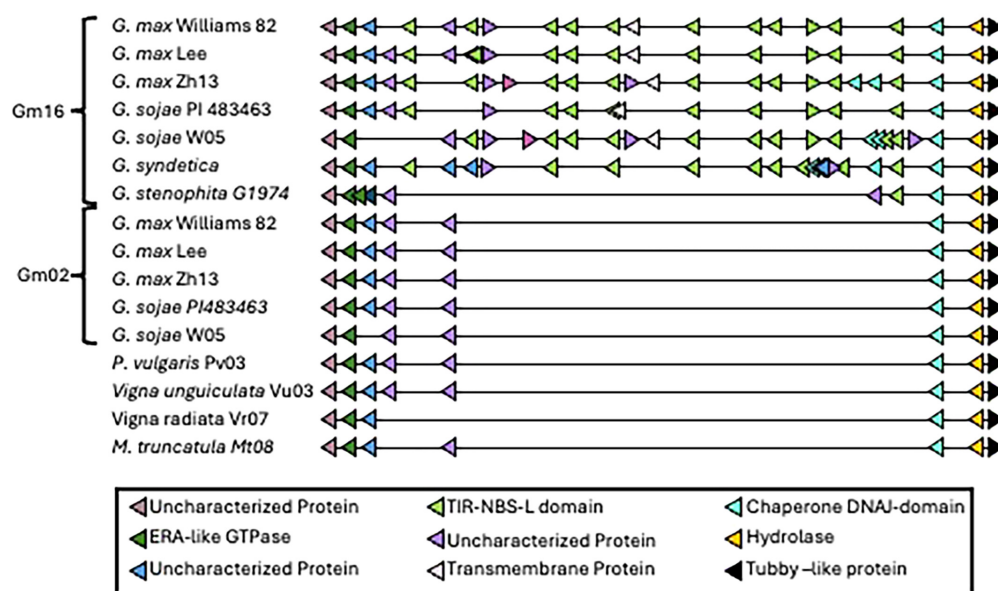


Fig. 4. Structural analyses of the *Rpp2* locus across legumes. The Legume Information System Genomic Context Viewer (Dash et al. 2016) was used to compare the region from Williams 82 Gm16:28,315,811 to 29,810,481 (version Wm82.a4.v1), which includes the *Rpp2* locus, with the syntenic region across multiple legume species. Within the *Glycine* genus, NLR genes are present in the *Rpp2* locus on chromosome Gm16 but varied in numbers. NLR genes are absent from the homologous region on Gm02. Similarly, NLR genes are absent from the *Rpp2* locus in more distantly related legumes, suggesting that the addition of NLR genes to the *Rpp2* locus is relatively recent. The relative location and orientation of predicted genes is depicted by colored arrowheads. The color of the arrowhead indicates sequence homology, with a description provided in the figure legend. To aid in clarity, genes found in fewer than five species are not shown.

amined the region surrounding this cluster for the presence of LTR retrotransposons (Fig. 5B). Only Glyma.19G054900 was located within an LTR retrotransposon, making it a possible progenitor of the *Rpp2* candidate genes.

