Functional Analysis of the Asian Soybean Rust Resistance Pathway Mediated by *Rpp2*

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Asian soybean rust is an aggressive foliar disease caused by the obligate biotrophic fungus *Phakopsora pachyrhizi*. On susceptible plants, the pathogen penetrates and colonizes leaf tissue, resulting in the formation of necrotic lesions and the development of numerous uredinia. The soybean *Rpp2* gene confers resistance to specific isolates of *P. pachyrhizi*. *Rpp2*-mediated resistance limits the growth of the pathogen and is characterized by the formation of red-dish-brown lesions and few uredinia. Using virus-induced gene silencing, we screened 140 candidate genes to identify those that play a role in *Rpp2* resistance toward *P. pachyrhizi*. Candidate genes included putative orthologs to known defense-signaling genes, transcription factors, and genes previously found to be upregulated during the *Rpp2* resistance response. We identified 11 genes that compromised *Rpp2*-mediated resistance when silenced, including *GmEDS1*, *GmNPR1*, *GmPAD4*, *GmPAL1*, five predicted transcription factors, an O-methyl transferase, and a cytochrome P450 monooxygenase. Together, our results provide new insight into the signaling and biochemical pathways required for resistance against *P. pachyrhizi*.

Asian soybean rust (ASR) is an aggressive foliar disease caused by the obligate biotrophic fungus, *Phakopsora pachyrhizi* Syd. & P. Syd. Unlike most rust fungi, *P. pachyrhizi* has an unusually broad host range, infecting over 95 plant species from more than 42 genera, including soybean and related *Glycine* spp. (Bromfield 1984; Ono et al. 1992; Rytter et al. 1984). In regions where the pathogen is established, it can cause significant yield losses of 10 to 80% (Bromfield 1984; Ogle et al.

1979; Patil et al. 1997). *P. pachyrhizi* was first discovered in Asia in 1902 and has since spread to all the major soybean-producing regions of the world (Goellner et al. 2010). The discovery of ASR in the continental United States in the fall of 2004 has raised concern from U.S. soybean producers and has generated new interest in the biology and epidemiology of the disease (Li et al. 2010; Schneider et al. 2005).

Susceptible soybean plants infected with virulent isolates of *P. pachyrhizi* are characterized by tan-colored lesions and sporulating uredinia, predominantly on the abaxial leaf surfaces (tan phenotype) (Bromfield 1984; Bromfield and Hartwig 1980; Miles et al. 2006). Microscopic studies have revealed the detailed process of infection, which starts when a urediniospore on the leaf surface produces a germ tube, followed by the formation of an appressorium. In contrast to other rust fungi that gain entry into the leaf mesophyll cells via stomata, *P. pachyrhizi* penetrates directly into epidermal cells through the formation of a penetration hypha that emerges from the appressorium (Bromfield 1984; Koch et al. 1983). This penetration results in the collapse of the breached epidermal cell. As the invading hypha emerges from the collapsed cell, a primary hypha is separated from the penetration hypha by a septum and typically branches into secondary hyphae in the leaf mesophyll. Haustorial mother cells then form at the tips of the primary and secondary hyphae, giving rise to haustoria that invade living mesophyll cells. At the late stages, intense colonization of mesophyll tissue occurs and newly developed uredia originate from the uredial primordia (Koch et al. 1983).

The adapted commercial cultivars of soybean grown in the United States and abroad are susceptible to ASR. Germplasm screening and genetic analysis has led to identification of seven loci that provide varying degrees of resistance to *P. pachyrhizi* (*Rpp*). These seven loci are referred to as *Rpp1* (Cheng and Chan 1968; Hartwig and Bromfield 1983; Hidayat and Somaatmadja 1977; McLean and Byth 1980), *Rpp1b* (Ray et al. 2009), *Rpp2* (Hidayat and Somaatmadja 1977), *Rpp3* (Bromfield and Hartwig 1980), *Rpp4* (Hartwig 1986), *Rpp5* (Garcia et al. 2008), and *Rpp?* (*Huuga*) (Monteros et al. 2007). *Rpp1* is unique among these genes in that it confers an immune response to particular avirulent fungal isolates for which there are no visible symptoms or uredinia formation on the leaves (Miles et al. 2006). In contrast, plants containing any of the other *Rpp* genes identified to date produce dark red-dish-brown (RB) lesions at the site of infection in response to avirulent isolates of the pathogen. The RB lesions are correlated with restricted fungal growth and limited sporulation on the leaf surfaces (Bonde et al. 2006; Garcia et al. 2008).

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Several approaches have been taken to investigate the effects of ASR on soybean mRNA transcript levels in compatible and incompatible interactions. A suppressive subtractive hybridization screen of *Rpp1* plants inoculated with virulent and avirulent isolates of *P. pachyrhizi* was utilized to identify the transcripts that were differentially expressed during the immune reaction (Choi et al. 2008). Another set of studies suggested that the differential expression of soybean genes in response to a virulent ASR isolate may be related to the growth stages of the plant (Panthee et al. 2007, 2009). Molecular interactions between ASR and soybean were also assessed by gene expression profiles in resistant *Rpp2* plants (PI230970) and susceptible *rpp2* plants (Embrapa 48) over a time course covering 6 to 168 h after inoculation (hai) (van de Mortel et al. 2007). Two distinct phases of soybean gene expression occur in response to ASR infection. During the first 24 hai, a nonspecific response was identified in which differential expression of soybean genes reached a maximum in the first 12 hai in the compatible and incompatible genotypes before returning to baseline levels. A second phase of differential gene expression was observed beginning at 72 hai in the *Rpp2* genotype but not until later time points in the *rpp2* genotype. The kinetics of gene expression changes suggested that race-specific recognition of the ASR isolate had occurred in *Rpp2* plants, leading to an effective defense response. Collectively, the studies related to soybean gene expression in response to *P. pachyrhizi* have led to the identification of several functional classes of genes that are upregulated during infection, including those associated with defense signaling, regulation of mRNA transcription (WRKY and MYB transcription factors) (TF), primary and secondary metabolism, and cell wall synthesis and reinforcement. Interestingly, all the studies have reported that genes involved in the phenylpropanoid biosynthetic pathway are highly upregulated during ASR infection, suggesting a role for products of this pathway in ASR defense (Choi et al. 2008; Panthee et al. 2009; van de Mortel et al. 2007).

