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Roles of small RNAs in soybean defense against *Phytophthora sojae* infection

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SUMMARY

The genus *Phytophthora* consists of many notorious pathogens of crops and forestry trees. At present, battling *Phytophthora* diseases is challenging due to a lack of understanding of their pathogenesis. We investigated the role of small RNAs in regulating soybean defense in response to infection by *Phytophthora sojae*, the second most destructive pathogen of soybean. Small RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), are universal regulators that repress target gene expression in eukaryotes. We identified known and novel small RNAs that differentially accumulated during *P. sojae* infection in soybean roots. Among them, miR393 and miR166 were induced by heat-inactivated *P. sojae* hyphae, indicating that they may be involved in soybean basal defense. Indeed, knocking down the level of mature miR393 led to enhanced susceptibility of soybean to *P. sojae*; furthermore, the expression of isoflavonoid biosynthetic genes was drastically reduced in miR393 knockdown roots. These data suggest that miR393 promotes soybean defense against *P. sojae*. In addition to miRNAs, *P. sojae* infection also resulted in increased accumulation of phased siRNAs (phasiRNAs) that are predominantly generated from

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SUPPORTING INFORMATION

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canonical resistance genes encoding nucleotide binding-leucine rich repeat proteins and genes encoding pentatricopeptide repeat-containing proteins. This work identifies specific miRNAs and phasiRNAs that regulate defense-associated genes in soybean during *Phytophthora* infection.

Keywords

Glycine max; *Phytophthora sojae*; oomycetes; microRNAs; phasiRNAs; defense gene regulation

INTRODUCTION

The genus *Phytophthora* consists of pervasive pathogens that are responsible for many devastating plant diseases. For example, the potato pathogen *Phytophthora infestans* was the causative agent of the Great Irish Famine (Bourke, 1964; Yoshida *et al.*, 2013), and *Phytophthora sojae* is the second most destructive pathogen of soybean, causing annual losses of approximately about 2 billion US dollars worldwide (Tyler, 2007; Ye *et al.*, 2011). Despite the significant importance of *Phytophthora* diseases in agriculture and forestry, our understanding of the molecular details of *Phytophthora* pathogenesis is rather limited.

A central feature of the arms race between plants and pathogens is activation of the plant immune response upon pathogen recognition, and subversion of this defense by virulence factors produced by successful pathogens. The first layer of plant defense is based on perception of pathogen- or microbe-associated molecular patterns (PAMP/MAMPs) by pattern recognition receptors (Boller and He, 2009; Monaghan and Zipfel, 2012). The activation of pattern recognition receptors initiates signal transduction and transcriptional reprogramming, leading to various antimicrobial responses such as cell wall thickening, production of reactive oxygen species, and secretion of antimicrobial compounds (Jones and Dangl, 2006; Torres *et al.*, 2006). Although PAMP-triggered immunity restricts the growth of the vast majority of potential pathogens encountered by plants, successful pathogens produce virulence factors to effectively suppress this basal defense. In particular, a multitude of secreted proteins, called effectors, are delivered into host cells to effectively subvert PAMP-triggered immunity (Jones and Dangl, 2006; Boller and He, 2009; Dodds and Rathjen, 2010). Because effectors often target key components of the defense system, they have been used as molecular probes to identify important genes/pathways in plant immunity.

Recent findings on the function of *Phytophthora* effectors suggest that small RNAs may play an important regulatory role in plant defense during *Phytophthora* infection. Small RNAs of 20–30 nucleotides are central players in RNA silencing, a universal gene regulation mechanism in eukaryotes (Ambros, 2004; Baulcombe, 2004). These non-coding small RNAs are processed from double-stranded RNA precursors and guide sequence-specific repression of target genes. Two effectors of the soybean pathogen *Phytophthora sojae* suppress small RNA biogenesis in plants, and expression of these *Phytophthora* suppressors of RNA silencing (PSRs) or a viral RNA silencing suppressor P19 in *Nicotiana benthamiana* led to hyper-susceptibility to *Phytophthora infestans* (Qiao *et al.*, 2013). These results indicate that small RNAs are required for effective plant defense against *Phytophthora*

infection. However, the specific small RNAs that regulate anti-*Phytophthora* defense are unknown.

Plants produce two major types of small RNAs: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Chen, 2010). miRNAs are encoded by endogenous *MIR* genes that encode single-stranded precursors that form stem-loop structures. siRNAs are derived from long dsRNA precursors that are produced by invading nucleic acids such as viruses and transgenes, or from endogenous loci such as repeats, transposable elements and genes (Voinnet, 2009; Chen, 2010). Both miRNAs and siRNAs affect the plant response to pathogen infection (Diaz-Pendon *et al.*, 2007; Sunkar *et al.*, 2007; Chen, 2009; Katiyar-Agarwal and Jin, 2010). The best known example is siRNAs, which target viral RNAs for degradation, and thereby restrict viral infection (Diaz-Pendon *et al.*, 2007). Changes in the accumulation of specific miRNAs have been reported in *Arabidopsis thaliana* during infection by the bacterial pathogen *Pseudomonas syringae* (Navarro *et al.*, 2006; Fahlgren *et al.*, 2007; Zhang *et al.*, 2011) or after treatments with bacterial flagellin (Navarro *et al.*, 2006; Li *et al.*, 2010). Functional analysis showed that miR393 and miR160 positively regulate defense against *P. syringae* (Navarro *et al.*, 2006; Li *et al.*, 2010), whereas miR398 and miR773 play a negative role (Li *et al.*, 2010). Furthermore, three *P. syringae* effectors were able to suppress the miRNA pathway in Arabidopsis (Navarro *et al.*, 2008), consistent with a regulatory role of miRNAs in anti-bacterial defense.

Changes in miRNA abundance were also observed in plants during infection by eukaryotic pathogens including fungi (Lu *et al.*, 2007; Campo *et al.*, 2013) and *Phytophthora* (Guo *et al.*, 2011). Using a miRNA microarray, the abundances of several miRNAs were found to be altered in soybean hypocotyls infected with *P. sojae* (Guo *et al.*, 2011); however, genome-scale profiling of small RNAs during *Phytophthora* infection has not been performed. In addition, the current miRBase (version 19, http://www.mirbase.org) lists more than 390 miRNAs produced by soybean, but their function in the defense response is unknown.

In this study, we investigated the role of small RNAs in soybean during *P. sojae* infection. Using Illumina sequencing, Northern blotting and quantitative RT-PCR, we identified specific miRNAs and siRNAs that were differentially accumulated in *P. sojae*-infected roots at an early infection stage. In particular, miR166 and miR393 were induced by *P. sojae* cell-wall components, and silencing of miR393 in soybean led to hyper-susceptibility to *P. sojae*. Furthermore, miR393 positively regulates the expression of isoflavonoid biosynthetic genes, potentially leading to a positive regulation of defense responses. In addition to miRNAs, *P. sojae*-infected roots also exhibited induced accumulation of secondary phased siRNAs (phasiRNAs), whose production is triggered by miRNAs. Interestingly, we identified increased accumulation of phasiRNAs generated from canonical plant resistance (R) genes encoding nucleotide binding-leucine rich repeat (NB-LRR) proteins and genes encoding pentatricopeptide repeat (PPR) proteins. These phasiRNAs may regulate a potentially large number of *NB-LRR* and *PPR* genes during *P. sojae* infection. Taken together, the results of this study suggest important regulatory roles of specific miRNAs and phasiRNAs on defense-associated genes in soybean against *P. sojae* infection.

