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Reduction of *Phakopsora pachyrhizi* infection on soybean through host- and spray-induced gene silencing

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Abstract

Asian soybean rust (ASR), caused by the obligate fungal pathogen *Phakopsora pachyrhizi*, often leads to significant yield losses and can only be managed through fungicide applications currently. In the present study, eight urediniospore germination or appressorium formation induced *P. pachyrhizi* genes were investigated for their feasibility to suppress ASR through a bean pod mottle virus (BPMV)-based host-induced gene silencing (HIGS) strategy. Soybean plants expressing three of these modified BPMV vectors suppressed the expression of their corresponding target gene by 45%–80%, fungal biomass accumulation by 58%–80%, and significantly reduced ASR symptom development in soybean leaves after the plants were inoculated with *P. pachyrhizi*, demonstrating that HIGS can be used to manage ASR. In addition, when the in vitro synthesized double-stranded RNAs (dsRNAs) for three of the genes encoding an acetyl-CoA acyltransferase, a 40S ribosomal protein S16, and glycine cleavage system H protein were sprayed directly onto detached soybean leaves prior to *P. pachyrhizi* inoculation, they also resulted in an average of over 73% reduction of pustule numbers and 75% reduction in *P. pachyrhizi* biomass accumulation on the detached leaves compared to the controls. To the best of our knowledge, this is the first report of suppressing *P. pachyrhizi* infection in soybean through both HIGS and spray-induced gene silencing. It was demonstrated that either HIGS constructs targeting *P. pachyrhizi* genes or direct dsRNA spray application could be an effective strategy for reducing ASR development on soybean.

KEYWORDS

dsRNA, HIGS, *Phakopsora pachyrhizi*, SIGS, soybean rust

1 | INTRODUCTION

Phakopsora pachyrhizi is an aggressive obligate pathogen and the causal agent of Asian soybean rust (ASR). Unlike other highly specialized rust fungi, *P. pachyrhizi* has a wide host range and is able to infect more than 150 species of plants from more than 53 genera, including soybean, related *Glycine* species, and other hosts in the Fabaceae (Hershman *et al*., 2011). Soybean yield losses of up to 80% in experimental trials have been reported in Asia (Hartman *et al*., 1991), 63% in Brazil during 2003, 60% in Paraguay during 2001 (Yorinori *et al*., 2005), up to 100% in South Africa (Caldwell *et al*., 2004), and up to 55% in the USA (Mueller *et al*., 2009).

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Currently, all commercial soybean cultivars are susceptible to *P. pachyrhizi*, and the only available method to control ASR is multiple fungicide applications (Miles *et al*., 2007). The level of ASR control depends on disease pressure and the timing of fungicide applications (Mueller *et al*., 2009). However, continued use of fungicides has led to not only the development of fungicide resistance among *P. pachyrhizi* populations, but also increased operation cost. For example, soybean producers in Brazil spent close to $2 billion per year on fungicides to control ASR (Godoy *et al*., 2015). Therefore, there is an urgent need to develop soybean varieties that are resistant or tolerant to ASR to reduce its potential to cause severe yield losses.

Soybean lines with resistance to *P. pachyrhizi* infection have been reported (Bromfield, 1984) and so far seven *resistance to P. pachyrhizi* (*Rpp*) genes have been discovered in different soybean germplasm accessions (Childs *et al*., 2018), such as *Rpp1* in PI 200,492 (McLean and Byth, 1980), *Rpp2* in PI 230,970 (Walker *et al*., 2014), *Rpp3* in PI 462,312 (Hartwig and Bromfield, 1983) and *Rpp4* in PI 459025B (Hartwig, 1986). However, developing rust-resistant soybean varieties through traditional plant breeding has been a slow, time-consuming process (Saurabh *et al*., 2014) and single-gene resistance can be overcome by the pathogen quickly (Grasso *et al*., 2006; Godoy, 2012; Schmitz *et al*., 2014).

Besides identifying ASR resistance-related genes and incorporating them into soybean to enhance its resistance to infection by *P. pachyrhizi*, recent advances in RNA interference (RNAi) offer another possibility of managing fungal diseases via host-induced gene silencing (HIGS). HIGS is an RNAi-based approach in which small interfering RNAs (siRNAs) homologous to genes of fungal origin are produced in the host plant and subsequently silence their targets in the pathogen during its infection of the host plant (Nowara *et al*., 2010). In the past several years, HIGS has been shown to be an effective alternative to fungicide applications in managing various plant diseases, such as reducing powdery mildew on barley and wheat through targeting the *Avra10* effector of *Blumeria graminis* f. sp. *tritici* (Nowara *et al*., 2010), suppressing wheat stripe rust through silencing a highly abundant *Puccinia striiformis* f. sp. *tritici* haustorial transcript (Yin *et al*., 2011), and reducing fusarium wilt disease in banana through expressing siRNAs targeting vital *Fusarium oxysporum* f. sp. *cubense* genes (Ghag *et al*., 2014), or aflatoxin contamination in maize by expressing a HIGS construct targeting the *Aspergillus flavus aflC* gene (Thakare *et al*., 2017; Sharma *et al*., 2018) or *aflM* gene (Raruang *et al*., 2020).

In addition, several studies have demonstrated that direct double-stranded RNA (dsRNA) application can be used to manage plant fungal diseases. For example, foliar spray of in vitro synthesized *CYP3* dsRNAs effectively reduced *F. graminearum* infection and resulted in much smaller head blight lesions on barley (Koch *et al*., 2016). External application of dsRNAs and small RNAs (sRNAs) targeting Dicer-like protein *DCL1* and *DCL2* genes of *Botrytis cinerea* on vegetables, fruits, and flower petals significantly suppressed grey mould (Wang *et al*., 2016). McLoughlin *et al*. (2018) demonstrated exogenously applied dsRNA protected plants against *Sclerotinia sclerotiorum* and *B. cinerea*.

The obligate biotrophic nature of *P. pachyrhizi* makes it difficult to study in vitro. The fungal genome has not been completely sequenced despite repeated attempts (Loehrer *et al*., 2014). To date, only limited transcriptomic information is available on *P. pachyrhizi*, such as expressed sequence tag analysis (Posada-Buitrago and Frederick, 2005), RNA-Seq studies of infected soybean leaves at different stages of infection (Tremblay *et al*., 2010, 2012, 2013), transcriptome analysis during appressorium and haustorial formation (Stone *et al*., 2012; Link *et al*., 2014), and secretome analysis of *P. pachyrhizi* (de Carvalho *et al*., 2017).