The availability of a virus-induced gene silencing (VIGS) system for use in soybean coupled with the completed genome sequence has made it possible to functionally analyze genes that may play a role in defense against *P. pachyrhizi* (Schmutz et al. 2010; Zhang et al. 2009, 2010). VIGS has been successfully employed as a functional genomics tool for rapidly and effectively testing the functions of genes in defense responses in many plant–pathogen interactions, including soybean (Chen et al. 2009; Ekengren et al. 2003; Fradin et al. 2009; Fu et al. 2009; Kachroo et al. 2008; Liu et al. 2002; Zhang et al. 2009). *Bean pod mottle virus* (BPMV)-based vectors have been used successfully for sequence-specific gene silencing and for stable protein expression in soybean (Kachroo et al. 2008; Zhang and Ghabrial 2006; Zhang et al. 2009, 2010). With respect to plant–pathogen interactions, BPMV-based VIGS vectors were used to silence putative orthologs of the *Arabidopsis* *AtRAR1*, *AtSGT1*, and *AtHSP90* genes in soybean that were shown to mediate three different modes of immunity, including pathogen-associated molecular pattern-triggered, effector-triggered, and systemic acquired immunity against *Pseudomonas syringae* (Fu et al. 2009). BPMV-based vectors are also effective tools for studying resistance to *Phakopsora pachyrhizi*, because VIGS combined with genetic mapping and gene expression analyses were used to identify the gene that confers the *Rpp4* resistance trait (Meyer et al. 2009).

The gene expression profiling experiments that have been conducted coupled with knowledge of genes that mediate defense signaling in other plants provides an extensive source of candidate genes to test for functions in soybean defenses to ASR. Here, we utilized a BPMV-based VIGS approach to identify genes that are necessary for *Rpp2*-mediated defense

against ASR. The *Rpp2* resistance trait was selected for these analyses based on the availability of a gene expression profiling dataset (van de Mortel et al. 2007). This gene expression information along with the sequences of soybean homologs of defense signaling genes was used to construct BPMV-based VIGS vectors targeting 140 genes potentially involved in the *Rpp2*-mediated defense response. The BPMV constructs were used to establish gene silencing in a soybean accession containing *Rpp2* (PI2309870). The silenced plants were tested with an avirulent isolate of *P. pachyrhizi* to determine which of the genes played a role in resistance. Using this experimental approach, we have identified 11 genes that are required for *Rpp2*-mediated resistance against ASR.

RESULTS

Silencing of soybean orthologs of defense-related genes.

We screened BPMV VIGS constructs targeting 140 genes for their ability to compromise *Rpp2*-mediated resistance and identified 11 genes that resulted in loss-of-resistance phenotypes when silenced (Table 1; Supplementary Table S1). We began our screen by focusing on putative soybean orthologs of *Arabidopsis* genes with known roles in defense against microbial pathogens. The amino acid sequences of the candidate *Arabidopsis* proteins were used to conduct tblastn (Altschul et al. 1990) searches against the soybean expressed sequence tag (EST) contigs assembled by The Institute for Genome Research (TIGR). Soybean sequences with the highest levels of identity to their *Arabidopsis* counterparts were used to construct VIGS vectors for silencing genes in soybean plants containing *Rpp2* (PI230970). If a silenced gene was required for resistance, we expected tan lesions indicative of a susceptible response to develop on silenced *Rpp2* plants following inoculation with the avirulent *P. pachyrhizi* isolate LA04-1. This would be consistent with our previous work using VIGS to compromise *Rpp4*-mediated resistance (Meyer et al. 2009).

Functional screening of known defense signaling genes resulted in identification of *GmEDS1* (*enhanced disease susceptibility 1*), *GmPAD4* (*phytoalexin-deficient 4*), and *GmNPR1* (*nonexpresser of PR genes 1*) as essential components of *Rpp2*-mediated defense signaling. Silencing of these genes resulted in localized chlorotic areas and tan lesions with visible uredinia on the leaf surface, indicative of a loss of resistance (Fig. 1). Total RNA was extracted from the leaves of plants displaying the tan lesion phenotypes in order to confirm systemic infection by BPMV constructs, to test whether mRNA transcript levels of the target genes were reduced, and to quantify the growth of *P. pachyrhizi*. Reverse-transcriptase

Table 1. Eleven genes showing loss-of-resistance phenotypes when silenced in *Rpp2* plants

Gene name	Gene ID	Plants with tan lesions (%) ^a
<i>GmEDS1</i>	Glyma06g19920	100
<i>GmPAD4</i>	Glyma13g04540	100
<i>GmPAL1</i>	Glyma02g47940	92
<i>GmO-MT</i>	Glyma07g05480	72
<i>GmCYP83E12</i>	Glyma03g03520	70
<i>GmWRKY36</i>	Glyma13g38630	50
<i>GmNPR1</i>	Glyma15g13320	50
<i>GmDBTF</i>	Glyma01g02990	60
<i>GmWRKY40</i>	Glyma08g15210	41
<i>GmWRKY45</i>	Glyma04g39620	37
<i>GmMYB84</i>	Glyma08g04670	37

^a Percentage of plants on which tan lesions were observed at 14 days after Asian soybean rust inoculation on at least one leaf in three independent VIGS trials (four to six plants were tested per trial).

Fig. 1. Loss of *Rpp2*-mediated resistance to *Phakopsora pachyrhizi* following silencing of 11 target genes identified by a virus-induced gene silencing (VIGS) screen. Plants were infected with *Bean pod mottle virus* (BPMV) VIGS vectors carrying approximately 300-bp cDNA fragments corresponding to the indicated target genes. Silenced *Rpp2* plants were inoculated with *P. pachyrhizi* isolate LA04-1 at 3 weeks after BPMV inoculation, and leaves were photographed 2 weeks later. Loss of resistance was observed when *GmEDS1*, *GmPAD4*, *GmNPR1*, *GmWRKY36*, *GmWRKY40*, *GmWRKY45*, *GmDBTF*, *GmMYB84*, *GmO-MT*, *GmPAL1*, and *GmCYP83E12* were silenced. Vector indicates *Rpp2* plants that were treated with the BPMV RNA2 without insert, which did not affect the reddish-brown lesion phenotype. Infected Williams 82 plants showing the susceptible tan phenotype served as a susceptible control.

polymerase chain reaction (RT-PCR) using primers to detect BPMV confirmed that the virus had spread systemically in experimental and empty vector control plants (data not shown). The relative abundance of mRNA transcripts for each target

Fig. 2. Downregulation of target gene expression by *Bean pod mottle virus* virus-induced gene silencing in leaves with *Rpp2* loss-of-resistance phenotypes. Total RNA was isolated from mock, vector control, and silenced plants at 2 weeks after *Phakopsora pachyrhizi* inoculation. Low-cycle reverse-transcriptase polymerase chain reaction (RT-PCR) (21 to 25 cycles) was performed using gene-specific primers, and products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The last panel shows the *Elf-1b* RT-PCR product that served as a control. At least two RT-PCR reactions were performed for every gene-silenced plant, with similar results.

gene was determined by low-cycle RT-PCR (Fig. 2) using primers designed to specifically amplify mRNA transcripts from each of the soybean target genes (Supplementary Table S2). In *Rpp2* plants displaying a loss-of-resistance phenotype, we observed a reduction in the abundance of the mRNA transcripts of the target genes when compared with mock- or empty BPMV vector-treated plants (Fig. 2). In both mock- and empty vector-inoculated plants, the transcripts were expressed at similar levels.