RESULTS

Small RNA profiling in soybean roots infected with *Phytophthora sojae*

In order to identify differentially accumulated small RNAs in *P. sojae*-infected soybean roots, we inoculated the susceptible cultivar Harosoy and the resistant cultivar Williams 82 with *P. sojae* strain P6497. Williams 82 carries the resistance gene *Rps1K*, which activates a defense response induced by the effector Avr1K (Dorrance *et al.*, 2004). Roots inoculated with sterile agar plugs served as mock-treated controls. Total RNAs were extracted from the infected root tissues at 8 h post-inoculation (hpi), when extensive hyphae penetration into the root cortex was observed (Figure S1). We hypothesize that the small RNAs that were altered at this early infection stage, before oospore development and tissue necrosis, may be involved in regulating basal defense response.

Four small RNA libraries (Harosoy mock, Harosoy infected, Williams 82 mock and Williams 82 infected) were constructed and subjected to Illumina sequencing. We obtained more than ten million high-quality reads from each library (Table S1). These reads were aligned to the soybean genome (http://www.phytozome.net/soybean.php), and then filtered to remove rRNAs, tRNAs and small nucleolar RNAs. The remaining sequences, representing potential small RNAs, were further analyzed for miRNA and siRNA identification. We did not observe significant differences between the percentage of reads that perfectly matched the soybean genome between libraries generated from Harosoy and Williams 82 (Table S1), indicating that these cultivars are highly similar in terms of their small RNA populations.

Among the four libraries, we identified 324 known soybean miRNAs belonging to 109 families (www.mirbase.org; Griffiths-Jones *et al.*, 2008). In addition, 54 sequences were predicted as potential novel miRNAs based on the established criteria (Meyers *et al.*, 2008) using a bioinformatic pipeline (Barrera-Figueroa *et al.*, 2011) and secondary structure prediction. To further confirm that these novel miRNAs are functional, we analyzed the cleavage of their potential targets using publically available degradome data from seven soybean libraries. The degradome analysis revealed cleavage signatures for 36 novel miRNAs with high confidence values ($P < 0.05$) (Table S2). In general, these miRNAs are predominantly 21 nt long (Figure S2), and showed a typical length distribution of miRNAs as previously observed (Voinnet, 2009).

The number of miRNAs in mock-treated roots of Harosoy appeared to be lower compared to that in *P. sojae*-infected roots. This may be due to the relatively low number of total small RNA reads in the mock-treated Harosoy library. Furthermore, no change was observed in the number of miRNAs detected from mock-treated or *P. sojae*-infected tissues of Williams 82 (Figure S2). Therefore, the number of expressed miRNA species was not significantly altered in *P. sojae*-infected tissues.

Identification of differentially accumulated miRNAs in *P. sojae*-infected soybean

We then determined the relative abundances of miRNAs in mock versus infected samples by comparing the normalized number of reads (per million) in each cultivar. Fourteen known and six potential novel miRNAs showed changes higher than twofold in one or both cultivars (Table S3). Northern blotting using independent samples verified the increased

accumulations of miR166, miR393, miR1507, miR2109 and miR3522, and the decreased accumulations of miR168, miR319 and miR482 in both cultivars (Figure 1a and Table S4). To determine whether the altered accumulation of these miRNAs was due to differential expression of the *MIR* genes, we examined the transcript abundances of the *MIR* transcripts (pri-miRNAs). Quantitative RT-PCR confirmed that the pri-miRNA abundance of most miRNAs showed changes consistent with the changes in abundance of the mature miRNAs (Figure 1b). The only exception is miR319, whose pri-miRNA did not show a significant difference in *P. sojae*-infected tissues.

In addition to known miRNAs, six novel miRNAs showed altered accumulation in the *P. sojae*-infected roots based on Illumina sequencing data (Table S3). Most of these novel miRNAs have low abundance and were undetectable using Northern blotting; nevertheless, we confirmed the increased accumulation of Gma13_15666134 and the decreased accumulation of Gma13_14875340 in both soybean cultivars during *P. sojae* infection (Figure 2a). Corresponding changes in their pri-miRNA abundance were also detected using quantitative RT-PCR (Figure 2b).

As miRNAs often repress their target gene expression by transcript cleavage, we examined the transcript levels of miRNA target genes in *P. sojae*-infected tissues. Potential target genes of each miRNA were predicted using PsRNATarget (http://plantgrn.noble.org/psRNATarget), and cleavage of these predicted targets was verified using the soybean degradome analyses (Table S2). Selected targets were then examined for transcript abundance using quantitative RT-PCR (Table S5). We observed a reduced abundance of the predicted target genes of all the miRNAs whose expression was induced by *P. sojae* (Figure S3). We also detected induced expression of two miR482 target genes in *P. sojae*-infected tissues, consistent with the reduced accumulation of miR482 (Figure S3). The target genes of miR168 and miR319 did not show significant changes at the transcript level, but it is possible that these genes are regulated by the two miRNAs at the translational level.

All the miRNAs that differentially accumulated in *P. sojae*-infected tissues exhibited similar changes in both soybean cultivars, indicating that these miRNAs may be involved in soybean basal defense rather than effector-triggered immunity.

Expression of miR166 and miR393 is induced by heat-inactivated *P. sojae* cells

In order to determine whether the observed changes in specific miRNAs were triggered by *P. sojae* PAMPs, we examined the abundance of these miRNAs in soybean roots treated with heat-inactivated *P. sojae* hyphae, sometimes referred to as the cell wall prep (West, 1981). *P. sojae* hyphae were boiled for 5 min, precipitated by centrifugation, and then used to inoculate soybean roots. Northern blotting showed that miR166 and miR393 were again significantly induced 8 h after the exposure of roots to the cell wall prep in both cultivars (Figure 3). The induction levels of miR166 and miR393 were very similar to those caused by *P. sojae* infection, suggesting that these two miRNAs were induced as a soybean defense response triggered by cell-wall component(s) or PAMPs, of P. *sojae*.

miR393 is required for soybean defense against *P. sojae*

We next examined the function of miR393 in soybean defense against *P. sojae*. Functional analyses of miRNAs are technically challenging especially when the miRNAs are generated from multiple *MIR* loci. Soybean has a tetraploid genome with 12 potential *MIR393* loci (Turner *et al.*, 2012). In order to evaluate the function of miR393, we generated *Agrobacterium rhizogenes*-induced transgenic roots with reduced levels of mature miR393 using the short tandem target mimic (STTM) technique (Tang *et al.*, 2012; Yan *et al.*, 2012). STTM transcripts contain two binding sites for a specific miRNA but cannot be degraded by the miRNA. When expressed in plant cells, the STTM transcripts trigger degradation of the miRNA.

Soybean cotyledons were inoculated with two *A. rhizogenes* strains each harboring one of two plasmids: pCAMBIA1300::*STTM393*, which carries *35S:STTM393*, or pEG104, which carries *35S*::*YFP*. Roots expressing *35S:STTM393* were isolated based on the presence of yellow fluorescence (Figure 4a). Northern blotting showed that the fluorescent roots, presumably expressing *STTM393*, exhibited an approximately 70% reduction in the level of miR393 compared to roots only expressing YFP (Figure 4a). Furthermore, the miR393 target transcripts showed increased accumulation in the *STTM393*-expressing roots, confirming that the *STTM393* construct effectively targeted miR393 for degradation (Figure S5a).