A shortlist of potential *P. pachyrhizi* target genes that have been generated through bioinformatic analysis of data from the above ASR transcriptomic studies and that might be involved in infection and pathogenicity were selected for suppressing ASR on soybean via HIGS. A bean pod mottle virus (BPMV)-based vector system developed by Zhang *et al*. (2010) was chosen to transiently express siRNA in soybean for this study. This vector system has been successfully used to test the candidate genes from soybean for their involvement in resistance to ASR (Meyer *et al*., 2009; Cooper *et al*., 2013; Liu and Whitham, 2013; Qi *et al*., 2016).

When soybean plants are infected by the BPMV-based vector system, dsRNAs formed during virus replication will trigger siRNA production. BPMV constructs that contain sequences from *P. pachyrhizi* genes can then produce siRNAs against target genes during *P. pachyrhizi* infection. The main objectives of this study were to determine whether genes involved in urediniospore germination or appressorium formation can be used to manage ASR through HIGS, and to examine the effectiveness of the dsRNAs for three of the genes in ASR reduction through spray-induced gene silencing (SIGS). In this study, eight genes from *P. pachyrhizi* were cloned into a BPMV-based HIGS vector system and introduced into soybean through biolistic and rubbing inoculation. Among them, three HIGS constructs significantly reduced *P. pachyrhizi* infection and pustule formation compared to the controls after being introduced into soybean plants in both in vitro and in planta assays. Significant reduction of *P. pachyrhizi* infection was also demonstrated after directly spraying in vitro synthesized dsRNAs targeting *P. pachyrhizi* genes encoding an acetyl-CoA acyltransferase (ATC), a 40S ribosomal protein S16 (RP_S16), and a glycine cleavage system H protein (GCS_H) onto detached soybean leaflets before *P. pachyrhizi* inoculation. To the best of our knowledge, this is the first report of suppressing *P. pachyrhizi* infection in soybean through HIGS and direct dsRNA application.

2 | RESULTS

2.1 | Construction of HIGS vectors and confirmation of successful HIGS construct expression

Eight *P. pachyrhizi* urediniospore germination or appressorium formation-related genes (Table 1) were selected based on bioinformatic analysis of the available transcriptomic data of *P. pachyrhizi* (Stone *et al*., 2012; Link *et al*., 2014; de Carvalho *et al*., 2017) and a segment

TABLE 1 List of candidate genes from *Phakopsora pachyrhizi* and primers used to construct HIGS vectors

Candidate gene	Accession no.	Putative function	Primer sequence		Amplicon size (bp)
ATC	EH237261.1	Acetyl-CoA acyltransferase	Forward	CGCGGATCCGGAAATGCGTCGCAAGTG	351
			Reverse	CGCGGATCCCTATCTCCTCCTTTCTAAT	
UN_1	EH249765.1	Hypothetical protein	Forward	CGCGGATCCAGAGACGAAGTCTTCCGT	202
			Reverse	CGCGGATCCATAGGAATGCCTAAGGGA	
UN_2	EH249689.1	Hypothetical protein	Forward	CGCGGATCCTTGTGGCTATCGGCTGTGT	192
			Reverse	CGCGGATCCTTTGAGCTGAATTGCGACA	
UN_3	EH236143.1	Hypothetical protein	Forward	CGCGGATCCAGCTTGCTCAAGAGAGTG	275
			Reverse	CGCGGATCCAGCCAGCTCTCCAGGCTT	
GCS_H	EH229082.1	Glycine cleavage system H protein	Forward	CGCGGATCCGATCAGAAGTCCGGCAACGA	304
			Reverse	CGCGGATCCTGATGCCGCTTTGACATCCT	
RP_S16	EH249914.1	40S ribosomal protein S16	Forward	CGCGGATCCTAGGTCCCGGTGTAGATGGG	319
			Reverse	CGCGGATCCAGGATTGGAAAGGCCAGGAA	
CRP_6	DN739834.1	Conidiation-related protein 6	Forward	CGCGGATCCTTGCAAGGACGGAAAGGGTT	257
			Reverse	CGCGGATCCCTTGGGTTCCATTCTCCGGG	
PHR	JK650639.1	Putative 3-hydroxy-3-methylglutaryl-coenzyme A reductase	Forward	CGCGGATCCCTAGTCTCTTATGGCGGCGG	326
			Reverse	CGCGGATCCGCGTTTGCTTGTCGTTCAGT	

Note: The restriction enzyme *Bam*HI site was incorporated in the primer and is underlined.

of 200–400 nucleotides (nt) in length for each of the genes was amplified and cloned into the BPMV vector (Zhang *et al*., 2013) (Figure 1a). Soybean seedlings developed mild mottling foliar symptoms 14 days after mechanical inoculation using primary inoculum prepared from plants bombarded with BPMV:EV (empty vector) whereas foliar photobleaching symptoms were observed on soybean seedlings inoculated with primary inoculum containing BPMV:PDS, which has a section of the sequence encoding soybean phytoene desaturase (PDS) in the modified BPMV, indicating that the expression of the target gene was specifically suppressed under the experimental conditions (Figure 1b). Mild mottling foliar symptoms were consistently observed on the second trifoliate leaves of soybean plants mechanically inoculated with eight primary inocula containing each of the eight HIGS constructs listed in Table 1 (Figure 1b). Furthermore, enzyme-linked immunosorbent assay (ELISA) confirmed the presence of BPMV in the leaf tissues inoculated with the empty BPMV vector or any one of the eight HIGS constructs. Taken together, the mottling foliar symptoms, photobleached leaves of the positive control, and the ELISA results indicate that the BPMV-HIGS constructs can be successfully delivered into soybean plants and expressed properly (Figure 1c).

2.2 | Detection of gene-specific sRNA in HIGS-treated soybean leaf samples

RNA-Seq was performed to examine whether the leaf samples expressing the HIGS constructs were associated with the production of gene-specific sRNAs. Leaf samples containing HIGS constructs targeting the *ATC*, *GCS_H*, and *RP_S16* genes were selected for sRNA library construction and sequencing, which yielded about 10 million reads per library. The sRNA sequencing data were analysed following the flowchart described in Figure 2. No detectable sRNA reads that matched the *ATC*, *GCS_H* or *RP_S16* gene sequences were observed in libraries prepared from mock-treated soybean leaves or BPMV:EV infected soybean leaves (Table 2). However, more than 1,000 sRNA reads in the soybean leaf samples expressing each of the HIGS constructs were aligned to their corresponding target gene sequences (Table 2), demonstrating that this transient HIGS approach was working as expected. These gene-specific sRNAs were found to distribute unevenly across the target gene sequence, with most of the sRNAs appearing to be generated from a few hotspots in the target sequence for *ATC* (Figure 3a), *GCS_H* (Figure 3c) or *RP_S16* (Figure 3e). Most of the sRNAs from leaf samples containing BPMV:ATC and BPMV:RP_S16 aligned to the antisense strand of the target sequence (Figure 3a,e) whereas the sRNAs from leaf samples containing BPMV:GCS_H appeared to align to both sense and antisense strands of the target sequence (Figure 3c). Further, the most abundant sRNA from these libraries was 21–22 nt in length (Figure 3b,d,f), which agrees with previous studies on sRNAs produced in tissues expressing HIGS constructs (Ghag *et al*., 2014; Jahan *et al*., 2015; Koch *et al*., 2016).