Rpp2C7_PI contains a novel repeat

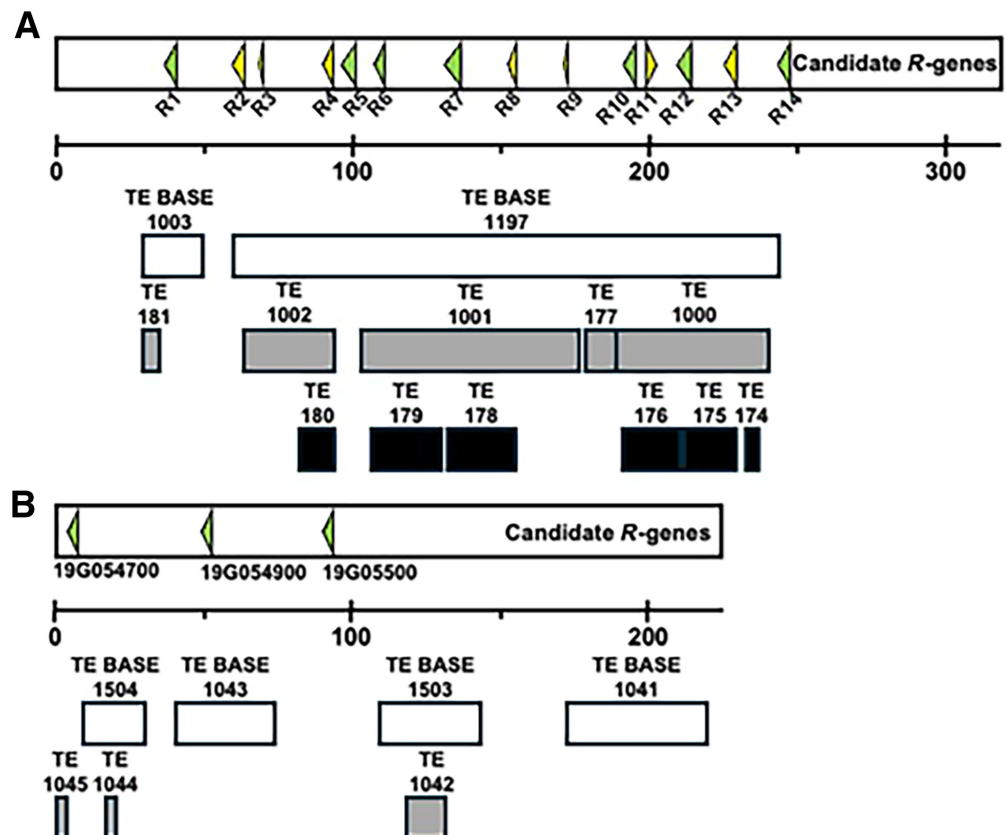
As stated previously, comparing the nucleotide sequence of *Rpp2C7_PI* with *Rpp2C6_Wms82* identified a 120-bp in-frame insertion at the very tail end of *Rpp2C7_PI*. We examined the insertion using Tandem Repeats Finder (Benson 1999) and identified two repeats with a consensus size of 63 bp, sharing 91% nucleotide identity. BLASTN (Camacho et al. 2009) of the repeats against *Rpp2C7_PI* revealed a truncated repeat of approximately 40 bp, immediately preceding the insertion (Fig. 6A). BLASTN (Camacho et al. 2009) of the two repeats against all full-length *Rpp2* candidate genes from PI 230970 and Williams 82 revealed that each contained the approximately 40-bp fragment but no additional repeats. Interestingly, *Rpp2C6_Wms82* had two copies of the 40-bp repeat fragment, separated by >1,600 bases. BLASTN (Camacho et al. 2009) against the Williams 82 reference genome (Wms82.a4.v1) identified no other sequences with homology to the insertion. To determine if the repeat had a broader biological function, we examined it at the protein level. We aligned the protein sequence corresponding to the repeated region for all full-length *Rpp2* candidate genes from Williams 82 and PI 230970 (Fig. 6). We used HMMER (version 3.3.2; <http://hmmer.org>) to build a hidden Markov model (HMM) from the alignment and *hmmsearch* (domain inclusion threshold and sequence inclusion threshold $E < 0.01$) to search the UniProtKB/TrEMBL database (version 8/01/2025; The UniProt Consortium 2025). The resulting alignment was then used to build a new HMM and initiate a new HMM search. We repeated this process iteratively. On the sixth search, the domain inclusion threshold and sequence inclusion threshold

were raised to $E < 1$. We then repeated the search, increasing the domain inclusion threshold and sequence inclusion threshold to $E < 10$. With this final step, we identified 142 sequences, representing 21 species, all taxonomically restricted to the 50-kb insertion clade of the Papilionoideae. The final HMM was converted to a logo using WebLogo version 2.8.2 (<https://weblogo.threeplusone.com/>), as shown in Figure 6B. Of the 142 sequences, 107 had annotations associated with *R* genes (resistance, TIR, or NB-ARC). The remaining proteins were annotated as uncharacterized. We identified nine sequences with two repeats; however, the repeats were separated by at least 359 amino acids. No sequences with three tandem repeats were identified, suggesting that this insertion in *Rpp2C7_PI* could be used as a perfect marker for selecting *Rpp2*.