We then examined the susceptibility of the miR393 knockdown roots to *P. sojae*. Transgenic roots expressing *STTM393* + YFP or YFP only were inoculated with zoospore suspensions of *P. sojae* strain P6497G, which constitutively expresses green fluorescence protein (GFP), facilitating microscopic analysis of infection development. The number of roots that supported hyphae penetration into root cortex and oospore development at the inoculated area were counted at 24, 36 and 48 hpi. We used both Harosoy and Williams 82 in this experiment. Note that, although Williams 82 is a resistant host, hyphae penetration and oospore development were still observed in a small proportion (approximately 30%) of the inoculated roots, with restricted infection in the inoculation areas (Figure 4b).

In both Harosoy and Williams 82, knocking down miR393 led to hyper-susceptibility to *P. sojae* P6497G. In Williams 82, a significantly greater number of inoculated miR393 knockdown roots allowed hyphal penetration and oospore development compared to roots that only expressed YFP (Figures 4b and S5b, and Table S6). The enhanced infection was supported by the greater *P. sojae* biomass determined by quantitative PCR amplifying the *P. sojae*-specific DNA region *PsCOX* (Figure 4c). Enhanced susceptibility of the miR393 knockdown roots was also observed in Harosoy, although it was not as significant as in Williams 82. This is expected because *P. sojae* P6497 is already a potent pathogen of Harosoy. Nonetheless, the infection in Harosoy roots was accelerated (Figure 4b), and the *P. sojae* biomass was also increased in *STTM393*-expressing roots (Figure 4c). These data suggest that miR393 is a positive regulator of soybean defense against *P. sojae* infection.

miR393 regulates the expression of isoflavonoid biosynthetic genes

We next investigated the potential mechanism by which miR393 enhances soybean resistance during *P. sojae* infection. In Arabidopsis, miR393 modulates secondary metabolism and enhances the production of antimicrobial compounds that specifically target biotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). A major group of antimicrobial metabolites in soybean is isoflavonoids. For example, glyceollins inhibit *P. sojae* growth *in vitro* (Lygin *et al.*, 2010). We therefore examined the potential effect of miR393 on isoflavonoid biosynthesis by evaluating the expression of *GmHID1* and *GmIFS1*, which encode enzymes catalyzing two consecutive steps in the isoflavonoid biosynthetic pathway (Lygin *et al.*, 2010).

Using quantitative RT-PCR, we found that both *GmHID1* and *GmIFS1* were significantly induced in *P. sojae*-infected soybean roots (Figure 4d). Remarkably, knocking down miR393 led to drastically reduced levels of *GmHID1* and *GmIFS1* even in *P. sojae*-infected roots (Figure 4d). These data demonstrate that miR393 positively regulates isoflavonoid biosynthetic pathway in soybean, which may contribute to the enhanced resistance to *P. sojae*.

miR1507 and miR2109 regulate NB-LRR genes and trigger phasiRNA production during *P. sojae* infection

Among the miRNAs that differentially accumulated in the *P. sojae*-infected soybean roots, miR1507 and miR2109 are predicted to target nucleotide-binding/leucine-rich repeat (*NB-LRR*) genes, which are canonical plant resistance (*R*) genes. In contrast to miR166 and miR393, miR1507 and miR2109 were only induced by *P. sojae* infection not by heat-inactivated hyphae (Figure 5a). Interestingly, miR1507 and miR2109 in *Medicago truncatula* are 22 nt long, and trigger the production of secondary siRNAs generated from their target *NB-LRR* loci (Zhai *et al.*, 2011). siRNAs generated from miRNA target transcripts are called phased siRNAs or phasiRNAs because they are in 21 nt register from one another (Chen, 2012). By triggering the production of phasiRNAs, miR1507 and miR2109 may potentially regulate a large number of *NB-LRR* genes.

Our sequencing data showed that miR1507 is exclusively 22 nt long in soybean with or without *P. sojae* infection. Degradome analysis verified four miR1507 targets including the *NB-LRR* genes *Glyma04g29220* and *Glyma06g39720* (Table S2). Interestingly, both genes were found to produce phasiRNAs, which showed higher abundance in *P. sojae*-infected roots (Figure 5c). Northern blotting confirmed the induced accumulation of phasiRNAs derived from *Glyma06g39720* in *P. sojae*-infected tissues (Figure 5b). These siRNAs are clearly ‘phased’ at 21 nt intervals from the predicted cleavage site of miR1507 (Figure 5d). phasiRNAs may act *in cis* to repress target loci or *in trans* to regulate a potentially large number of genes. Consistent with the induction of both miR1507 and the phasiRNAs, *Glyma04g29220* and *Glyma06g39720* exhibited reductions in transcript abundance compared with the miR1507-targeted non-*NB-LRR* gene *Glyma06g45100* (Figure 5e), from which production of phasiRNAs was not detected (Figure 5c). These data suggest that miR1507-dependent phasiRNA production is induced to regulate *NB-LRR* genes during *P. sojae* infection.

miR2109 was thought to be 22 nt long in *M. truncatula*, but predominantly 21 nt long in soybean (Zhai *et al.*, 2011). However, our sequencing data and Northern blots detected both 21 and 22 nt forms of miR2109 in soybean (Figure 5a, b). The presence of the 22 nt form raised the possibility that miR2109 may also trigger the production of phasiRNAs. Indeed, one *NB-LRR* gene (*Glyma03g14900*) targeted by miR2109 was found to generate phasiRNAs, especially during *P. sojae* infection (Figure 5b,c). The transcript level of *Glyma03g14900* was also significantly lower than that of the other miR2109-targeted locus *Glyma01g06750* (Figure 5e), from which we did not detect phasiRNA production (Figure 5c). These data indicate that expression of *Glyma03g14900* may be regulated by both miR2109 and phasiRNAs.

Genome-scale analysis of phasiRNAs in soybean during *P. sojae* infection

Because phasiRNA production from specific miR1507 and miR2109 target loci was enhanced in *P. sojae*-infected tissues, we performed a genome-scale analysis on phasiRNA-generating (*PHAS*) loci in roots infected with *P. sojae*. We found that phasiRNAs were predominantly generated from genes encoding NB-LRR proteins and PPR proteins (Figure 6a). Intriguingly, *P. sojae* infection led to increased numbers of *PHAS* loci in both susceptible and resistant cultivars, especially from *PPR* loci. Consistent with enhanced accumulation of phasiRNAs (Figure 6b), we observed reduced expression of four *PPR* genes in Harosoy and Williams 82 at 8 hpi (Figure 6c). Interestingly, three of the four *PPR* genes that were repressed at 8 hpi were de-repressed at 24 hpi in Harosoy, but remained repressed in Williams 82 (Figure 6c). These data revealed a potential correlation between low levels of *PPR* gene expression and enhanced resistance to *P. sojae*. These data suggest that phasiRNAs may play a role in soybean defense by regulating the expression of specific *NB-LRR* and *PPR* genes during *P. sojae* infection.

DISCUSSION

This study comprises a genome-scale small RNA profiling and functional analysis of small RNAs during *Phytophthora* infection. We identified specific soybean small RNAs, including miRNAs and phasiRNAs, that differentially accumulated in *P. sojae*-infected roots at an early infection stage, and observed that the small RNA changes led to altered expression of defense-associated genes. These findings suggest that small RNAs are important regulators of soybean defense against *P. sojae*.

miR166 and miR393 are rapidly induced by treatment with heat-inactivated hyphae, indicating that the induction is probably triggered by PAMPs. *Phytophthora* cell-surface peptides, β-glucans and PEP-13 have been reported to elicit defense responses, including accumulation of phytoalexins and activation of the salicylic acid signaling pathway (Brunner *et al.*, 2002; Fellbrich *et al.*, 2002; Day and Graham, 2007). The induction of miR166 and miR393 by a yet to be determined cell-wall component(s) supports a role of these miRNAs in establishing PAMP-triggered immunity in soybean.

miR166 is a conserved miRNA that targets class III homeodomain leucine zipper (HD-ZIPIII) genes and regulates organ polarity, vascular development and lateral root development (Hawker and Bowman, 2004). Consistent with the induction of miR166, we

also detected reduced expression of HD-ZIPIII genes in *P. sojae*-infected roots. The repression of HD-ZIPIII genes may contribute to the sequestering of soybean growth so that more energy may be devoted to defense.