Because the loss-of-resistance phenotype in the *Rpp2* plants was expected to be accompanied by an increase in fungal growth, we used quantitative RT-PCR (QRT-PCR) to determine relative levels of *P. pachyrhizi* accumulation in control and VIGS-treated plants. The amount of *P. pachyrhizi* α -tubulin transcript in each sample was normalized to the soybean *ubiquitin* gene to provide an estimate of the relative ratio of fungal mRNA to leaf mRNA in each sample. An increase in the ratio is an indicator of fungal growth (Meyer et al. 2009; van de Mortel et al. 2007). In *GmEDS1*- and *GmPAD4*-silenced plants, the accumulation of *P. pachyrhizi* α -tubulin transcript was four times higher than the vector-treated *Rpp2* plants (Fig. 3). Similarly, the accumulation of α -tubulin transcripts in *GmNPR1*-silenced plants was 2.5 times higher compared with empty vector-treated *Rpp2* plants. Collectively, these results show a correlation between the increase in fungal growth and the appearance of tan lesions in the *Rpp2* genetic background, demonstrating that silencing *GmEDS1*, *GmPAD4*, and *GmNPR1* resulted in loss of resistance.

Silencing WRKY TF in *Rpp2* plants compromises disease resistance.

Previous gene expression studies of compatible and incompatible interactions between ASR and *Rpp2* plants led to the identification of several different WRKY TF that were differentially regulated during infection (van de Mortel et al. 2007). The WRKY TF represent a well-established plant gene super-

Fig. 3. Accumulation of Asian soybean rust (ASR) α -tubulin mRNA transcripts in vector control and the virus-induced gene silencing-treated PI230970 plants that had tan lesion phenotypes. Transcript levels of *Phakopsora pachyrhizi* α -tubulin in ASR-infected leaves were plotted relative to soybean *ubiquitin-3* expression levels as determined by quantitative reverse-transcriptase polymerase chain reaction. Values sharing the common letters are not significantly different at $P < 0.05$.

family, and several individual WRKYs have demonstrated roles in various plant defense-signaling pathways (Rushton et al. 2010). In the current study, we silenced 64 soybean WRKY TF to test their involvement in *Rpp2*-mediated resistance. Here, we have used the naming conventions for soybean WRKY TF as defined by Zhou and associates (2008). The screen resulted in the identification of three WRKY TF (*GmWRKY36*, *GmWRKY40*, and *GmWRKY45*) which compromised *Rpp2* resistance when silenced, as evidenced by the tan phenotype (Fig. 1). An additional gene, referred to in this study as

GmDBTF, was also selected for silencing based upon its expression profile in the previous microarray study (van de Mortel et al. 2007). Although *GmDBTF* was previously annotated as a WRKY TF, the predicted protein lacks the signature WRKY DNA-binding domain. However, it does contain a C₂H₂ domain similar to those found in other WRKY proteins (Rushton et al. 2010; Zhou et al. 2008). Plants inoculated with BPMV constructs containing a portion of the *GmDBTF* gene also had compromised *Rpp2* resistance (Fig. 1). Low-cycle RT-PCR analysis using gene-specific primers for the individual

Fig. 4. Expression of genes containing W-boxes in their promoters in *GmWRKY36*- and *GmDBTF*-silenced plants. These genes were selected based on the observation that promoter sequences contained four or more W-boxes. Low-cycle reverse-transcriptase polymerase chain reaction (RT-PCR) (21 to 25 cycles) was performed using gene-specific primers, and RT-PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The last panel shows the *Elf-1b* RT-PCR product that served as a control. At least two RT-PCR reactions were performed for every gene-silenced plant, with similar results.

Table 2. Genes that are upregulated in *Rpp2* resistance responses and have four or more W-boxes in their promoters

Gene ID ^a	Annotated function	W-box positions ^b
Glyma01g43420.1	<i>GmWRKY12</i>	-23, -416, -726, -883, -974
Glyma10g34540.1	<i>GmPROT1</i> (proline transporter)	-30, -100, -311, -815, -899
Glyma11g29720.1	<i>GmWRKY19</i>	-76, -350, -789, -929, -964
Glyma02g39210.1	<i>GmSIZF</i> (salt induced zinc finger ankyrin repeats)	-849, -855, -897, -972
Glyma06g01110.1	<i>GmZincGata</i> Zn GATA TF	-212, -337, -434, -552
Glyma15g01990.1	Hypothetical protein with CYC RICH DOMAIN	-42, -228, -480, -485, -565
Glyma08g14270.1	Hypothetical protein	-480, -521, -806, -943
Glyma13g27060.1	<i>GmFMO</i> (flavin-monooxygenase)	-115, -174, -244, -867
Glyma07g17170.1	<i>GmLACBp</i> laccase binding	-429, -496, -599, -747, -840
Glyma04g01090.1	<i>GmGata</i> (GATA TF)	-89, -300, -415, -504, -626
Glyma02g47940.1	<i>GmPAL1</i>	-113, -463, -741, -778
Glyma13g30570.1	<i>GmNUDX15</i> (nudix hydrolase ATNUDX15)	-345, -869, -910, -959
Glyma02g39870.1	<i>GmWRKY39</i>	-175, -244, -261, -528
Glyma07g09960.1	<i>GmCYP450</i>	-195, -535, -689, -777, -822
Glyma02g16740.1	Phenazine biosynthesis	-4, -100, -881, -967
Glyma15g06510.1	Oligopeptide transporter-4	-95, -341, -349, -924
Glyma13g00580.1	Serine-type peptidase subtilisin like	-81, -361, -531, -768
Glyma12g06460.1	Zinc finger protein c3hc4	-32, -186, -270, -991
Glyma06g46550.1	Strictosidine synthase	-118, -590, -635, -670
Glyma06g47560.1	Derlin-2, putative Rhomboid superfamily	-108, -169, -209, -217
Glyma20g25990.1	Hypothetical protein	-171, -303, -401, -821
Glyma15g04160.1	GRAS domain pyroglutamyl peptidase	-355, -733, -745, -885
Glyma07g31700.1	Protein kinase	-352, -536, -579, -793
Glyma03g00830.1	MATE efflux family protein	-48, -257, -479, -545
Glyma15g18880.1	Hypothetical protein (MAPEG superfamily)	-69, -436, -518, -795
Glyma09g24170.1	Hypothetical protein	-69, -436, -518, -795
Glyma02g34940.1	Hypothetical protein	-304, -812, -822, -832

^a Genes were identified from gene expression data published by van de Mortel and associates (2007).