Discussion

In the current study, we used the molecular markers used to fine-map *Rpp2* (Yu et al. 2015) and primers developed from the corresponding region in the Williams 82 genome to build and sequence a BAC contig corresponding to the *Rpp2* locus in the resistant line PI 230970. Sequencing the BAC contig identified 14 genes with homology to the TIR-NBS-WH-LRR *R* gene family. Of these, seven appeared to be full-length and functional. Based on the unique *Rpp2* haplotype identified by Yu et al. (2015), only four of these were candidates for *Rpp2* (*Rpp2C5_PI*, *Rpp2C6_PI*, *Rpp2C7_PI*, and *Rpp2C10_PI*). While developing the contig, we took advantage of previous research (Zhang et al. 2009) that had generated three VIGS constructs from Williams 82 that could be used to target the *Rpp2* locus. Each of the constructs was able to compromise resistance to *P. pachyrhizi* isolate LA04-1 in PI 230970, confirming that one of the four genes above is responsible for *Rpp2*-mediated resistance. RNA-seq analyses confirmed that silencing *Rpp2* repressed defense

Fig. 5. The *Rpp2* candidate genes are located in an island of nested long terminal repeat (LTR) retrotransposons. Research by Lexa et al. (2020) identified nested LTR retrotransposons throughout the soybean genome (version Wm82.a2.v1). In **A**, we used BLASTN (Camacho et al. 2009) to compare LTR retrotransposons found in the *Rpp2* locus in Williams 82 against the *Rpp2* locus sequenced from PI 230970. The white blocks represent the original LTR retrotransposons, interrupted by more recent LTR insertions. The gray blocks represent the next generation of LTR retrotransposon insertions, and the black blocks represent the newest generation of LTR retrotransposon insertions. In **B**, we used the same approach to examine nested LTR transposons surrounding Glyma.19G054700, Glyma.19G054900, and Glyma.19G05500 in Williams 82 (version Wm82.a4.v1), which are the genes mostly closely related to the *Rpp2* TIR-NLRs. LTR retrotransposon names refer to names assigned by Lexa et al. (2020).



pathways. Sequencing of RT-PCR products from mock- and *P. pachyrhizi*-inoculated PI 230970 revealed that *Rpp2C7_PI* was the only *Rpp2* candidate gene expressed in pathogen-inoculated tissue and accounted for 98% of sequenced RT-PCR products in mock-inoculated tissue. Combined, these data strongly support *Rpp2C7_PI* as the sole candidate for *Rpp2*.

In previous research, we used microarray technology to conduct a time course comparing PI 230970 (*Rpp2*) with Embrapa-48 (susceptible) at 6, 12, 18, 24, 36, 48, 72, 96, and 168 h after mock inoculation or inoculation with a Brazilian field isolate of *P. pachyrhizi* (van de Mortel et al. 2007). Gene expression followed a biphasic pattern, with both genotypes responding similarly to *P. pachyrhizi* within the first 12 h and gene expression returning to mock-inoculation levels from 24 to 72 h after inoculation. At 72 h after inoculation, PI 230970 reestablished differential gene expression earlier and with greater magnitude than Embrapa-48, likely reflecting pathogen recognition and activation of *Rpp2*-mediated defenses. This includes genes associated with the phenylpropanoid pathway, which leads to the formation of defense compounds such as phytoalexins, antimicrobial compounds, lignan, and lignin. We queried our RNA-seq data for genes with the best BLASTP matches to genes involved in the phenylpropanoid pathway in Arabidopsis (Biala and Jasiński 2018; El Houari et al. 2021; Yadav et al. 2020). We identified 21 DEGs associated with the phenylpropanoid pathway, all of which were repressed in response to *Rpp2* silencing ($\log_2FC = -1.79$ to -6.59 ; Supplementary Table S6). These include homologs of *AtPAL1* (Phenylalanine ammonia lyase 1), *AtPAL2* (Phenylalanine ammonia lyase 2), *At4CL2* (4-coumarate:CoA ligase 2), *At4CL3* (4-coumarate:CoA ligase 3), *AtCCR2* (Cinnamoyl CoA reductase 2), *AtCAD1* (Cinnamoyl alcohol dehydrogenase 1), *AtCAD9* (Cinnamoyl alcohol dehydrogenase 9), *AtCHS* (Chalcone synthase), *AtCHI* (Chalcone flavanone isomerase), *AtCOMT1* (Caffeate O-methyltransferase 1), *AtCCoAOMT1* (Caffeoyl coenzyme A O-methyltransferase

1), *AtDFR* (Dihydroflavonol 4-reductase), and *F5H* (Ferulic acid 5-hydroxylase 1).

Pandey et al. (2011) were also interested in using a VIGS approach to identify components of *Rpp2*-mediated resistance. Of the 11 genes they targeted, only *GmPAL1* (Glyma.02G309300, Wms82.a4v1) was also differentially expressed in our study. Silencing *GmPAL1* (Glyma.02G309300) resulted in loss of resistance to *P. pachyrhizi* isolate LA04-1 in PI 230970 plants. If *GmPAL1* is required for *Rpp2*-mediated resistance, silencing *Rpp2* should repress *GmPAL1*, as observed in our study (Glyma.02G309300, $\log_2FC = -2.08$). Pandey et al. (2011) also silenced the *GmMYB84* transcription factor, repressing components of the phenylpropanoid pathway, including *GmPAL1*, *Gm4CL*, and *GmCHS*. These genes were also repressed in response to *Rpp2* silencing.