The function of miR393 in plant immunity has been studied in Arabidopsis, where over-expression of *MIR393* led to enhanced resistance to the bacterial pathogen *Pseudomonas syringae* (Navarro *et al.*, 2006) and the oomycete pathogen *Hyaloperonospora parasitica* (Robert-Seilaniantz *et al.*, 2011). Using the *STTM393* construct, we effectively reduced mature miR393 levels in soybean roots and showed that miR393 is a positive regulator of soybean defense against *P. sojae*. Despite several trials, we were unable to over-express *MIR393* in hairy roots due to transgene silencing (Figure S6). Nonetheless, these transgenic roots, with lower abundance of miR393, exhibited hyper-susceptibility to *P. sojae*. This experiment confirmed the phenotype observed in *STTM393*-expressing roots and reinforced our conclusion that miR393 is required for full resistance of soybean to *P. sojae*. Taken together, it is evident that miR393 plays a conserved role in plant basal defense upon recognition of a broad range of microbial pathogens.

miR393 is known to repress auxin signaling by targeting the TIR/AFB auxin receptors (Chen, 2009; Vidal *et al.*, 2010; Si-Ammour *et al.*, 2011); however, the mechanism by which miR393 regulates plant defense has not yet been fully elucidated. An interesting finding in Arabidopsis was that miR393 regulates genes involved in secondary metabolic pathways, and may redirect the metabolic flow toward production of compounds that are more effective at inhibiting biotrophic and hemibiotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). Although Arabidopsis and soybean produce different antimicrobial compounds, we found that the miR393 knockdown soybean plants showed drastically reduced expression of *GmHID1* and *GmIFS1*, which encode key enzymes for isoflavonoid biosynthesis. Isoflavonoids are important antimicrobial compounds (Bednarek and Osbourn, 2009). In soybean, glyceollin production is induced by *P. sojae*, and application of glyceollins inhibits *P. sojae* growth *in vitro* (Lygin *et al.*, 2010). We also observed significant induction of *GmHID1* and *GmIFS1* in soybean roots by *P. sojae* infection, which is abolished in miR393 knockdown roots. We propose that miR393-mediated soybean defense against *P. sojae* may be, at least in part, due to its positive regulation of the isoflavonoid biosynthetic pathway.

In addition to conserved and known soybean miRNAs, we also identified 54 potential novel miRNAs and found cleavage signatures from 36 of them. Importantly, two of these potential miRNAs, Gm13_14875340 and Gm13_15666134, exhibited altered accumulation during *P. sojae* infection. The novel miRNA Gm13_14875340 was repressed in soybean during *P. sojae* infection; consistently, expression of its target gene *Glyma04g00510* was enhanced. *Glyma04g00510* encodes a protein that shows sequence similarity to the minichromosome maintenance 10 in Arabidopsis and rice (*Oryza sativa*), which has been shown to be involved in DNA replication (Shultz *et al.*, 2007). It remains to be determined whether and how this novel miRNA contributes to the soybean defense response to *P. sojae*.

Another class of soybean small RNAs that were significantly induced by *P. sojae* was phasiRNAs. Analyses of small RNA populations in legumes during mutually beneficial interactions with rhizobia and mycorrhizae revealed that the majority of the phasiRNA-

generating (*PHAS*) loci are *NB-LRR* genes (Zhai *et al.*, 2011). In *Medicago truncatula*, these *PHAS* loci are mainly targeted by three 22 nt miRNA families, i.e. miR1507, miR2109 and miR2118. We also observed that a significant proportion of *PHAS* loci were *NB-LRR* genes, which are targets of miR1507 and miR2109. Importantly, both miR1507 and miR2109 were induced during *P. sojae* infection. Furthermore, we also observed induced production of phasiRNAs in *P. sojae*-infected tissues from specific *NB-LRR* loci. These phasiRNAs probably regulate a potentially large number of *NB-LRR* genes during *P. sojae* infection.

NB-LRRs are immune receptors that recognize specific pathogen proteins, such as effectors, and elicit a robust defense response (Meyers *et al.*, 2002; McHale *et al.*, 2006). The activation of NB-LRR proteins is often associated with programmed cell death, which restricts pathogen spread from the infection sites; on the other hand, mis-regulation of NB-LRRs leads to tissue damage and auto-immune diseases (McHale *et al.*, 2006; Eitas and Dangl, 2010). The induction of miR1507, miR2109 and their associated phasiRNAs leads to decreased expression of multiple *NB-LRR* genes at the early infection stage of *P. sojae*. Twenty-two nucleotide miRNAs and their derived phasiRNAs have also been shown to repress the tobacco *N* gene, which encodes an NB-LRR protein that confers resistance to tobacco mosaic virus (Li *et al.*, 2012). It is possible that these miRNAs and phasiRNAs function to fine tune the expression of *NB-LRR* genes, and the precise regulation of *NB-LRR* gene expression is important for effective, but not overly induced, defense against pathogen infections.

In addition to *NB-LRR* loci, *P. sojae* infection also induced the production of phasiRNAs from *PPR* genes. Negatively correlated with the increased abundance of phasiRNAs, multiple *PPR* genes were repressed at the early infection stage in soybean. PPR proteins belong to a large sequence-specific RNA binding protein family that regulates genes with key functions in mitochondria and chloroplasts (Kotera *et al.*, 2005; Prikryl *et al.*, 2010). In Arabidopsis, *PPR* genes are regulated by *trans*-acting siRNAs (tasiRNAs) generated from the *TAS1* and *TAS2* loci, which are targets of the 22 nt miR173 (Howell *et al.*, 2007). It was recently reported that siRNA-mediated regulation of *PPR* genes is conserved in eudicots: *PPR* genes are regulated either by phasiRNAs generated from *PPR PHAS* loci or by tasiRNAs generated from the *TAS* loci (Xia *et al.*, 2013). Furthermore, miR1509, which belongs to the evolutionarily conserved miR173 family, was proposed to regulate *PPR* genes in soybean by triggering tasiRNA production from intermediate *TAS*-like genes (Xia *et al.*, 2013). Our data showed that phasiRNAs produced from *PPR* genes were triggered by a 23 nt miRNA, miR1508 (Figure S7). This result is consistent with a recent degradome analysis, which suggested miR1508 as a trigger for phasiRNA production in soybean (Hu *et al.*, 2013). It is worth noting that the level of miR1508 was not altered by *P. sojae* infection at 8 hpi (Figure S7); therefore, *P. sojae* infection may specifically induce the production of phasiRNAs from the *PPR* loci without altering the biogenesis of their parent miR1508. These data raised the interesting possibility that the phasiRNA biosynthetic pathway may be specifically induced in soybean during *P. sojae* infection as a defense response.