^b Nucleotide position of W-boxes with respect to the known or predicted ATG start codon.

GmWRKY genes and *GmDBTF* was performed to confirm a reduction of the targeted gene transcripts in silenced plants (Fig. 2), and levels of fungal mRNA were measured and found to correlate with the observed tan phenotypes (Fig. 3). The accumulation of fungal α -tubulin transcripts was 1.5 to 2.0 times higher in *GmDBTF*-, *GmWRKY40*-, and *GmWRKY45*-silenced plants compared with the empty vector control plants and 3 times higher in *GmWRKY36*-silenced plants.

The members of the WRKY TF superfamily are characterized by a conserved DNA-binding region known as the WRKY domain, which mediates recognition and binding to DNA elements referred to as W-boxes (C/TTGACT/C) (Pandey and Somssich 2010). Some WRKY TF regulate the expression of pathogenesis-related (*PR*) genes (Eulgem and Somssich 2007; Eulgem et al. 2000) that often contain multiple W-boxes within their promoters (Maleck et al. 2000). *GmPR1* (Glyma13g32540) was induced in *Rpp2* plants when challenged with *P. pachyrhizi* (van de Mortel et al. 2007); therefore, we tested expression of *GmPR1* in *Rpp2* plants silenced with *GmWRKY36* or *GmDBTF*. Interestingly, we observed that the levels of *GmPR1* expression in response to isolate LA04-1 were lower in plants silenced for *GmWRKY36* compared with *GmDBTF* and controls (Fig. 4). To further identify genes that are potentially regulated by these WRKY TF, we compiled a list of 635 unique soybean genes that were previously shown to be induced during an incompatible *Rpp2*–ASR interaction (van de Mortel et al. 2007). We then searched the promoter regions of these genes and identified 27 with four or more W-boxes (Table 2). The expression of a subset of these potential target genes was analyzed in the leaves of *Rpp2* plants silenced for either *GmWRKY36* or *GmDBTF* and that displayed a tan phenotype. *GmWRKY36* and *GmDBTF* were selected for this analysis because they showed the strongest loss-of-resistance phenotypes of the TF tested. Additionally, we also wanted to

test the putative role of *GmDBTF* as a TF by studying genes it may regulate. The RT-PCR results showed that the expression of *GmWRKY12*, *GmWRKY19*, and *GmPROT1* (proline transporter) was compromised when either *GmWRKY36* or *GmDBTF* was silenced (Fig. 4). The expression of some of these genes was differentially affected depending on whether *GmWRKY36* or *GmDBTF* was silenced. The expression of *GmFMO* (flavine mono-oxygenase), *GmO-MT*, and *GmPR1* was only compromised in *GmWRKY36*-silenced *Rpp2* plants whereas the expression of *GmPAL1* was only compromised in *GmDBTF*-silenced plants. These results indicate that both *GmWRKY36* and *GmDBTF* contribute to *Rpp2*-mediated resistance, presumably by regulating the expression of these and other genes during ASR infection.

The phenylpropanoid pathway contributes to *Rpp2*-mediated resistance.

The phenylpropanoid pathway provides precursors for lignin biosynthesis and for the production of antimicrobial metabolites. Lignin is a complex phenolic compound produced by plants to fortify cell walls and is also produced in response to pathogens (Bhuiyan et al. 2007, 2009; Vance et al. 1980). Two key steps in the production of lignin include the conversion of phenylalanine to *trans*-cinnamic acid by phenylalanine ammonia lyase (PAL) and the transfer of a methyl group from caffeic acid to ferulate by *o*-methyltransferases (*O*-MT). In soybean, *GmO-MT* and *GmPAL1* were previously shown to be upregulated during *Rpp2*-mediated resistance, suggesting their possible involvement in the process of lignification (van de Mortel et al. 2007). Our low-cycle RT-PCR data for *GmO-MT* and *GmPAL1* indicated the down regulation of these genes in *GmWRKY36*- and *GmDBTF*-silenced *Rpp2* plants, respectively (Fig. 4). Based on these observations, we speculated that both *GmO-MT* and *GmPAL1* may play a role in *Rpp2*-mediated resistance. We then tested the effect of silencing these genes on the phenotypic response in *Rpp2* plants challenged with *P. pachyrhizi*. Our results demonstrated a loss of resistance when

Fig. 5. Total lignin content in *Rpp2* plants in which *GmO-MT* and *GmPAL1* were silenced. The relative abundance of acetyl bromide soluble lignin in *Rpp2* plants infected with empty *Bean pod mottle virus* vector was set to 100% for comparison with the lignin content of leaves treated with virus-induced gene silencing constructs for silencing *GmO-MT* or *GmPAL1*. Leaves were harvested at 14 days after *Phakopsora pachyrhizi* inoculation, and only leaves with loss-of-resistance phenotypes were collected for the *GmO-MT* and *GmPAL1* samples. Values sharing the common letters are not significantly different at $P < 0.05$.

Fig. 6. Expression of phenylpropanoid pathway genes in *GmMYB84*-silenced plants. Genes were selected based on the criteria that promoter sequences contain at least one AC element, and they are known or predicted to have roles in the phenylpropanoid pathway. Low-cycle reverse-transcriptase polymerase chain reaction (RT-PCR) (21 to 25 cycles) was performed using gene-specific primers, and RT-PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The last panel shows the *Elf-1b* RT-PCR product that served as a control. At least two RT-PCR reactions were performed for every gene-silenced plant, with similar results.

either of the genes was silenced in *Rpp2* plants (Fig. 1). Low-cycle RT-PCR using gene-specific primers confirmed a reduction in the transcript levels of *GmO-MT* and *GmPAL1* in leaves showing the loss-of-resistance phenotype (Fig. 2). As expected, loss of resistance was accompanied by increased uredinia formation on the silenced leaves. QRT-PCR analysis of *P. pachyrhizi* α -tubulin showed a fourfold increase in *GmPAL1*-silenced leaf tissue and a threefold increase in *GmO-MT*-silenced plants (Fig. 3). O-MT have central roles in phenylpropanoid biosynthetic pathways through their involvement in modifications of chalcone, isoflavonoids, and metabolic precursors of lignin (Ferrer et al. 2008). We detected a 30% decrease in the total lignin content in *GmO-MT*-silenced plants compared with empty vector-treated control plants (Fig. 5). These results implicate this particular soybean O-MT in lignin accumulation and indicate that lignin production is an important component of *Rpp2*-mediated resistance. Interestingly, no significant changes in the lignin content were observed in *GmPAL1*-silenced plants compared with empty vector-treated control plants.