Recently, Shao et al. (2024) developed an NLR-ID Toolkit to help identify and characterize novel NLRs from soybean that confer resistance to agronomically important viruses. Twenty-seven NLRs were screened for antiviral activity by transient expression in *Nicotiana benthamiana* and co-infection with a GFP-labeled virus. Eight members of the *Rpp2-like* family significantly reduced the viral titer for two or more viruses, including soybean mosaic virus, tobacco mosaic virus, plum pox virus, cabbage leaf curl virus, barley stripe mosaic virus, and tobacco rattle virus. *Rpp2C1_Wms82* was effective against plum pox virus and tobacco rattle virus, and *Rpp2C2_Wms82* was effective against plum pox virus and cabbage leaf curl virus. Cui et al. (2017) also demonstrated that cleavage of *Rpp2C2_Wms82* RNA by miR1510 reduced resistance to *Phytophthora sojae*.

Comparing the *Rpp2* gene cluster on chromosome Gm16 with the homologous region on chromosome Gm02 and syntenic regions in other legumes (Fig. 4) revealed no *R* genes, suggesting that acquisition of *R* genes in the *Rpp2* cluster occurred since the separation of soybean and its closest relative. This mirrors what we found for the *Rpp1* (Pedley et al. 2019) and *Rpp4* (Meyer et al. 2009) gene clusters. To determine where the *Rpp2* genes