If phasiRNAs are regulators of soybean defense, successful pathogens may have evolved virulence factors to target their biogenesis. Indeed, an effector PSR2 was identified as an essential virulence protein that promotes *P. sojae* infection of soybean. Over-expression of

PSR2 in Arabidopsis led to reduced accumulation of tasiRNAs from the *TAS1* and *TAS2* loci, which also regulate *PPR* genes (Qiao *et al.*, 2013). *PSR2* is expressed in *P. sojae* at later infection stages after 16 hpi (Qiao *et al.*, 2013). Consistent with the expression profile of *PSR2*, the *PPR* genes were de-repressed at 24 hpi in the susceptible Harosoy roots, which may be due to the phasiRNA suppression activity of PSR2. In contrast, the *PPR* genes remained repressed at 24 hpi in the resistant Williams 82 roots, which do not allow effective *P. sojae* infection. We therefore propose that soybean employs phasiRNAs to regulate the expression of defense-associated genes, such as *NB-LRR* and *PPR* genes, and that *P. sojae* has evolved effectors, such as PSR2, to disrupt this defense mechanism.

In summary, *Phytophthora* diseases cause enormous damage to agriculture and forestry. Consistent with our recent findings on *Phytophthora* effectors that inhibit small RNA biogenesis, small RNA profiling in *P. sojae*-infected soybean revealed important regulatory roles of specific miRNAs and phasiRNAs in plant immunity. Further investigations including time-course analysis of small RNA profiling and functional characterization of specific small RNAs, especially phasiRNAs, will help elucidate mechanistic details of small RNA-mediated defense regulation during *Phytophthora* infection.

EXPERIMENTAL PROCEDURES

Plant growth conditions

Soybean (*Glycine max*) seeds were surface-sterilized and pre-germinated as previously described (Morgan *et al.*, 2010). Germinated seeds were transferred to sterile pouches (Mega International, http://www.mega-international.com) infused with B&D nutrient solution (1000 μM $CaCl_2 \cdot 2H_2O$, 500 μM KH_2PO_4, 10 μM Fe-citrate, 250 μM $MgSO_4 \cdot 7H_2O$, 1.5 mM K_2SO_4, 1 μM $MnSO_4 \cdot H_2O$, 2 μM H_3BO_4, 0.5 μM $ZnSO_4 \cdot 7H_2O$, 0.2 μM $CuSO_4 \cdot 5H_2O$, 0.1 μM $CoSO_4 \cdot 7H_2O$, 0.1 μM $Na_2MoO_4 \cdot 2H_2O$), and grown in a growth chamber (22°C, 12 h photoperiod) for 2 weeks before inoculation of the roots with mycelial plugs of *P. sojae* isolate P6497 or P6497G.

For hairy root induction, germinated seeds were planted into sterile vermiculite (Crop Production Services, http://www.cpsagu.com) supplemented with Peter's complete nutrient solution (1 g L^{-1}; Crop Production Services) and grown in a growth chamber (22°C, 12 h photoperiod) for 10 days. Cotyledons were then collected for *Agrobacterium* inoculation.

Phytophthora sojae culture conditions

P. sojae isolate P6497 was cultured in 10% v/v V8 medium (Erwin and Ribeiro, 1996). The GFP-labeled strain P6497G was grown in 10% V8 medium supplemented with 40 μg mL^{-1} geneticin. Both strains were grown at 25°C in the dark for 3–4 days before using the mycelium or zoospores for inoculation.

Small RNA library construction

Four small RNA libraries were constructed from *P. sojae*-infected or mock-treated root tissues using the susceptible cultivar Harosoy and the resistant cultivar Williams 82. Soybean roots were inoculated by overlaying V8 medium agar plugs with growing *P. sojae* hyphae as

previously described (Zhou *et al.*, 2009). Total RNAs were extracted from inoculated tissues 8 h after inoculation. Roots treated with sterile agar plugs were used as the mock control. RNAs in the range of 18–30 nt were recovered from polyacrylamide gels and subjected to library construction using a TruSeq small RNA sample preparation kit (Illumina, http://www.illumina.com).

Identification of small RNAs and target prediction

The small RNA libraries were sequenced using an Illumina Genome Analyzer IIx. More than 10 million high-quality reads were obtained from each library. These sequences were mapped to the soybean genome (US Department of Energy Joint Genome Institute *Glycine max* gene index, version 8, http://soap.genomics.org.cn) using the Short Oligonucleotide Analysis Package (SOAP) alignment software (Li *et al.*, 2009). Reads that perfectly aligned to the genome were filtered to remove known repeat sequences in plants, i.e. rRNAs, tRNAs, snRNAs and snoRNAs. The remaining sequences were further analyzed for miRNA and phasiRNA identification.

Known miRNAs were identified by aligning to annotated miRNAs (www.mirbase.org, release 19) (Griffiths-Jones *et al.*, 2008) using FASTA version 3.6 (Pearson, 2000). miRNAs with fewer than two nucleotide differences were classified in the same family.

Small RNA targets were predicted using the plant small RNA analysis server PsRNATarget (http://plantgrn.noble.org/psRNATarget) using the soybean genome sequence with an expectation cutoff threshold of 3.0. The complementary length range was set to 20 bp, and the allowed energy to open the secondary structure around the miRNA target site was set to 25 unpaired energy.

Identification of novel miRNAs

Candidate novel miRNAs were analyzed to meet the following criteria: (i) the free energy of the secondary RNA structure of their precursor transcript, predicted using UNAFold (Markham and Zuker, 2008), is less than −35 kcal mol^{-1}; (ii) there are fewer than four mismatches between the putative miRNA and miRNA*; (iii) there is no more than one asymmetrical bulge in the stem region, and the size of each asymmetrical bulge is less than two nucleotides; (iv) the strand bias of putative miRNAs is such that small RNA reads mapping to the positive strand of the hairpin DNA segment account for at least 80% of all mapped reads; (v) putative miRNAs sequences that map to the miRNA and miRNA* loci account for at least 75% of the reads within the loci (Meyers *et al.*, 2008; Thakur *et al.*, 2011). Representative secondary structures of potential novel miRNAs are presented in Figure S4. Normalized reads of the novel miRNAs and their corresponding miRNA* are presented in Table S8.

Degradome analysis of 5′ RACE soybean libraries

Target genes of known and potentially novel miRNAs were identified using seven soybean degradome libraries that are publically available from the National Center for Biotechnology Information Gene Expression Omnibus datasets (GSM825574, GSM647200, GSM848963,

GSM848964, GSM848965, GSM848966 and GSM848967) using the software package CleaveLand3.0 (Addo-Quaye *et al.*, 2009).

Identification of phasiRNAs

phasiRNA analysis was performed as described previously (De Paoli *et al.*, 2009). The number of identified 21 nt in-phase signatures was counted within 210 bp (10 phases) using the soybean sRNA database at http://mpss.udel.edu/soy_sbs/. Genomic regions with phasing scores >15 were considered *PHAS* loci. These regions were then mapped to the soybean genome to determine gene identities.

Northern blotting

Total RNAs were extracted from root tissues using Trizol (Invitrogen, http://www.lifetechnologies.com). Approximately 5 μg of total RNAs were used to detect miRNAs using oligonucleotide probes that were end-labeled with γ-^{32}P ATP. phasiRNAs were detected using radiolabeled probes created from 300 to 500 bp PCR products encompassing the phasiRNA-generating regions.

Quantitative RT-PCR

Quantitative RT-PCR was performed using iQ™ SYBR® Green Supermix and a CFX96 real-time PCR detection system (Bio-Rad, http://www.bio-rad.com). Total RNA was extracted using Trizol, treated with DNase I (Fermentas, http://www.thermoscientificbio.com/fermentas), and used for cDNA synthesis using M-MLV reverse transcriptase (Promega, http://www.promega.com). Gene-specific primers (Table S7) were designed to flank the predicted miRNA-binding site on the target genes. *GmUBI* was used as the internal control.