The involvement of *GmMYB84* in *Rpp2* resistance.

Collectively, the MYB proteins compose a superfamily of TF involved in the regulation of several plant-specific developmental and physiological responses, including phenylpropanoid metabolism (Mehrtens et al. 2005; Stracke et al. 2001). In the current screen, we tested VIGS constructs targeting 20 different MYB genes and, of these, silencing *GmMYB84* in *Rpp2* plants resulted in a loss-of-resistance phenotype (Fig. 1). Low-cycle RT-PCR analysis using gene-specific primers for

Fig. 8. Proposed framework for the *Rpp2*-mediated defense response. *GmEDS1*, *GmPAD4*, and *GmNPR1* control the upstream defense network. *GmWRKY* TFs, *GmDBTF*, and *GmMYB84* function at an intermediate level to regulate pathogenesis-related genes as well as those of the phenylpropanoid pathway. The red-brown text indicates genes that were shown by virus-induced gene silencing to be required for *Rpp2* resistance.

Fig. 7. Expression analysis of virus-induced gene silencing target genes that showed loss-of-resistance phenotypes following Asian soybean rust infection in PI230970 (*Rpp2*) and Williams82 (*rpp2*) plants. Total RNA was isolated from *Phakopsora pachyrhizi*-inoculated leaves that were collected at the time points indicated. Low-cycle reverse-transcriptase polymerase chain reaction analysis was performed for the expression analysis by using gene-specific primers.

GmMYB84 confirmed a reduction in transcript level in the VIGS plants compared with the mock and vector control plants (Fig. 2). An increase of 2.0 to 2.5 times in *P. pachyrhizi* α -tubulin transcripts in leaves showing loss-of-resistance phenotypes further demonstrated that fungal growth was increased (Fig. 3).

Genes that are regulated by MYB proteins contain AC elements (-GCCTACC- and -ACCTACA-) in their promoters, and a variety of phenylpropanoid genes are regulated by these TF (Hernandez et al. 2004; Mellway et al. 2009). Phylogenetic analysis of the predicted protein sequence for *GmMYB84* indicates that it belongs to the R2R3 class of MYB proteins and has a conserved DNA binding domain (data not shown). R2R3 MYB proteins from *Petunia* $\times$ *hybrida* and *Vitis vinifera* have been shown to play roles in the regulation of phenylpropanoid biosynthesis (Deluc et al. 2006; Quattrocchio et al. 2006). We tested the expression of six genes that are known or predicted to be involved in the phenylpropanoid pathway using low-cycle RT-PCR. Our results showed that four of the six genes tested were downregulated in *GmMYB84*-silenced plants, including PAL (*GmPAL1*), 4-coumarate-CoA-ligase (*Gm4-CL*), cinnamate-4-hydroxylase (*GmC4H*), and chalcone synthase (*GmCHS*) (Fig. 6). Expression levels of *GmO-MT* and *GmIOMT* (isoflavone O-methyl transferase) were unaffected in *GmMYB84*-silenced plants. These results suggest the potential role of *GmMYB84* as a regulator for some phenylpropanoid genes but not for *GmO-MT* or *GmIOMT*.

Silencing of *GmCYP83E12* results in a loss-of-resistance phenotype.

Cytochrome P450 monooxygenases (CYP450s) mediate a wide range of oxidative reactions involved in the biosynthesis of plant secondary metabolites, including phenylpropanoid metabolism (Ferrer et al. 2008). VIGS of *GmCYP83E12* in *Rpp2* plants resulted in a mix of tan and RB phenotypes (Fig. 1). The mixed phenotypes could result from nonuniform silencing, or another possibility could be that this gene makes a minor contribution to *Rpp2*-mediated resistance. Gene-specific RT-PCR confirmed the reduction in transcript levels of this gene (Fig. 2). Quantification of *P. pachyrhizi* α -tubulin demonstrated a significant increase in fungal growth (1.5 times) compared with empty vector controls (Fig. 3).

Expression profiles of VIGS target genes following *P. pachyrhizi* infection.

To determine whether the genes that compromised *Rpp2*-mediated resistance when silenced are differentially regulated during *P. pachyrhizi* infection, we tested the mRNA expression after 0, 12, 24, 48, 72, 144, 192 h in PI230970 (*Rpp2*) and susceptible Williams 82 plants. Low-cycle RT-PCR was performed to evaluate the relative accumulation of the mRNA transcripts in the two genotypes after inoculation. We observed rapid induction of transcript accumulation for all the candidate genes except for *GmWRKY40* and *GmWRKY45* in the *Rpp2* plants compared with the susceptible Williams 82 control (Fig. 7). It is possible that the changes in expression of *GmWRKY40* and *GmWRKY45* are outside the resolution of the low-cycle RT-PCR.

DISCUSSION

Soybean resources such as the genome sequence (Schmutz et al. 2010), extensive EST collections (Shoemaker et al. 2002), and microarray expression profiling data (van de Mortel et al. 2007) were used in conjunction with an efficient VIGS tool to begin developing a genetic framework for *Rpp2*-based resistance against ASR. We used VIGS to test the possible

roles of 140 candidate soybean genes in *Rpp2*-mediated resistance to ASR. The candidate genes were selected on the basis of sequence similarity to genes required for plant defenses in model plants and differential regulation as determined by DNA microarray analyses of the *Rpp2*-mediated resistance response to ASR (van de Mortel et al. 2007). Based on the loss-of-resistance phenotypes, we identified 11 different genes that contribute to *Rpp2*-based resistance (Fig. 8). When the loss-of-resistance phenotypes are considered in the context of molecular and biochemical information as well as knowledge from model plant-pathogen interactions, we can begin to develop the network of genes that mediate *Rpp2* signaling and that contribute directly in ASR resistance (Fig. 8; Table 1).