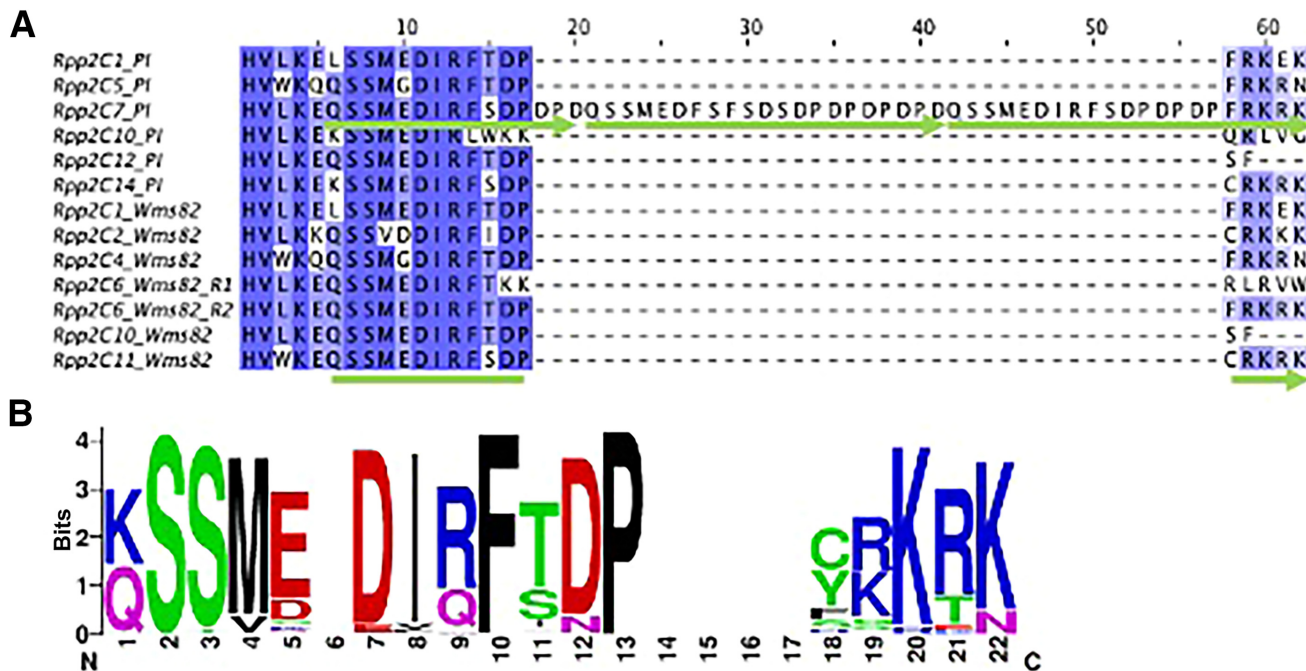


Fig. 6. *Rpp2C7_PI* carries a unique insertion. **A**, Comparing the sequence of *RPP2C7_PI* relative to the other full-length candidate NLR proteins revealed a 40-amino-acid insertion. The insertion comprises two imperfect repeats of the preceding sequence (green arrows), each containing the conserved domain “QSSMED.” To further investigate the function of the repeat, we aligned the repeat motif from all the *RPP2* candidate proteins and built a hidden Markov model (HMM) (version 3.3.2, <http://hmmer.org>). We iteratively searched the HMM six times against UniProtKB/TrEMBL database (version 8/01/2025; The UniProt Consortium 2025), continually refining the HMM. The final HMM is shown in **B**. The height of each amino acid is reflective of its conservation across 142 sequences identified. All 142 sequences were restricted to the 50-kb insertion clade of the Papilionoideae.

might have originated from, we used BLASTN ($E < 10$) to compare the *Rpp2* R genes from Williams 82 and PI 230970 with all predicted transcripts in the Williams 82 reference genome. We identified 36 *Rpp2*-like genes (including the 11 from the *Rpp2* locus), located on chromosomes Gm03, Gm04, Gm09, Gm13, Gm16, and Gm19. Only 15 of the corresponding R proteins outside the *Rpp2* locus were full-length. For comparison, we used the HMM designed to characterize the repeat fragment found in *Rpp2C7_PI* to screen all predicted proteins in the Williams 82 reference genome. Of the 29 proteins identified, 19 corresponded to the *Rpp2*-like genes, including seven from the *Rpp2* locus (*data not shown*). These findings confirm that the *Rpp2*-like gene family is relatively new and restricted to the 50-kb insertion clade of the Papilionoideae.

Zhao et al. (2015) and Fei et al. (2018) recently identified and characterized miR1510, a 22-nucleotide micro RNA (miRNA) targeting NLRs within Phaseoleae species. Of the 11 miR1510 targets predicted by Fei et al. (2018), four (Glyma.16G159100, Glyma.19G05470, Glyma.19G054900, and Glyma.19G055000) are members of the *Rpp2* family. Cui et al. (2017) were interested in examining miRNAs associated with defense responses in soybean. They predicted that miR1510a/b could target 10 different NLR genes. Of these, six belonged to the *Rpp2* family, including four from the *Rpp2* locus (Glyma.16G135200 [*Rpp2C1_Wms82*], Glyma.16G135500 [*Rpp2C2_Wms82*], Glyma.16G137200 [*Rpp2C9_Wms82*], Glyma.16G137600 [*Rpp2C11_Wms82*], Glyma.19G022700, and Glyma.19G054700). They confirmed that *Rpp2C2_Wms82* mRNA was directly cleaved by miR1510. Given that miR1510 is taxonomically restricted to the Phaseoleae and the *Rpp2* family is restricted to the 50-kb insertion clade of the Papilionoideae, this provides clear evidence of the relatively recent evolution of the *Rpp2* family and its regulation.

In summary, we have identified *Rpp2C7_PI*, a TIR-NBS-WH-LRR, as the single candidate gene for *Rpp2*. The *Rpp2* gene family, and their regulation by miRNAs, is taxonomically restricted within legumes. Genes within the *Rpp2* locus and the *Rpp2* family confer resistance to viral, fungal, and oomycete pathogens, making this cluster agronomically important for soybean breeders and growers.

Materials and Methods

Rpp2 BAC contig development

A BAC library was developed from high molecular weight genomic DNA isolated from young leaf tissue of the SBR-resistant soybean accession PI 230970 (*Rpp2*) by the Clemson University Genomics Institute (Luo and Wing 2003). The genomic DNA was partially digested with *Hind*III, cloned into pIndigoBAC536 (*Hind*III), and transformed into *Escherichia coli* DH10B (Invitrogen). Estimated genome coverage was 10 $\times$, with an average insert size of 120 kb. To enable PCR screening of the BAC library, the 104 plates were replicated to develop plate, row, and column pools as described by Marek and Shoemaker (1997). DNA was extracted using the plasmid buffer set for DNA extraction (Qiagen, cat. 19046) and ethanol precipitation.