Construction of the STTM393 construct

The STTM393 construct carries two tandem gma-miR393 binding sites linked by an 88 nt spacer (Tang *et al.*, 2012; Yan *et al.*, 2012). The gma-miR393 binding site contains perfect complementary sequences of miR393 with the exception of the ‘CUA’ bulges that prevent miR393-mediated cleavage. The construct was cloned into the vector pCAMBIA1300 (Tang *et al.*, 2012), and the recombinant plasmid was then introduced in *Agrobacterium rhizogenes* strain K599 for soybean transformation.

Hairy root induction from soybean cotyledons

Soybean cotyledons were inoculated with *A. rhizogenes* strain K599 carrying pEG104 (Earley *et al.*, 2006) or a 1:1 mixture of *A. rhizogenes* strains carrying pCAMBIA1300::*STTM393* and pEG104, respectively. *A. rhizogenes* was grown in Luria–Bertani medium supplemented with kanamycin at 25°C for 2 days. Inoculum was prepared by resuspending the cells in 10 mM $MgSO_4$ with a final OD_{600} of 0.3.

Soybean cotyledons were removed from 10-day-old seedlings grown in vermiculite, and inoculated with 20 μl of *A. rhizogenes* cell suspensions as described previously (Subramanian *et al.*, 2005; Kereszt *et al.*, 2007). Inoculated cotyledons were placed in sterile Petri dishes containing 1% agar, and incubated in a growth chamber at 22°C with a 16 h

photoperiod. Hairy roots were monitored for yellow fluorescence production using a Leica MZ FLIII fluorescence stereomicroscope (http://www.leica-microsystems.com) over a period of 4 weeks. Fluorescent roots were excised for Northern blotting and *P. sojae* infection.

P. sojae infection assay of hairy roots

Hairy roots expressing yellow fluorescence were removed from the cotyledons and inoculated with zoospores of *P. sojae* strain P6497G, a derivative of *P. sojae* strain P6497. Strain P6497G carries the plasmid pTOR::*AcGFP1* and constitutively expresses GFP under the control of the *Ham34* promoter (Whisson *et al.*, 2007).

Detached hairy roots were immersed in the zoospore suspension (approximately 5000 zoospores ml^{-1}) for 5 min. Inoculated roots were incubated in 1% agar plates at room temperature in the dark for 48 h. Disease progression was monitored using a Zeiss Imager A1 fluorescence microscope (http://www.zeiss.com). *P. sojae* biomass was determined by quantitative PCR using primers designed to amplify the *Cox* region that is specific to *P. sojae* (Dou *et al.*, 2008; Grünwald *et al.*, 2011).

Statistical analysis

Statistical analyses were performed using JMP 8.0 (SAS Institute Inc., www.sas.com).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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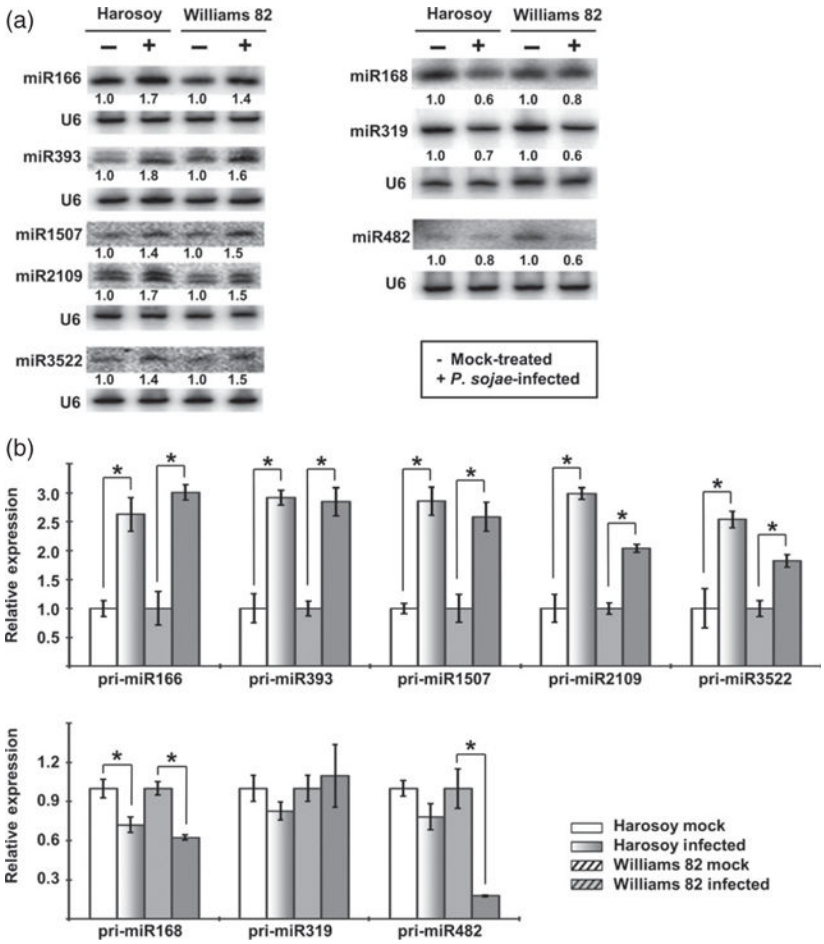


Figure 1.

Specific known soybean miRNAs differentially accumulated in roots infected by *P. sojae*.

(a) Northern blots showing miRNA abundances in mock-treated and *P. sojae*-infected tissues at 8 hpi. U6 served as the loading control. The numbers below each blot indicate the relative abundance of the miRNAs.

(b) Transcript abundances of pri-miRNAs in *P. sojae*-infected tissues. Relative expression levels were determined by quantitative RT-PCR using *GmUBI* as the internal standard. Values are means ± SD from two independent biological replicates. Asterisks indicate statistically significant differences ($P < 0.01$).

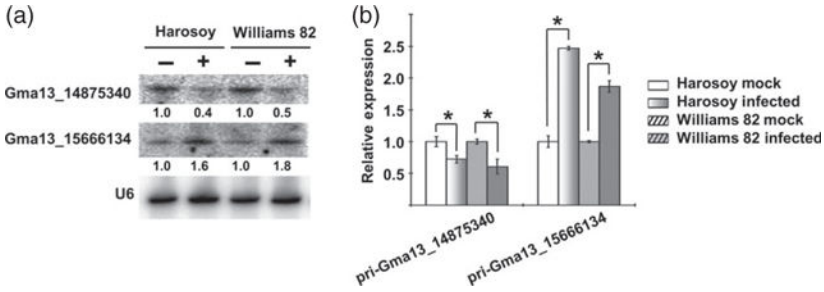


Figure 2.

Two novel miRNAs differentially accumulated in *P. sojae*-infected soybean roots.

(a) Northern blots showing altered miRNA abundance in *P. sojae*-infected tissues at 8 hpi. U6 served as the loading control. The numbers below each blot indicate the relative abundance of the miRNAs

(b) Transcript abundances of pri-miRNAs determined by quantitative RT-PCR using *GmUBI* as the internal standard. Values are means ± SD from two independent biological replicates. Asterisks indicate statistically significant differences ($P < 0.01$).