The *Rpp2* locus has been mapped to linkage group J but the gene has not been cloned (Silva et al. 2008). Three different clusters of putative resistance (*R*) genes have been identified based upon the genomic sequence of susceptible cv. Williams 82. Cluster 1 encodes homologs of the *Vf* locus in apple, which encodes receptor-like leucine-rich repeat (LRR) proteins that confer resistance to apple scab; and clusters 2 and 3 encode two different families of Toll interleukin I receptor (TIR) nucleotide-binding site (NBS) LRR proteins (M. A. Graham, unpublished). Interestingly, we found that *EDS1* and *PAD4*, which participate in both basal and R-protein-mediated resistance to various pathogens in other plant species (Aarts et al. 1998; Fradin et al. 2009; Glazebrook 2001; Parker et al. 1996; Peart et al. 2002; Xiao et al. 2005), were necessary for *Rpp2* function. In most cases, R proteins requiring the functions of *EDS1* and *PAD4* belong to the TIR-NBS-LRR family (Meyers et al. 1999). *EDS1* and *PAD4* encode lipase/esterase-like proteins (Falk et al. 1999; Jirage et al. 1999) and function within the same defense pathways that regulate salicylic acid (SA) accumulation (Feys et al. 2001; Zhou et al. 1998).

Some *R* genes, such as *RPW8* and *HRT* from *Arabidopsis*, are dependent upon *EDS1* but are independent of *NPR1* (Chandra-Shekara et al. 2004; Xiao et al. 2005). Our finding that silencing *GmNPR1* caused a susceptible phenotype demonstrates that *Rpp2* initiates an *NPR1*-dependent signaling pathway. This observation further suggests that SA has a role in mediating *Rpp2* resistance, and it is consistent with previous studies demonstrating that *EDS1* and *PAD4* are involved in a defense augmentation loop that is regulated by SA (Rusterucci et al. 2001), and that *NPR1* is central to SA signal transduction (Cao et al. 1997). The ability of *GmNPR1* to restore proper resistance responses to *Arabidopsis npr1* mutants shows that its function is conserved between soybean and *Arabidopsis* (Sandhu et al. 2009).

We used two different VIGS constructs in our efforts to silence *GmNPR1*. The first construct, named 1023, which was not as effective, targeted bases 756 to 1,045 of the *GmNPR1* open reading frame. The second construct, named *NPR1-3'*, that was used to obtain the loss-of-function phenotypes, targeted bases 1,245 to 1,539 proximal to the 3' end of the open reading frame. These results are consistent with a previous observation that target sequences near the 3' end of the open reading frame are most effective in the BPMV vector (Zhang et al. 2010). For example, the 3' end of the open reading frame of the *GmPDS* gene was more effective as a target sequence than the 5' end of the open reading frame (Zhang et al. 2010). In addition, these results demonstrate that the inability of a BPMV VIGS clone to alter a phenotype does not necessarily eliminate a gene as being necessary for expression of that phenotype. Unfortunately, it was not practical to test multiple constructs for each of the 140 target genes selected for this study. Repeated *GmNPR1*-silencing experiments yielded the mixed RB and tan phenotype. This partial breakdown in resistance might also reflect the fact that utilizing VIGS does not ensure a

uniform silencing of the targeted gene. Another possibility is that *GmNPR1* may have a limited role in *Rpp2*-based defense signaling, although this seems unlikely.

Downstream of *GmEDS1*, *GmPAD4*, and *GmNPR1* in our model of the *Rpp2* resistance pathway are WRKY and MYB TF (Fig. 8). WRKY TF are plant-specific TF well known for their roles in regulating various physiological processes, including defense responses (Eulgem et al. 2000; Maleck et al. 2000). Here, we screened 64 *GmWRKY* TF for their roles in *Rpp2*-mediated resistance and, of these, constructs targeting *GmWRKY36*, *GmWRKY40*, and *GmWRKY45* caused loss-of-resistance phenotypes. A potential complication with gene silencing is that genes sharing sufficient sequence identity may be unintentionally silenced (Jackson et al. 2003). A comparison of the nucleotide sequences of *GmWRKY36* with *GmWRKY45* and *GmWRKY40* showed that they share identity of only 38 and 40%, respectively, at the nucleotide level. Additionally, they do not share smaller 21- to 24-bp stretches of identity, which renders off-target silencing improbable (Xu et al. 2006). The putative ortholog of *GmWRKY36* in *Arabidopsis* is *WRKY6*. *AtWRKY6* regulates expression of pathogen defense-associated genes such as *PR1*, and it is involved in regulation of genes involved in senescence (Robatzek and Somssich 2002). Our results suggest a conserved role for *GmWRKY36* and *AtWRKY6* in pathogen defense, although this remains to be tested.

GmWRKY40 and *GmWRKY45* share 84% identity at the nucleotide level, and low-cycle RT-PCR indicates that expression of both genes is reduced in the presence of either VIGS construct (data not shown). This observation demonstrates that soybean genes with at least 84% nucleotide identity can be co-silenced by the BPMV vector system. Thus, it is not possible at this time to conclude definitively whether *GmWRKY40*, *GmWRKY45*, or both are required for *Rpp2* resistance. Reduced mRNA expression of these WRKY TF resulted in robust loss-of-resistance phenotypes in *Rpp2* plants. Previous work suggested that *GmWRKY40* and *GmWRKY45* are moderately induced when soybean is subjected to drought and salt stress (Zhou et al. 2008), suggesting a possible role in abiotic stress responses. Our results clearly show that these TF have a functional role in defense against ASR. Studies in poplar have also supported a role for WRKY TF in rust disease resistance. For example, poplar *WRKY23* is involved in resistance to poplar rust by causing deregulation of genes that disrupt redox homeostasis and cell wall metabolism (Levee et al. 2009). The closest soybean homolog to *Populus tremuloides* *WRKY23* is *GmWRKY51*, construct no. 70) but no phenotypic change was observed on *Rpp2* plants treated with the corresponding VIGS construct.

We found that soybean defense responses were disrupted in *GmWRKY36*-silenced plants. *GmPR1* was not induced in *GmWRKY36*-silenced plants that were infected with *P. pachyrhizi*, suggesting that it is involved in regulating expression of PR genes in soybean. The presence of multiple W-boxes in the promoter region of *GmPR1* indicates that it is a potential target for *GmWRKY36*. Based on misexpression of *GmPR1* in *GmWRKY36*-silenced plants, we also examined the regulation of other rust-responsive genes with W-boxes in their promoters. RT-PCR analysis demonstrated that a subset of genes possessing W-boxes was regulated by *GmWRKY36* while others were unaffected. Interestingly, silencing *GmDBTF* and *GmWRKY36* affected the expression of *GmWRKY12*, *GmPROT1*, and *GmWRKY19*. However, only *GmDBTF* silencing affected expression of *GmPAL1*, and only *GmWRKY36* silencing affected expression of *GmO-MT*, *GmSIZF*, *GmFMO*, and *GmPR1*. These results suggest that *GmWRKY36* and *GmDBTF* may function cooperatively to regulate the expression of some genes and function independently of one another

to regulate the expression of others. It will be interesting to further characterize the groups of genes that fall into these three regulatory classes and decipher the underlying mechanisms controlling their mRNA expression.