To identify all possible *Rpp2* candidates from PI 230970 (*Rpp2*) in our analyses, the sequence (Gm16:29,253,139 to 29,441,257, version Wm82.a2.v1; Schmutz et al. 2010) corresponding to the genomic interval fine-mapped by Yu et al. (2015) was compared with the Williams 82 reference genome sequence version Wm82.a4.v1 (Schmutz et al. 2010) using BLASTN (Camacho et al. 2009). This identified a region spanning 188,256 kb (Gm16:29,448,705 to 29,636,961) and included 10 potential R genes. Scanning this region with the SoyBase genome browser identified one additional R gene located 31.7 kb downstream. Therefore, we expanded the region of interest to 220 kb

(Gm16:29,448,705 to 29,668,593, Wm82.a4) to include all 11 R gene homologs. This region was used to develop five primer pairs, distributed across the region, that could be used in contig development (Fig. 1; Supplementary Table S1). Primers were developed using Oligo 7.0 (Molecular Biology Insights). BAC-end sequences for BAC PI_230970_53A08 were used to develop one additional primer pair to confirm BAC orientation.

For each of the four identified BACs, the Qiagen large-construct kit (Qiagen, cat. 12462) was used to isolate high-quality DNA that was sheared and cloned using the TOPO Shotgun subcloning kit (Invitrogen, Thermo Fisher Scientific, cat. K7000-01) and One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, cat. C404003). Subclone DNA was isolated using Qiagen miniprep solutions (P1, P2, P3, cat. 19051, 19052, and 19053) and Whatman 96-well unifilters and uniplates (cat. 7770-0062 and 7701-1750). Sequencing was performed using the Applied Biosystems Big Dye version 3.1 chemistry protocol and Hi-Di formamide (cat. 4311320 and 4337457) on an Applied Biosystems 3730 DNA Analyzer with a 96-capillary array. Sequences for a given BAC were assembled independently. Sequencher version 5.4.6 (Gene Codes Corporation) was used to remove vector sequences and low-quality DNA. To improve assembly accuracy, forward and reverse reads for the same subclone were assembled prior to whole BAC assembly. Assembling all subclones from a BAC required a minimum match percentage of 100% and a 100-bp overlap. The Williams 82 reference genome (version Wm82.a4.v1; Schmutz et al. 2010) was used to orient contigs when necessary to fill gaps. To fill gaps with the sequence from PI 230970, PCR primers were developed from the ordered contigs and used to amplify DNA from a given BAC or PI 230970 genomic DNA. PCR products were cloned and sequenced using Platinum *Taq* DNA polymerase High Fidelity (Invitrogen, cat. 11304-011), the TOPO TA cloning kit (Invitrogen, cat. 45-0641), and One Shot TOP10 electrocompetent *E. coli* (Invitrogen, cat. C404052). Following completion of each BAC, the sequences of all BACs were combined into a single contig, providing further assembly validation. The sequences of the markers used to map *Rpp2* (Yu et al. 2015) were compared with the assembled *Rpp2* contig using BLASTN (Camacho et al. 2009) to orient the contig (Fig. 1; Supplementary Table S2).

Rpp2 locus annotation in PI 230970

The 318,346-bp sequence corresponding to the *Rpp2* contig from PI 230970 (GenBank accession PX270335) was divided into 2,000-bp fragments. Each fragment was compared against proteins from UniProt (The UniProt Consortium 2023) and The Arabidopsis Information Resource (TAIR v.11, www.arabidopsis.org) using BLASTX (Camacho et al. 2009) to identify potential coding sequences and sequences homologous to known proteins. BLASTN (Camacho et al. 2009) against Wm82.a4.v1 primary transcripts, FGENESH (Salamov and Solovyev 2000), and NetPlantGene2 Server (Hebsgaard et al. 1996) were used to predict primary transcripts and exon/intron boundaries in candidate R genes of interest. InterProScan5 (Jones et al. 2014) was used to scan predicted R proteins for functional domains.

VIGS of *Rpp2* candidate genes

Three primer pairs targeting the same approximately 400 bp of the L-like domain of three different NLR genes from the Williams 82 *Rpp2* locus were developed (Supplementary Table S3). PCR products were directionally cloned in the antisense orientation into RNA2 of the BPMV VIGS vector pBPMV-1A-D35 as described previously (Zhang et al. 2009). The orientation and identity of the VIGS inserts were confirmed by sequencing. Constructs BPMV:*Rpp2C1_Wms82*, BPMV:*Rpp2C6_Wms82*, and

BPMV:*Rpp2C11_Wms82* corresponded to Glyma.16G135200 (419-bp insert), Glyma.16G136600 (309-bp insert), and Glyma.16G137600 (441-bp insert), respectively, with 100% nucleotide identity.