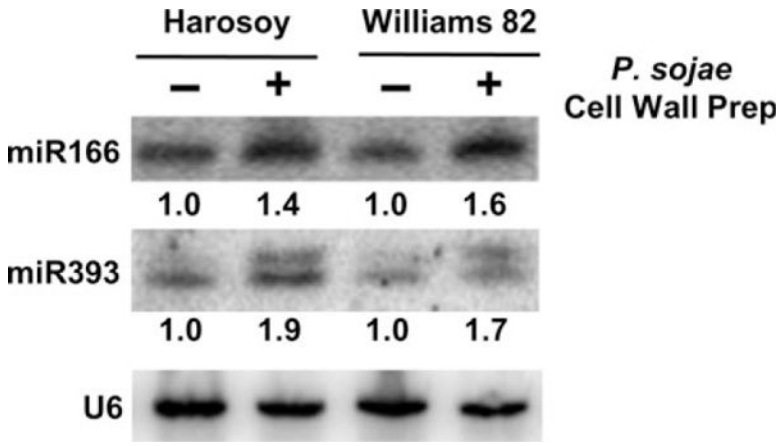
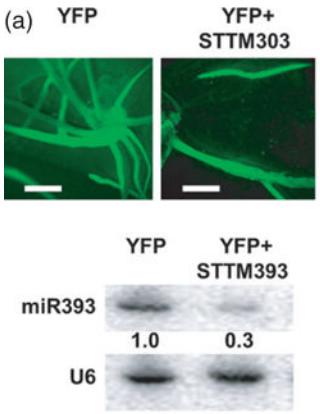


Figure 3.

Expression of miR166 and miR393 was induced by heat-inactivated *P. sojae* cells.

The abundances of miR166 and miR393 were determined by Northern blotting 8 h after exposing the roots to heat-inactivated hyphae (+) or water (−). U6 served as the loading control. This experiment was repeated four times with similar results.



(b)

Harosoy	24 hpi		36 hpi		48 hpi	
	YFP	STTM393	YFP	STTM393	YFP	STTM393
No. of roots infected	0/11	11/12	11/11	11/12	11/11	11/12
No. of roots developed Oospores	0/11	2/12	3/11	5/12	7/11	8/12
Williams 82	**24 hpi**		**36 hpi**		**48 hpi**	
	YFP	STTM393	YFP	STTM393	YFP	STTM393
No. of roots infected	0/22	10/17	3/22	15/17	8/22	15/17
No. of roots developed Oospores	0/22	6/17	1/22	11/17	4/22	12/17

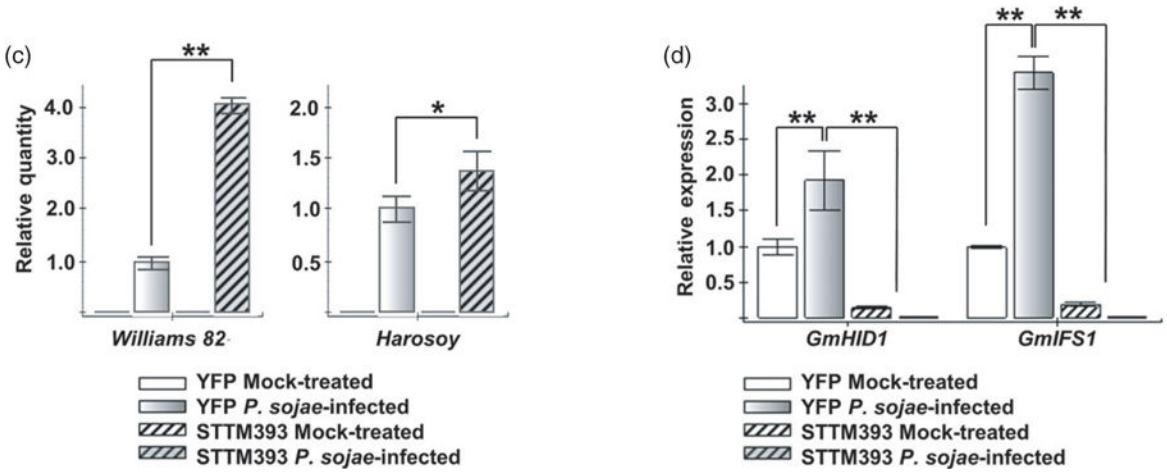


Figure 4.

miR393 regulates soybean defense against *P. sojae* infection.

(a) miR393 levels were greatly decreased in Williams 82 roots expressing the *STTM393* construct. Hairy roots were induced by *A. rhizogenes* carrying *35S*-YFP or *35S*-YFP + *35S*-STTM393. Transgenic roots were selected on the basis of production of yellow fluorescence. The images were taken at 4 weeks post-inoculation. Scale bar = 4 mm. The abundance of miR393 was determined by Northern blotting using U6 as the control. The numbers below the blot indicate relative abundance.

(b) Infection progress of *P. sojae* in soybean roots expressing *YFP* or *STTM393*. Roots allowing hyphae penetration or oospore development were counted at 24, 36 and 48 hpi. This experiment was repeated three times with similar results. Data from the other two experiments are presented in Table S6.

(c) *STTM393*-expressing roots were hyper-susceptible to *P. sojae*. *P. sojae* biomass was evaluated by quantitative PCR amplifying *PsCOX* at 48 hpi. *GmUBI* was used as the internal control. Values are means ± SD from two independent biological replicates. Asterisks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$).

(d) Transcript abundances of *GmHID1* and *GmIFS1* in *STTM393*-expressing roots determined by quantitative RT-PCR using *GmUBI* as the internal control. Values are means ± SD from two independent biological replicates. Asterisks indicate statistically significant differences (**$P < 0.01$).