PAL1 catalyzes the first step of the pathway for the synthesis of phenolic phytochemicals known collectively as phenylpropanoids. Phenylpropanoid pathway genes that are induced during defense responses can play important roles in disease resistance in many plant species (Graham et al. 2007; Soria-Guerra et al. 2010). Several genes involved in the phenylpropanoid pathway, including *GmPAL*, were highly upregulated in *Rpp2* soybean plants following ASR infection (van de Mortel et al. 2007). Furthermore, recent metabolic profiling revealed increased accumulation of isoflavonoids and flavonoids in all the resistant soybean genotypes during ASR infection when compared with susceptible genotypes (Lygin et al. 2009). *Rpp2* plants in which *GmPAL1* was silenced had a susceptible phenotype suggesting a role for phenylpropanoids in ASR defense. Interestingly, some phenolic compounds derived from the phenylpropanoid pathway inhibited germination of *P. pachyrhizi* spores (Lygin et al. 2009). Collectively, these observations point to an essential role of the phenylpropanoid pathway in *Rpp2* resistance.

The process of lignification requires metabolites from a branch point in the phenylpropanoid pathway. The accumulation of lignin or lignin-like phenolics is well studied during plant defense responses (Nicholson and Hammerschmidt 1992; Vance et al. 1980). O-methyl transferases are involved in methylation of ferulic and sinapic acids, both of which are precursors for lignin biosynthesis. Silencing of *GmO-MT* compromised *Rpp2*-based disease resistance, and biochemical analysis demonstrated that the total lignin content was reduced. However, no significant changes in total lignin content were observed in *GmPAL1*-silenced leaf tissues despite showing a loss-of-resistance phenotype. This might be due to the presence of other *PAL* coding sequences in the soybean genome. Sequence searches revealed two additional *PAL* coding genes (Glyma03g338801 and Glyma10g06600), raising the possibility that each has a distinct role in phenylpropanoid metabolism. Biochemical studies of *Atpal1* and *Atpal2* mutants in *Arabidopsis* suggested overlapping and independent functions of these genes (Rohde et al. 2004). In *Populus tremuloides*, *PtPAL1* was connected with the biosynthesis of condensed tannin and other phenolics, whereas *PtPAL2* is mostly associated with lignin biosynthesis (Kao et al. 2002). These studies show that *PAL1* paralogs may have distinct and overlapping functions in phenylpropanoid pathways, and suggest that further studies of the roles of specific *PAL1* isoforms are warranted in soybean.

The *GmO-MT* transcript levels were reduced in *GmWRKY36*-silenced plants, suggesting that *GmO-MT* transcription is regulated by *GmWRKY36*. A role of this soybean WRKY in regulating expression of a lignin biosynthetic gene would be consistent with other transgenic and biochemical studies that have implicated WRKY TF in regulating lignification (Naoumkina et al. 2008). Consistent with this idea, overexpression of rice *OsWRKY89* caused greater lignification and enhanced resistance to rice blast fungus (Wang et al. 2007). Our data, combined with knowledge from other studies, support the idea that upregulation of phenylpropanoid-related pathways and lignin reinforcement of cell walls are plant defense mechanisms that are critical for *Rpp2*-mediated disease resistance to ASR.

Another gene we identified that is required for *Rpp2*-based resistance is *GmCYP83E12*, which belongs to the CYP450 family of monooxygenases. CYP450s are involved in primary metabolism as well as biosynthesis of plant secondary metabolites, including phytoalexins such as those derived from phe-

nylpropanoid precursors (Ehrling et al. 2008; Frank et al. 1996). Two *CYP83* subfamily members in *Arabidopsis* are involved in plant defense by catalyzing reactions on aldoxime moieties that are needed for biosynthesis of aci-Nitro compounds leading to the accumulation of both thiol- and indole-derived glucosinolates (Bak and Feyereisen 2001). One of the members of *CYP83*, *AtCYP83B1*, is involved in the indole pathway, which branches from the phenylpropanoid pathway. The functions of both *CYP83* family members can affect flux through the phenylpropanoid pathway (Hemm et al. 2003). It is possible that *GmCYP83E12* is associated with the production of antimicrobial metabolites, possibly derived from the phenylpropanoid pathway, though additional studies are needed to establish this.

The MYB TF were implicated in the regulation of genes that are involved in the phenylpropanoid pathway (Mehrtens et al. 2005; Mellway et al. 2009; Stracke et al. 2001). These genes are notably upregulated in soybean by wounding, elicitor treatments, or challenge by various pathogens (Graham and Graham 1994, 1996; Zabala et al. 2006; Zou et al. 2005). Out of the 20 different MYB genes we tested, only the VIGS construct targeting *GmMYB84* compromised *Rpp2*-mediated resistance. Phylogenetic analysis for the predicted *GmMYB84* protein sequence showed that it belongs to the R2R3 class of MYB proteins and has high similarity to the *Petunia* PH4 protein, which is involved in anthocyanin synthesis (Quattrocchio et al. 2006). MYB TF are known to regulate phenylpropanoid genes by binding to AC elements in their promoter regions (Hernandez et al. 2004). Based on this information, we hypothesized that *GmMYB84* might act as regulator of genes in the phenylpropanoid pathway. We demonstrated that expression of *GmPAL1*, *Gm4-CL*, *GmC4H*, and *GmCHS* genes was affected in *GmMYB84*-silenced *Rpp2* plants, which supported our hypothesis. Consistent with the silencing results, these genes have at least one AC element in their promoter sequences, providing further evidence that they could be potential targets for *GmMYB84*. However, the regulation of *GmPAL1* is expected to be complex, given that *GmDBTF* also was necessary for its expression. At this time, we do not know whether *GmMYB84* and *GmDBTF* act in concert or independently to regulate *GmPAL1* expression; therefore, we have indicated their roles in regulation of *Rpp2* resistance in parallel in Figure 8. Furthermore, a role for *GmDBTF* in expression of other phenylpropanoid pathway genes dependent upon *GmMYB84* was not tested.