2.3 | HIGS constructs reduced *P. pachyrhizi* infection in greenhouse conditions

ASR development on the control and HIGS construct-treated soybean plants was visually assessed 2 weeks after inoculation with *P. pachyrhizi*

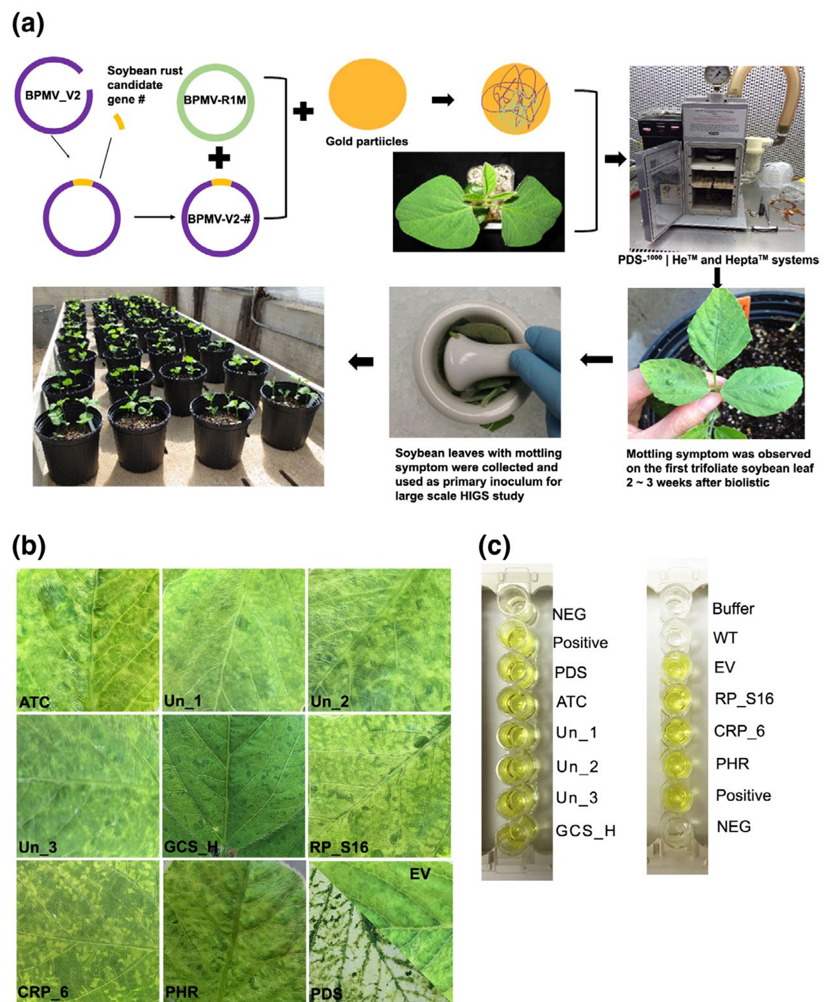

FIGURE 1 Suppression of *Phakopsora pachyrhizi* gene expression in soybean through a BPMV-based transient host-induced gene silencing (HIGS) approach. (a) Workflow of primary viral inoculum generation for various HIGS constructs. (b) Visual mottle symptoms on soybean leaves expressing various HIGS constructs. (c) Enzyme-linked immunosorbent assay (ELISA) confirming the presence of bean pod mottle virus (BPMV) in leaf samples. The genes used in the HIGS constructs encode various proteins with putative functions, such as acetyl-CoA acyltransferase (ATC), glycine cleavage system H protein (GCS_H), 40S ribosomal protein S16 (RP_S16), conidiation-related protein 6 (CRP_6), putative 3-hydroxy-3-methylglutaryl-coenzyme A reductase (PHR) or unknown functions (Un_1, 2, and 3). The gene *PDS* (soybean phytoene desaturase) was used as a positive control to show the bleached leaf phenotype. The BPMV that was supplied with the ELISA kit was used as a positive control. Water (WT) and buffer were included as negative controls

and the representative foliar symptoms of three repeated studies are presented (Figure 4a). Significant reduction (α = 0.01) in pustule number (by more than 41.6%–61.2%) was observed for soybean leaves treated with HIGS constructs targeting *P. pachyrhizi ATC*, unknown 3 (*Un_3*), *GCS_H*, and *RP_S16* genes compared to the BPMV:EV treatment (Figure 4b). In addition, *P. pachyrhizi* biomass accumulation in soybean leaves containing HIGS constructs targeting *P. pachyrhizi ATC*, *Un_3*, *GCS_H*, and *RP_S16* genes was reduced by 70.2%, 57.8%, 63.7%, and 63.6%, respectively, compared to the *P. pachyrhizi* biomass accumulation in soybean leaves containing the BPMV:EV without HIGS target genes (Figure 4c). The *P. pachyrhizi* biomass in soybean leaves inoculated with other HIGS constructs did not show significant differences compared to the control leaves inoculated with the BPMV:EV. The corresponding transcript was also found to be reduced by 49.6%, 75.2%, and 46.3% (α = 0.01), respectively, in the leaf samples containing HIGS construct targeting the *P. pachyrhizi ATC*, *GCS_H*, and *RP_S16* genes, respectively, when compared to the control leaf sample containing BPMV:EV (Figure 4d). The transcript level for *Un_3* in the leaf samples inoculated with HIGS construct BPMV:Un_3 did not change significantly compared to BPMV:EV control.

2.4 | HIGS constructs reduced *P. pachyrhizi* infection in detached leaf assays

Similar suppression in ASR severity as determined by pustule number per cm^2 of leaf was observed on detached soybean leaves expressing BPMV:ATC, BPMV:GCS_H, and BPMV:RP_S16, and was about 58.1%–70.8% of that of the BPMV:EV control (Figure 5a,b). Soybean leaves expressing BPMV:ATC, BPMV:GCS_H, and BPMV:RP_S16 also showed significant reduction (α = 0.01) in *P. pachyrhizi* biomass in inoculated soybean leaves, which was about 20.3%, 34.7%, and 25.4%, respectively, of that in the control leaves expressing BPMV:EV (Figure 5c). The expression of the corresponding *P. pachyrhizi ATC*, *GCS_H*, and *RP_S16* genes was also reduced to 45.1%, 20.4%, and 54.6%, respectively, of that in the control leaves treated with BPMV:EV. However, the pustule number per cm^2, *P. pachyrhizi* biomass, and target gene expression in soybean leaves expressing BPMV:Un_3 did not show significant differences compared to those in control leaves (Figure 5a,b), which is different from what was observed in the greenhouse study.