To generate the inoculum for silencing experiments, BPMV RNA1 (pBPMV-IA-R1M) and RNA2 DNA clones were co-bombarded on Williams 82 leaves 2 weeks after sowing (Zhang et al. 2009). To generate the inoculum for the negative empty vector control, empty vector RNA2 was co-bombarded with RNA 1. Three to five weeks later, BPMV-infected leaf tissue was collected, lyophilized, and stored at -20°C . *Rpp2*-resistant (PI 230970) and -susceptible (Williams 82) plants were germinated in a growth chamber at the U.S. Department of Agriculture–Agricultural Research Service, Foreign Disease-Weed Science Research Unit at Fort Detrick, Maryland. Two weeks post-germination, the first true leaves were dusted with carborundum and rub-inoculated with the lyophilized inoculum as described previously (Whitham et al. 2016). Eight plants were treated with one of the three test constructs or an empty vector control. Four weeks later, BPMV-infected plants were transferred to the Plant Pathogen BSL-3 containment facility at Fort Detrick (Melching et al. 1983), inoculated with *P. pachyrhizi* isolate LA04-1, and placed in a dew chamber overnight, as described previously (Pandey et al. 2011). VIGS and *P. pachyrhizi*-infected plants were transferred to a BSL-3 greenhouse and evaluated for resistance (red-brown lesions) or susceptibility (tan lesions) 2 weeks later. The experiment was independently replicated three times.

Assessment of *Rpp2* silencing

To assess *Rpp2* candidate gene silencing, 1-cm-diameter leaf disks were excised from the fourth trifoliate of BPMV-infected plants with a cork borer 2 weeks after inoculation with *P. pachyrhizi*. Collected tissue was immediately frozen in liquid nitrogen and stored at -80°C . Frozen leaf disks were processed with a Qiagen TissueLyser II in 2-ml Eppendorf Safe-Lock tubes with 2-mm Zirconium oxide beads (Next Advance) at 30 Hz for 90 s. RNA was extracted using the Qiagen Plant RNeasy kit and treated with TURBO DNase (Invitrogen, Thermo Fisher Scientific). First-strand cDNA synthesis was performed with the Transcriptor High Fidelity cDNA Synthesis kit with an anchored-oligo(dT)₁₈ primer (Roche). qRT-PCR was performed on cDNA from experimental and control plants using *Rpp2*-specific oligonucleotide primers KP1236 and KP1237 (Supplementary Table S4). Primers were designed to amplify a 352-bp region of the TIR domain of all seven full-length *Rpp2* candidate genes from PI 230970. Primer annealing sites were 100% identical across all seven genes. The amount of fungal growth was assessed by measuring the constitutively expressed *P. pachyrhizi* α -tubulin gene using primers KP1045 and KP1046 (Supplementary Table S4). Soybean *Rpp2* candidate gene and *P. pachyrhizi* α -tubulin transcript levels were normalized to the soybean *ubiquitin-3* gene (GenBank accession D28123), which is not differentially expressed in plants infected with *P. pachyrhizi* (van de Mortel et al. 2007). *Ubiquitin-3* expression was analyzed with primers KP267 and KP268 (Supplementary Table S4). All qRT-PCRs were conducted with TB Green Advantage qPCR Premix (Takara Bio). Reaction parameters consisted of an initial 180-s denaturation at 95°C and 40 reaction cycles (95°C for 15 s and 60°C for 30 s), followed by a dissociation curve, taking a fluorescent measurement at every half-degree between 65 and 95°C . All qRT-PCR experiments were conducted on a CFX Opus 96 Real-Time PCR System (Bio-Rad). Fold changes in gene expression were calculated from the difference in threshold cycle (Ct) using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001). Three experimental replicates were per-

formed for each qRT-PCR experiment using three independent biological replicates.