In conclusion, our results show that there is significant interplay between members of soybean TF families in controlling genes that are necessary for resistance to ASR. This study is a step toward establishing the genetic and biochemical hierarchies that regulate and mediate ASR resistance in soybean. The *Rpp2* resistance pathway model that was developed also builds upon knowledge available from model plant systems but many gaps remain. For example, *AtNPR1* interacts with TGA TF in *Arabidopsis*, which is necessary for activating defense responses (Fan and Dong 2002); however, at this time, we have not identified a member of the TGA TF family that participates in *Rpp2*-mediated resistance. This *Rpp2* pathway model, combining data from soybean and model plants, has allowed us to develop some interesting new hypotheses to explore in order to better understand soybean disease defense pathways.

MATERIALS AND METHODS

BPMV VIGS constructs and *Rpp2* loss-of-resistance screening.

PCR products were amplified from corresponding EST clones or genomic DNA and were then cloned into RNA2 of

the BPMV VIGS vector (Zhang et al. 2009). The orientation and identity of the VIGS inserts were confirmed by sequencing using a vector-specific forward primer 1548F (Zhang et al. 2009). To generate inoculum for the VIGS experiments, BPMV RNA1 (pBPMV-IA-R1M) and the recombinant RNA2 DNA clones were co-inoculated by gold particle bombardment on leaves of Williams82 plants at 14 days after sowing, as previously described (Zhang et al. 2009, 2010). BPMV-infected leaf tissue was collected at 3 to 5 weeks after bombardment, lyophilized, and stored at -20°C .

Seed of PI2130970 (*Rpp2*) were germinated in a growth chamber and, 2 weeks later, the primary leaves of four to six seedlings were dusted with Carborundum and subsequently rub inoculated with lyophilized leaf tissue corresponding to each VIGS construct suspended in 50 mM potassium phosphate buffer, pH 7.0. BPMV-inoculated plants were maintained in the growth chamber at 20°C with a 16-h photoperiod. Three weeks following BPMV infection, plants were transferred to the United States Department of Agriculture Plant Pathogen BSL-3 containment facility at Fort Detrick (Melching et al. 1983). The plants were inoculated with 1.1×10^5 spores of *P. pachyrhizi* isolate LA04-1 resuspended in sterile water containing 0.5% Tween-20 and then placed in a dew chamber overnight. Plants were then moved to a greenhouse and evaluated for resistant (RB lesions) and susceptible (tan lesions) phenotypes at 2 weeks after inoculation with *P. pachyrhizi*. In addition to the BPMV vector-inoculated plants, controls included mock-inoculated plants (the same experimental conditions as the VIGS-treated plants but rub inoculated with buffer instead of the BPMV inoculum), plants that were inoculated with a BPMV vector lacking an insert, and plants that were not treated prior to inoculation with *P. pachyrhizi*. All of the control plants were infected with *P. pachyrhizi* as described for the experimental plants. For each candidate gene that resulted in a loss of resistance, three independent replicates of the experiments were performed to ensure that phenotypes were consistent.

Tissue collection, total RNA isolation, and RT-PCR analysis.

The third and fourth trifoliolate leaves of four plants were collected from mock, empty vector, and silenced leaves at 2 weeks after *P. pachyrhizi* inoculation. The leaves were immediately frozen in liquid nitrogen and stored at -80°C . Leaf tissue was ground in liquid nitrogen, and RNA was extracted using 4 ml of Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA was precipitated with 2-propanol and the RNA was dissolved in 50 μl of diethyl pyrocarbonate-treated water containing RNaseOUT (2 U/ μl) (Invitrogen) and stored at -80°C . All RNA samples were treated with RNase-free DNase at 37°C for 30 min using the DNasefree kit (Ambion, Austin, TX, U.S.A.) in order to ensure the presence of only RNA amplicons prior to cDNA synthesis. First-strand cDNA synthesis was performed with 2 μg of RNA and the Transcriptor first strand cDNA synthesis kit (Roche, Indianapolis, IN, U.S.A.). To test the silencing of the respective genes, low-cycle RT-PCR (21 to 25 cycles) was performed with gene-specific primers. Elongation factor 1b (*Elf-1b*; Glyma02g44460) was included as an internal control for each set of PCR reactions.

Assessment of fungal mRNA accumulation.

Fungal growth was assessed by quantifying the constitutively expressed ASR α -tubulin gene by TaqMan QRT-PCR (Meyer et al. 2009; van de Mortel et al. 2007). DNA-free RNA (2 μg) was used for the cDNA synthesis using the Transcriptor first-strand cDNA synthesis kit RT-PCR (Roche). PCR cycling at 95°C for 10 s and data collection for 30 s at the extension

temperature of 60°C for 45 cycles was performed. Expression data were normalized to the soybean *Ubiquitin-3* gene (GenBank accession number gi 456713, dbj D28123.1), which showed no evidence for differential expression following ASR infection (van de Mortel et al. 2007).

Determination of acetyl bromide soluble lignin content.

Plant material was homogenized in 80% (vol/vol) ethanol, heated for 1 h at 80°C, and then centrifuged at 12,000 × *g* for 30 min. The pellet was resuspended in 80% ethanol and additionally homogenized using a Polytron Homogenizer (Fisher, Pittsburgh, PA, U.S.A.) at high speed for 2 to 3 minutes, and the procedure was repeated. The alcohol-insoluble pellet (AIS) was washed three times with 85% acetone and air dried. The dry AIS was suspended in 0.5% aqueous sodium dodecyl sulfate overnight, and the residue was recovered and washed with water by filtration, washed with a 1:1 mixture of chloroform and methanol, rinsed with acetone, and air-dried. Starch was removed by incubation with α-amylase (Type IIA; Sigma-Aldrich, St. Louis) for 48 h at 30°C. The remaining cell wall material (CWM) was washed with 100% acetone, air dried, and used for analyses. Acetyl bromide soluble lignin (ABSL) in soybean samples was determined according to Fukushima and Hartfield (2004). CWM (1 mg) were placed in glass vials and 100 µl of freshly prepared 25% (vol/vol) acetyl bromide was added. Samples were incubated at 50°C for 2 h, with occasional mixing. After cooling, 400 µl of 2 M sodium hydroxide and 70 µl of freshly prepared 0.5 M hydroxylamine hydrochloride were added and, after shaking, the volume was brought to 2 ml with acetic acid and mixed again by inversion. The solution (200 µl) was pipetted into a UV-specific 96-well plate and read in a microplate reader at 280 nm against the blank, which contained all reagents except the CWM. The specific absorption coefficient (SAC), 20 g⁻¹ cm⁻¹, was earlier reported for purified HCl-dioxane lignin from soybean (Lygin et al. 2009). ABSL concentration was determined using the following equation: percent lignin content = (absorbance × 100)/SAC × sample concentration (g⁻¹) × 0.7 cm (Sasaki et al. 1996), where 0.7 cm represents the path length of the light.

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