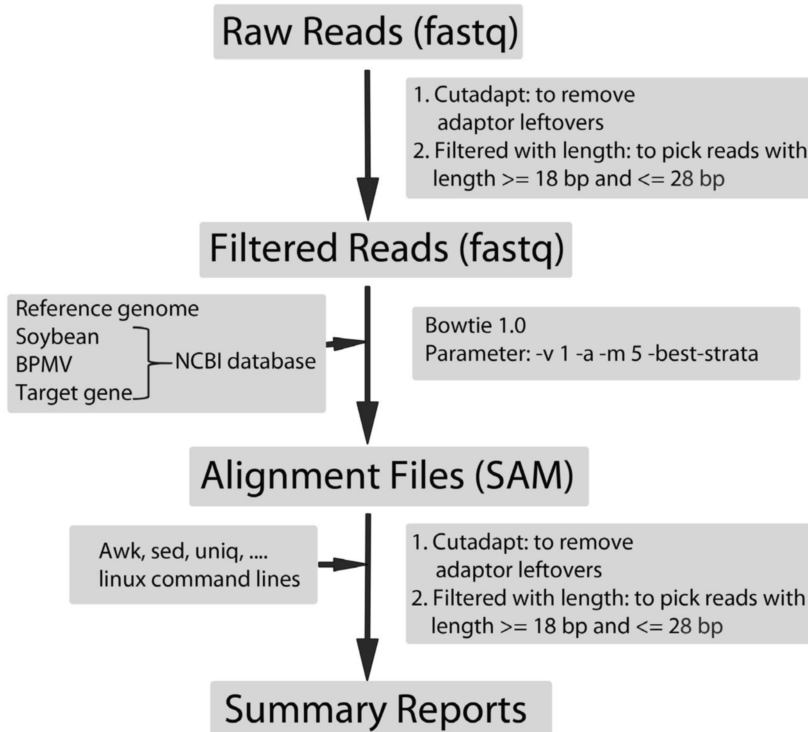

FIGURE 2 Analysis workflow used to identify gene-specific small RNAs expressed in small RNA libraries prepared from HIGS- and empty vector (EV)-treated soybean leaf samples. The bioinformatics pipeline used in the small RNA analysis was modified from Tian *et al.* (2017)

TABLE 2 Number of small RNA reads matched to the intended target sequences in each of the libraries

	Number of reads aligned to reference sequences[b]			
Libraries sequenced[a]	BPMV	ATC	GCS_H	RP_S16
WT	552	2	4	1
BPMV:EV	388,893	2	2	1
BPMV:ATC	47,517	1,562	1	1
BPMV:GCS_H	44,721	5	3,146	1
BPMV:RP_S16	84,249	1	2	1,889

[a]Leaves from soybean plants (Williams 82) were collected 2 weeks after rubbing inoculation with buffer (WT), bean pod mottle virus (BPMV) empty vector (EV), BPMV:ATC, BPMV:GCS_H, or BPMV:RP_S16. ATC, acetyl-CoA acyltransferase; GCS_H, glycine cleavage system H protein; RP_S16, 40S ribosomal protein S16.

[b]Information on reference sequences is provided in Table 1.

2.5 | Direct dsRNAs spray on detached soybean leaves reduced ASR development

Soybean leaves sprayed with dsRNA targeting *P. pachyrhizi ATC*, *GCS_H*, and *RP_S16* genes before being inoculated with *P. pachyrhizi* were found to have a lower number of pustules per cm^2 of leaf than the negative control 2 weeks after inoculation (Figure 6a). The control soybean leaves sprayed with water developed an average of 9.1 pustules per cm^2, whereas the detached soybean leaves sprayed with dsRNA of *ATC*, *GCS_H*, and *RP_S16* developed an average of 1.5, 1.6, and 1.6 pustules per cm^2, respectively, which are significantly lower values than for the control (Figure 6b). Soybean leaves sprayed with dsRNA of *ATC*, *GCS_H*, and *RP_S16* also showed significant reduction ($\alpha = 0.01$) in *P. pachyrhizi* biomass, which was about 17.0%, 20.9%, and 25.1%, respectively, of that in the detached soybean leaves sprayed with water (Figure 6c). The expression of corresponding *P. pachyrhizi* genes for *ATC*, *GCS_H*, and *RP_S16* was also reduced to 32.9%, 32.4%, and 28.2%, respectively, of that in detached soybean leaves treated with H_2O based on quantitative reverse transcription PCR (RT-qPCR) (Figure 6d).

3 | DISCUSSION

Lack of resistance in commercial soybean varieties and the slow process in breeding lines with high level of resistance to ASR have created an urgent need to develop alternative approaches to fungicide applications to reduce environmental pollution, operating cost to farmers, and the chance of the pathogen becoming fungicide resistant (Childs *et al.*, 2018). The emergence of RNAi and cross-kingdom RNAi, which has been employed to manipulate gene expression to improve specific traits or to suppress pathogen infection and disease development in crops (Baulcombe, 2004; Saurabh *et al.*, 2014), offers a new potential for enhancing soybean resistance to ASR. Enhanced crop resistance to various fungal and oomycete pathogens using HIGS through either stable or transient expression of genes from pathogens or even direct spray application of dsRNAs targeting pathogens has been reported (Nowara *et al.*, 2010; Chen *et al.*, 2016; Wang and Jin, 2017; Zhu *et al.*, 2017; Song and Thomma, 2018). This study examined the feasibility of using HIGS and SIGS as an effective alternative approach to