Expression of *Rpp2* candidate genes in response to *P. pachyrhizi* infection

The primers used to confirm silencing of *Rpp2* candidate genes, KP1236 and KP1237 (Supplementary Table S4), were used to amplify genomic DNA of PI 230970 and Williams 82. As stated above, the primer annealing sites were identical across all seven full-length *R* genes in PI 230970. In addition, primer annealing sites were identical for four (*Rpp2C5_Wms82*, *Rpp2C6_Wms82*, *Rpp2C10_Wms82*, and *Rpp2C11_Wms82*) of the seven full-length *R* genes in Williams 82 (Supplementary Fig. S2). Small sequence differences in the priming sites were not expected to impact amplification of *Rpp2C1_Wms82*, *Rpp2C2_Wms82*, and *Rpp2C4_Wms82*. Importantly, these primers were selected because the amplified products would contain sequence differences unique to each *R* gene. The primers were used to amplify genomic DNA of PI 230970 and Williams 82. PCR products were cloned into pCR4-TOPO (Invitrogen, Thermo Fisher Scientific) and transformed into TOP10 Electrocompetent *E. coli* (Invitrogen, Thermo Fisher Scientific). Cloned DNA was prepared using the Wizard SV 96 Plasmid DNA purification system (Promega) and sequenced and analyzed at the U.S. Department of Agriculture–Agricultural Research Service CICGRU facility as described above. To assign subclones to specific *R* genes, BLASTN (Camacho et al. 2009) was used to compare consensus sequences for each individual clone with all *R* gene transcripts from the *Rpp2* locus in PI 230970 or Williams 82. Because the *Rpp2* *R* genes would be equally distributed in genomic DNA, differences in amplification efficiency would be reflected in the number of sequenced genomic clones corresponding to each *R* gene. Paired sequences of more than 100 genomic clones from each genotype were used to determine application efficiency for each *R* gene. To measure baseline expression of *Rpp2* candidate genes in PI 230970 and Williams 82, we collected leaves from mock-inoculated and infected PI 230970 and Williams 82 plants 7 days after inoculation. Leaf tissue was frozen in liquid nitrogen and stored at -80°C . RNA was isolated using the Qiagen Plant RNeasy kit, followed by Turbo DNase treatment (Ambion, Thermo Fisher Scientific). First-strand cDNA synthesis was performed with the Transcriptor High Fidelity first-strand cDNA synthesis kit (Roche). cDNA was used for PCR amplification and cloning as described above. Over 100 clones were sequenced for each genotype-by-treatment combination, and clones were assigned to *R* genes as described above. To correct expression differences due to amplification efficiency, the number of genomic clones for each gene was used to determine the amplification efficiency correction factor for each *Rpp2C* gene within each genotype by dividing the expected gene counts by observed gene counts (Supplementary Table S5). Because the primers amplified nine genes from genomic DNA of Williams 82, the expected representation for each gene would be 0.11. In PI 230970, eight genes were amplified from genomic DNA; therefore, the expected representation for each gene would be 0.125.

RNA-seq of *Rpp2* silenced plants

Three biological replicates of RNA from PI 230970 plants inoculated with VIGS construct BPMV:*Rpp2C6_Wms82* or the empty vector and inoculated with *P. pachyrhizi* isolate LA04-1 were used for RNA-seq analyses. The Iowa State University DNA facility prepared and sequenced RNA-seq libraries using the Illumina HiSeq 2500 platform, generating 100-bp reads. Raw sequences have been deposited at the National Center for Biotechnology Information Sequence Read Archive as BioProject accession PRJNA1314848. Adaptor sequences, low-quality

bases, and reads fewer than 50 bp in length were removed using Trimmomatic (Bolger et al. 2014). Reads were aligned to the Williams 82 reference genome sequence (version Wm82.a4.v1) using STAR version 2.7.10 (Dobin et al. 2013). SAMtools version 1.17 (Li et al. 2009) was used to identify uniquely mapping reads. Mapping files (.bam) were imported into RStudio version 4.4.1 (Posit Team 2024). rtracklayer (Lawrence et al. 2009) was used to import the gene feature file (GFF) corresponding to the reference genome, and summarizeOverlaps (Lawrence et al. 2013) was used to count the number of reads per sample aligning to each gene. ggplot2 (Wickham 2009) was used to visualize sample replicates to ensure technical reproducibility. Following data visualization, one BPMV:*Rpp2C6_Wms82* sample was removed from further analysis. Prior to data normalization and identification of DEGs, counts assigned to any *R* genes in the Williams 82 *Rpp2* locus were removed, as they could be of viral origin. All genes with counts per million <1 across all biological replicates were removed from further analysis. EdgeR (Robinson et al. 2010) was used to normalize data using TMM (Trimmed Mean of M values) and to conduct a single-factor comparison to identify DEGs, comparing BPMV:*Rpp2C6_Wms82* with empty vector samples using a false discovery rate < 0.05. DEGs were annotated using the SoyBase database Genome Annotation Report page (<https://legacy.soybase.org/genomeannotation/>), and significantly (corrected *P* value < 0.05) overrepresented biological process GO terms were identified using the SoyBase GO Term Enrichment tool (https://legacy.soybase.org/goslimgraphic_v2/dashboard.php).

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