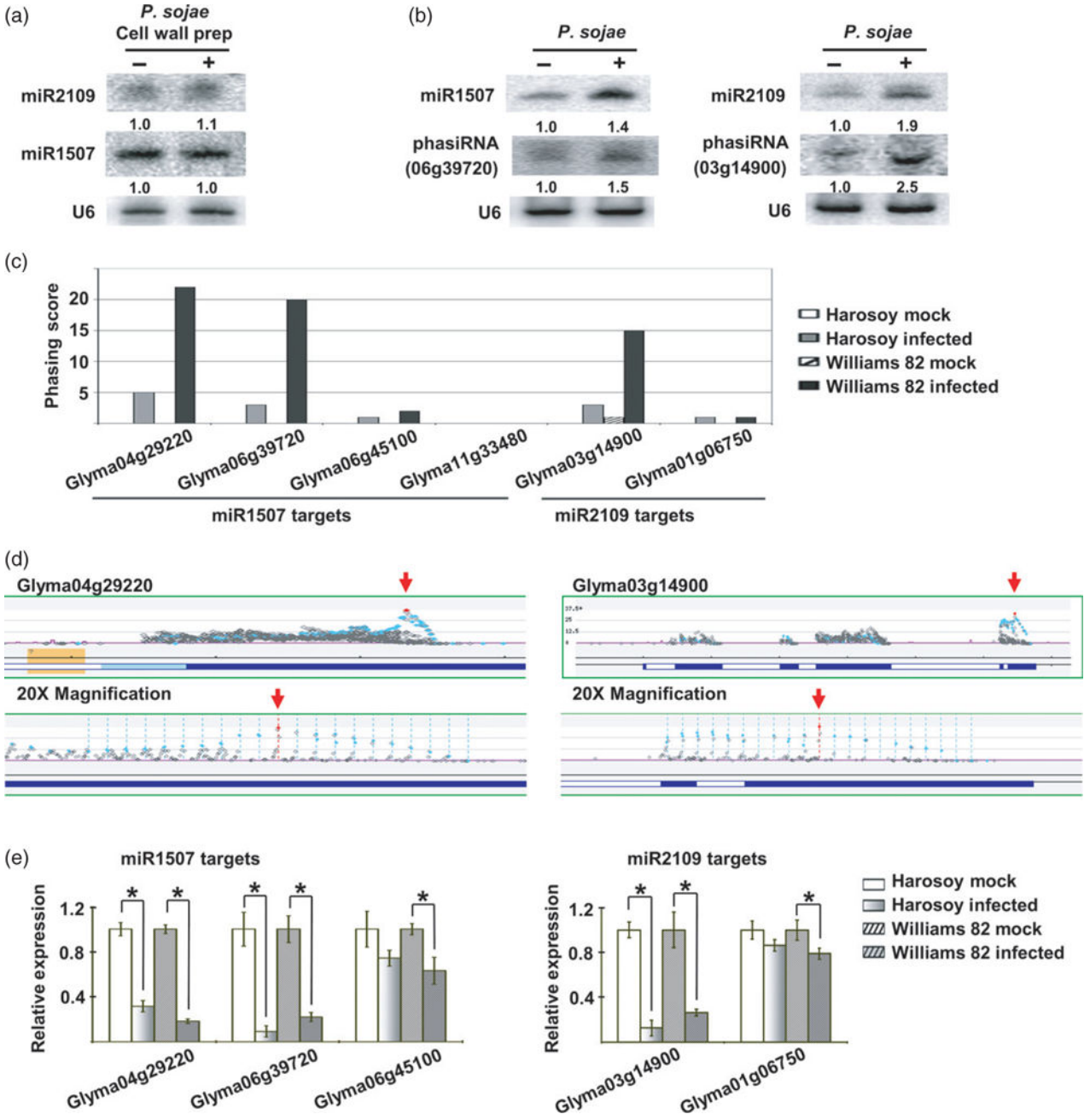


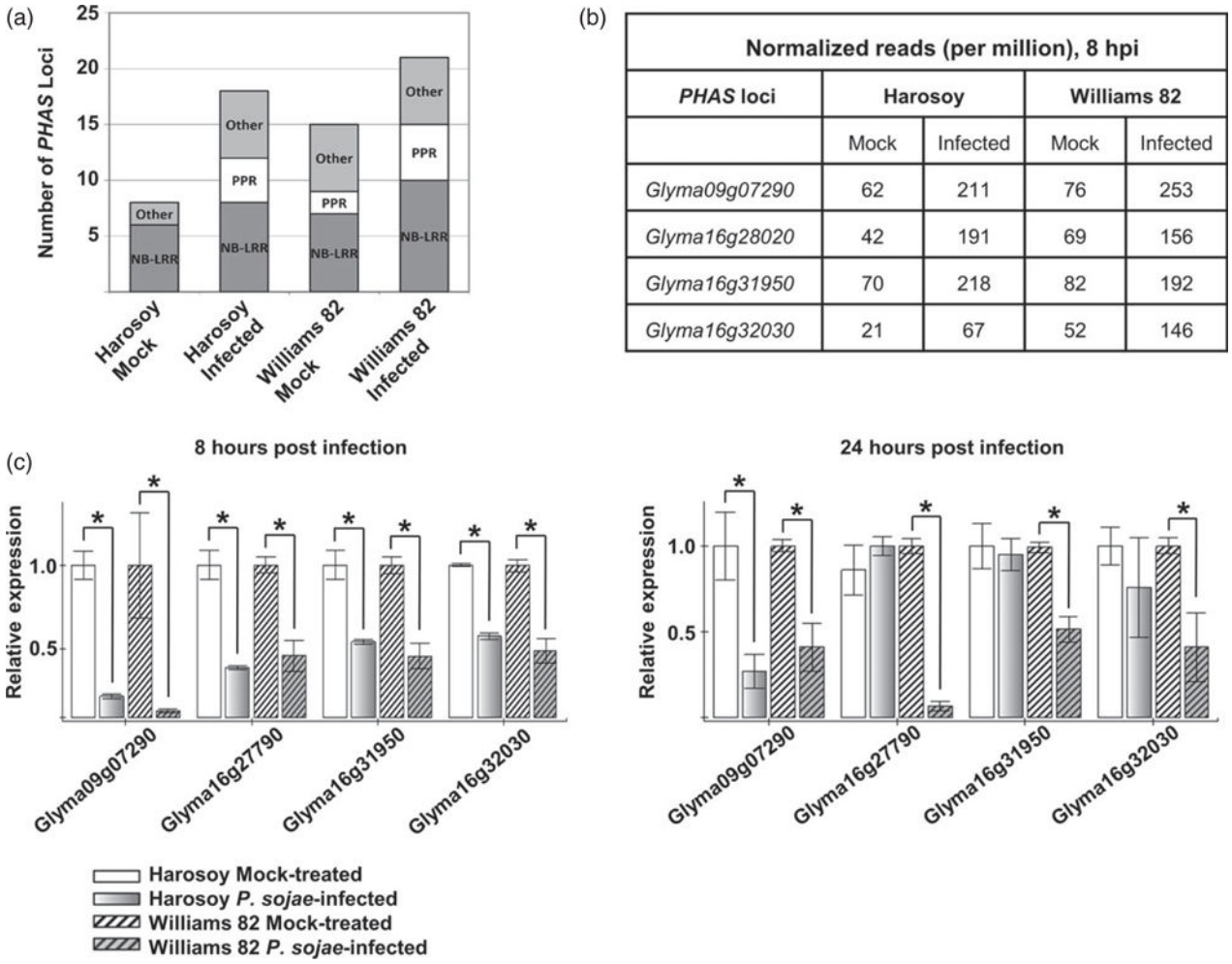
Figure 5.

phasiRNAs were induced from two 22 nt miRNAs that target *NB-LRR* genes in *P. sojae*-infected roots.

(a) Abundances of miR1507 and miR2109 were unaltered in Williams 82 roots exposed to *P. sojae* cell wall prep at 8 hpi. U6 served as the loading control. This experiment was repeated at least three times with similar results.

(b) Northern blotting showing the abundances of miRNA1507, miR2109 and the phasiRNAs derived from their target *NB-LRR* loci in Williams 82 roots inoculated with *P. sojae* at 8 hpi. This experiment was repeated twice with similar results.

(c) phasiRNA production from miR1507 and miR2109 target genes.
(d) phasiRNAs generated from the miR1507 target *Glyma04g29220* and the miR2109 target *Glyma03g14900* show 21 nt in-phase signatures following the cleavage sites (indicated by the red arrows) of their parent miRNA. phasiRNAs are shown as blue dots in a ten-cycle interval of 21 nt.
(e) Transcript abundances of miR1507 and miR2109 target genes evaluated by quantitative RT-PCR using *GmUBI* as the internal control. Values are means ± SD from two independent biological replicates. Asterisks indicate statistically significant differences (* $P < 0.01$).



Normalized reads (per million), 8 hpi				
PHAS loci	Harosoy		Williams 82	
	Mock	Infected	Mock	Infected
Glyma09g07290	62	211	76	253
Glyma16g28020	42	191	69	156
Glyma16g31950	70	218	82	192
Glyma16g32030	21	67	52	146

Figure 6.

phasiRNAs were induced from pentatriopeptide repeat (*PPR*) genes during *P. sojae* infection.

(a) Functional classification of *PHAS* loci in soybean roots.

(b) Enhanced accumulation of phasiRNAs generated from *PPR* loci in *P. sojae*-infected roots.

(c) Transcript levels of *PPR* genes in *P. sojae*-infected roots at 8 and 24 hpi. Relative transcript abundance was evaluated by quantitative RT-PCR using *GmUBI* as the internal control. Values are means ± SD from two independent biological replicates. Asterisks indicate statistically significant differences (* $P < 0.01$).