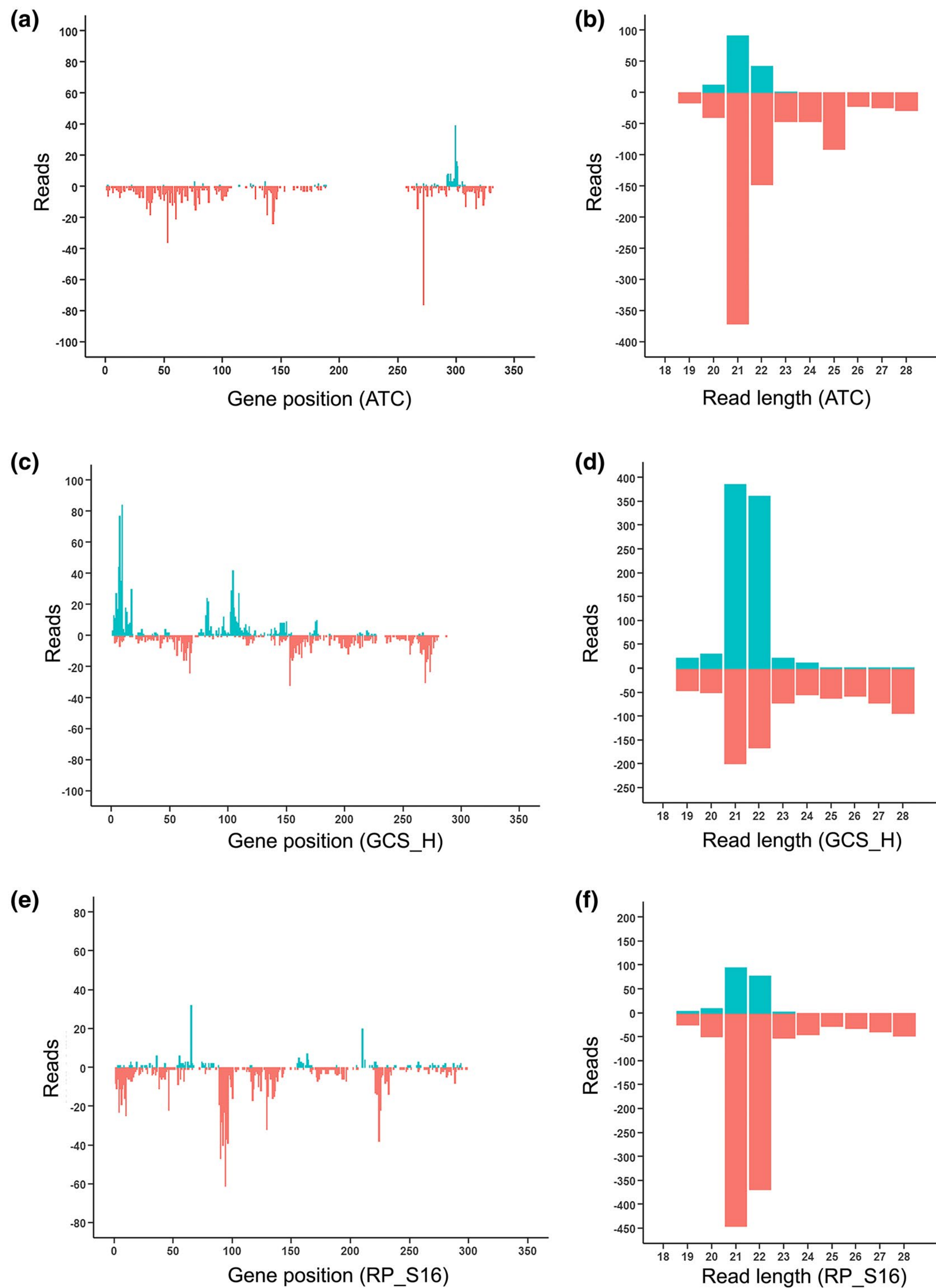

FIGURE 3 Small RNA (sRNA) profiling of *Phakopsora pachyrhizi* target genes in soybean leaf tissue that had been rub-inoculated with modified BPMV containing either EV or HIGS constructs. Number of reads that mapped to various positions of the target genes (a), (c), and (e) and their corresponding read length distributions (b), (d), and (f) of gene-specific sRNAs identified through RNA-Seq analysis of the sRNA libraries prepared from leaf samples treated with BPMV:ATC (a) and (b), BPMV:GCS_H (c) and (d) or BPMV:RP_S16 (e) and (f). ATC, acetyl-CoA acyltransferase; GCS_H, glycine cleavage system H protein; RP_S16, 40S ribosomal protein S16

conventional soybean breeding and fungicide application for ASR disease management.

In the present study, eight genes from *P. pachyrhizi* that were up-regulated either in urediniospore germination or appressorium formation were selected and screened for their potential in suppressing ASR development through the BPMV-based HIGS vector system. Three of the HIGS constructs that target *ATC*, *GCS_H*, and *RP_S16* reduced the endogenous *P. pachyrhizi* transcript abundance

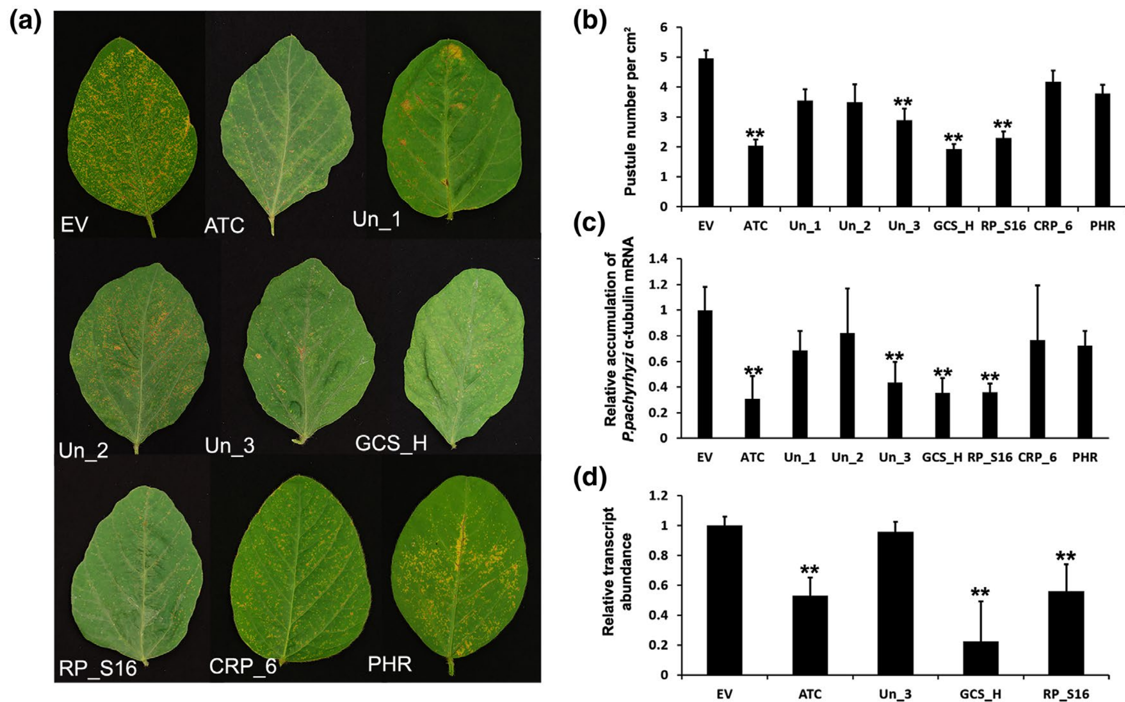

FIGURE 4 Responses of soybean plants (Williams 82) that had been rub-inoculated with modified BPMV containing either EV or HIGS constructs prior to inoculation with *Phakopsora pachyrhizi* in a greenhouse. (a) Differences in soybean rust symptom development 2 weeks after *P. pachyrhizi* inoculation among soybean plants that had been inoculated with modified BPMV containing HIGS construct targeting various *P. pachyrhizi* genes encoding acetyl-CoA acyltransferase (ATC), glycine cleavage system H protein (GCS_H), 40S ribosomal protein S16 (RP_S16), conidiation-related protein 6 (CRP_6), putative 3-hydroxy-3-methylglutaryl-coenzyme A reductase (PHR), and other hypothetical proteins of unknown functions (Un_1, Un_2, Un_3) or with modified BPMV only containing an EV. (b) Pustule numbers per cm^2 of soybean leaves that had been inoculated with modified BPMV containing HIGS construct targeting *P. pachyrhizi* genes or EV. (c) Relative expression of *P. pachyrhizi α-tubulin* mRNA to soybean *ubiquitin* following BPMV and *P. pachyrhizi* inoculation. (d) Relative levels of target gene expression in soybean leaves 2 weeks post-inoculation with *P. pachyrhizi*. Values are expressed relative to the endogenous *P. pachyrhizi* reference gene *Cyt B*, with the EV set at 1. Bars represent mean values ± *SD* of one representative experiment with five biological replicates. Asterisks indicate statistical significance: $^*p < .05$, $^{**}p < .01$ (Student's *t* test)

by at least 45.4% and fungal growth (biomass) by at least 57.8% in infected soybean leaves compared to control soybean leaves infected by the BPMV:EV in both greenhouse and detached leaf studies. However, when the construct BPMV:Un_3 was screened under more controlled laboratory detached leaf assay conditions, it did not significantly suppress ASR symptom development as in greenhouse conditions, indicating that the detached leaf assay, which allows a large number of candidate genes to be screened simultaneously, may be more reliable with fewer false positives.

The presence of gene-specific siRNAs in soybean leaves treated with the HIGS constructs has also been demonstrated through analysis of sRNA-Seq data. In addition, these gene-specific siRNAs could only be found in soybean leaves inoculated with the corresponding HIGS constructs, indicating the successful expression of these constructs in soybean plants. This result further confirms that the reduction of *ATC*, *GCS_H*, and *RP_S16* transcript abundance in respective HIGS construct inoculated leaves was due to the presence of gene-specific siRNA expressed by those HIGS constructs.

Several recent studies have demonstrated that direct spraying of dsRNA can reduce *F. graminearum* infection on barley leaves (Koch *et al.*, 2016) and *S. sclerotiorum* infection in canola roots (McLoughlin *et al.*, 2018). In the present study, direct spraying of dsRNA resulted in a reduction in the number of pustules per cm^2 of leaf, fungal biomass, and endogenous target gene expression by at least 68% compared to the control. The results from this study and previous reports demonstrate that this novel way of exploring RNAi is effective and has great potential for fungal disease control.

However, it is still unclear how the siRNA produced in the host cells or the dsRNA applied in vitro travels to the pathogen cells to suppress the expression of its target genes. Cai *et al.* (2018) reported that host plants secrete extracellular sRNA-containing vesicles during pathogen infection, which have been observed in the extrahaustorial matrix (Micali *et al.*, 2011). In addition, transport of RNA between plants and fungi appears to be bidirectional. sRNAs from *B. cinerea* have been shown to target host defence genes in *Arabidopsis* and tomato (Weiberg *et al.*, 2013). *Verticillium dahliae* recovered from infected cotton plants contained 28 miRNAs from cotton, implying that host-derived sRNAs were transmitted into the pathogen during infection (Liu *et al.*, 2016). Furthermore, the transportation of endogenous sRNAs from host cells into fungal cells may not be a simple concentration-dependent diffusion process but rather a more selective movement (Cai *et al.*, 2018).

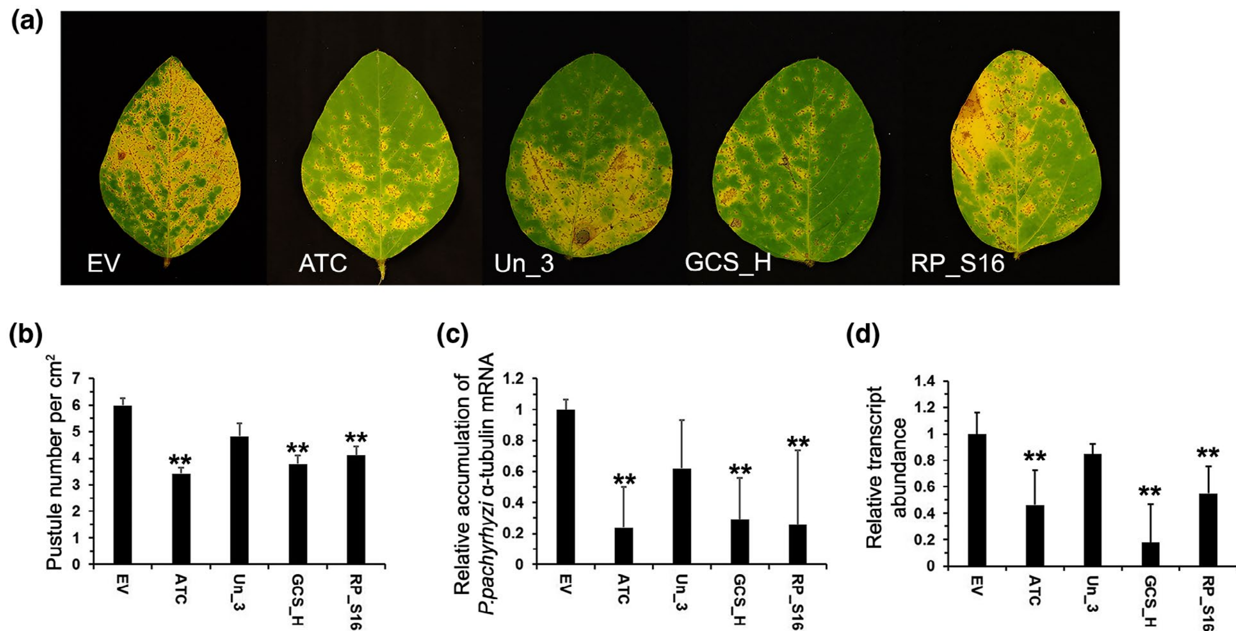

FIGURE 5 Responses of detached soybean (Williams 82) leaves that had been rub-inoculated with modified BPMV containing either EV or HIGS constructs prior to inoculation with *Phakopsora pachyrhizi* urediniospores. (a) Differences in soybean rust symptom development 2 weeks after *P. pachyrhizi* inoculation among the detached soybean leaves that had been inoculated with modified BPMV containing EV or HIGS constructs targeting various *P. pachyrhizi* genes that encode an acetyl-CoA acyltransferase (ATC), a glycine cleavage system H protein (GCS_H), a 40S ribosomal protein S16 (RP_S16), or a hypothetical protein of unknown function (Un_3). (b) Pustule numbers per cm^2 of soybean leaves that had been inoculated with modified BPMV containing HIGS constructs targeting *P. pachyrhizi* genes or EV. (c) Relative expression of *P. pachyrhizi* α*-tubulin* mRNA to soybean *ubiquitin* following BPMV and *P. pachyrhizi* inoculation. (d) Relative levels of target gene expression in soybean leaves inoculated with modified BPMV containing either EV or HIGS constructs 2 weeks after *P. pachyrhizi* inoculation. Values are expressed relative to the endogenous *P. pachyrhizi* reference gene *Cyt B*, with expression in the EV-treated leaves set at 1. Bars represent mean values ± *SD* of one representative experiment with five biological replicates. Asterisks indicate statistical significance: $^*p < .05$, $^{**}p < .01$ (Student's *t* test)

Several studies have revealed that the effects of HIGS are generally not observed until after the formation of haustoria, and silencing is more effective against genes that are highly expressed in haustoria than genes expressed in other cell types (Nowara *et al.*, 2010; Yin *et al.*, 2011; Panwar *et al.*, 2013). Therefore, the transfer of siRNA into pathogens is thought to occur across haustoria or similar feeding structures and the plant extracellular vesicles are the most likely candidates for delivering RNAs into pathogens (Nowara *et al.*, 2010; Micali *et al.*, 2011; Panwar *et al.*, 2013). In the soybean–*P. pachyrhizi* pathosystem, the HIGS signal molecules are likely to travel from host to the fungus via the haustorial interphase utilizing the mechanism of vesicle-mediated (exosomes) transport (Qi *et al.*, 2018). However, further experimental evidence is needed to support this hypothesis. Only three out of eight genes suppressed *P. pachyrhizi* infection in the present study, which is probably because *P. pachyrhizi* infection cannot be suppressed by silencing certain genes that are not critical during the initial infection process. It is also possible that sRNAs generated from the other five genes might not be able to be effectively transported into *P. pachyrhizi*. Further study is necessary to dissect the mechanism of siRNA or dsRNA transportation between soybean and *P. pachyrhizi*, which will help to select better candidate genes.

Once the candidate genes that are effective in reducing target gene expression, fungal biomass accumulation, and number of pustules per cm^2 of leaf in the infected soybean leaves have been identified, one of the next natural steps is to develop stable transgenic soybean plants expressing a HIGS construct that targets multiple *P. pachyrhizi* genes simultaneously to obtain durable protection of soybean plants against *P. pachyrhizi*. However, managing soybean fungal diseases through a transgenic approach has its own limitations: acquiring regulatory approval of transgenic materials before going to market is a time-consuming process and the negative public perceptions about genetically modified organisms in general are another concern. Therefore, the current study also explored the potential of SIGS in managing ASR following the successful studies on managing other plant fungal diseases using dsRNA as reported by Koch *et al.* (2016) and Wang *et al.* (2016). Spraying dsRNAs has been widely used previously as “oral insecticides” to control plant pests (San Miguel and Scott, 2016). SIGS can be designed to suppress multiple genes to achieve simultaneous control of a wide range of pests and pathogens without the need for a genetic engineering process (Koch *et al.*, 2016; Wang and Jin, 2017). The present study also demonstrated that direct spraying of dsRNAs specific to pathogen genes, such as *P. pachyrhizi ATC*, *GCS_H*, and *RP_S16*, can be an effective means to manage soybean rust disease. Future studies should focus on improving the efficacy and duration of plant protection by the sprayed dsRNAs

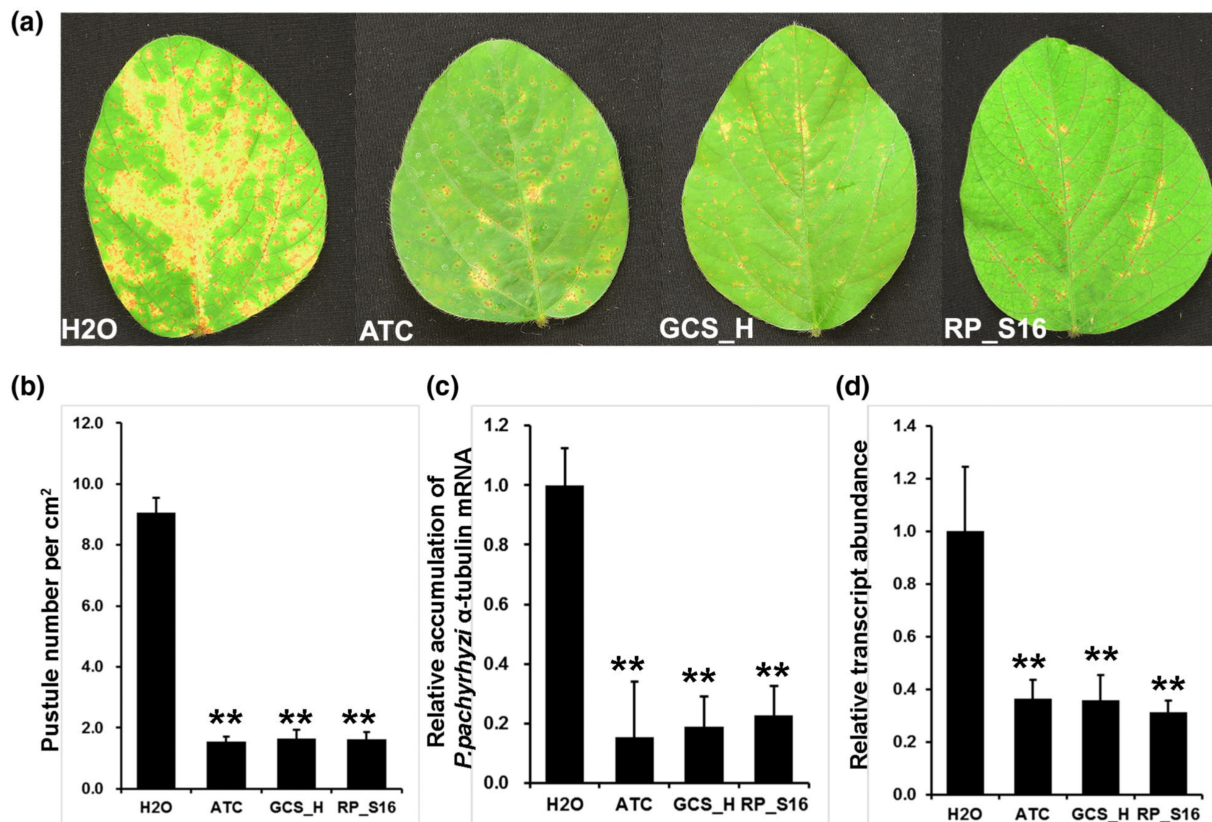

FIGURE 6 Responses of detached soybean (Williams 82) leaves that had been pretreated with or without dsRNAs of *Phakopsora pachyrhizi* genes prior to inoculation with *P. pachyrhizi*. (a) Differences in rust symptom development on soybean leaves 2 weeks after inoculation with H_2O (negative control) or with dsRNAs targeting various *P. pachyrhizi* genes. ATC, acetyl-CoA acyltransferase; GCS_H, glycine cleavage system H protein; RP_S16, 40S ribosomal protein S16. (b) Pustule numbers per cm^2 of leaf 2 weeks post inoculation with *P. pachyrhizi* of soybean leaflets that had been pretreated with water or dsRNAs targeting various *P. pachyrhizi* genes. Bars represent mean values ± *SD* of one representative experiment with five biological replicates. Asterisks indicate statistical significance: $^*p < .05$, $^{**}p < .01$ (Student's *t* test)

with the use of nontoxic, degradable, layered double hydroxide clay nanosheets (Mitter *et al.*, 2017).

In summary, a BPMV-based HIGS system was evaluated in the present study to explore the feasibility for ASR control. Among the eight selected *P. pachyrhizi* genes, three of them (*ATC*, *GCS_H*, and *RP_S16*) reduced the number of pustules per cm^2 of leaf, fungal biomass accumulation, and target gene expression in the soybean leaves containing the corresponding HIGS constructs. This suppression was probably due to the presence of gene-specific sRNAs produced by inoculated BPMV constructs. In addition, direct spraying of dsRNAs (*ATC*, *RP_16S*, and *GCS_H*) significantly reduced the number of pustules per cm^2 of leaf, fungal biomass, and endogenous target gene transcript abundance in detached leaf assays. Our study demonstrated that HIGS or SIGS using genes from *P. pachyrhizi* can be a new and effective approach for managing ASR on soybean and possibly other fungal pathogens. To the best of our knowledge this is the first report of suppressing *P. pachyrhizi* infection in soybean through HIGS and direct dsRNA treatment. Future studies are needed to fine-tune the procedures to improve the effectiveness of direct spray application of dsRNA for ARS management under field conditions.

4 | EXPERIMENTAL PROCEDURES

4.1 | Selection of fungal candidate genes and HIGS vector construction

The available *P. pachyrhizi* cDNA sequences that were up-regulated in germinating urediniospores or during appressorium formation (Posada-Buitrago and Frederick, 2005; Link *et al.*, 2014) were searched through BLAST analysis to generate a shortlist of eight genes that might be involved in infection and pathogenicity (Table 1). Based on the cDNA sequences, a 200–400 bp fragment of each gene was selected and tested by the SI-FI software tool (http://labtools.ipk-gatersleben.de/) to avoid off-targets in the soybean transcriptome. Gene-specific primers containing a *Bam*HI restriction site at each end were designed for cloning into pBPMV-IA-V2, which was modified from pBPMV-IA-R2 with multiple cloning sites (Zhang *et al.*, 2009).

Total RNA from *P. pachyrhizi* was isolated from germinated urediniospores (average viability 70%–80%) that were previously collected from a soybean field in 2013 at Ben Hur Research Station (Baton Rouge, USA) using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA synthesis was performed with 2 μg of total RNA and a High-Capacity cDNA

TABLE 3 List of primers used for real-time quantitative reverse transcription PCR and in vitro double stranded RNA synthesis

Gene product	Primer name and sequence (5′–3′)		Amplicon size (bp)
Glycine max Ubiquitin-3	U3F	GTGTAATGTTGGATGTGTTCCC	107
	U3R	ACACAATTGAGTTCAACACAAACCG	
P. pachyrhizi α*-tubulin*	TUBF	CCAAGGCTTCTTCGTGTTTCA	67
	TUBR	CAAGAGAAGAGCGCCAAACC	
TaqMan probe	5′ FAM–3′ Blackhole1	TCGTTTGGAGGCGGATCGGTTCA	
	CytB_1_F	TCAAGACGCATCCAATTCTAGGTC	89
	CytB_1_R	GTGTTACACCCGTGATAATCTGAATGAT	
	PPATC_F	GAGGAGCTGCAAATGGGTGA	115
	PPATC_R	GAATGGGGATGGCAGCATCA	
	PP_Un_3_F	CGGATCTCAGGATCTCACACG	91
	PP_Un_3_R	GCCAGCTCTCCAGGCTTAAA	
	PP_GCS_H_F	AGCCGTCGAGAGTGTCAAAG	110
	PP_GCS_H_R	AGATTGGCCTGGTCGCTTAG	
	PP_RP_S16_F	AAATTTGGAGGAAAGGGAGCACG	89
	PP_RP_S16_R	ACAGCAGGAAATAAAAACCCAAACC	
P. pachyrhizi ATC dsRNA	ATC_T7_F	GCGTAATACGATCCATCATAGGGAGAGTTTCCATCGATGATGGCATTC	741
	ATC_T7_R	GCGTAATACGATCCATCATAGGGAGA CTAGCAAGCCAAACCATAAGC	
P. pachyrhizi GCS_H dsRNA	GCS_H_T7_F	GCGTAATACGATCCATCATAGGGAGAAAACCATCGTAATCTGCCCG	420
	GCS_H_T7_R	GCGTAATACGATCCATCATAGGGAGATCCTCTCCTTCACAATGAGCT	
P. pachyrhizi RP_S16 dsRNA	RP_S16_T7_F	GCGTAATACGATCCATCATAGGGAGACCAGCCTCACAAAACGTTCA	500
	RP_S16_T7_R	GCGTAATACGATCCATCATAGGGAGAAACCCAAACCTCCATCAACG	

Reverse Transcription Kit (Applied Biosystems). The primer pairs listed in Table 1 were used for PCR amplification with cDNA as templates, and the PCR products were cloned into pCR2.1-TOPO (Invitrogen). Inserted sequences were confirmed by sequencing using M13 forward and reverse primers before being cloned into the pBPMV-IA-V2 vector.

4.2 | BPMV virus inoculum production

To generate virus inoculum for the HIGS experiments, plasmid DNA of BPMV RNA1 (pBPMV-IA-R1M, which induces moderate symptoms on inoculation compared to wild-type RNA1 clone pBPMV-IA-R1) and the recombinant RNA2 (pBPMV-IA-V2 containing *P. pachyrhizi* genes) were used to coat gold particles (cat. #165-2263, Bio-Rad) and bombarded into the primary leaves of Williams 82 soybean plants 14 days after sowing, as previously described (Zhang *et al.*, 2009). BPMV-infected leaf tissue was collected at 3–5 weeks after bombardment, lyophilized and stored at 4 °C before being ground in 20 ml of 50 mM potassium phosphate (pH 7.0) for 3 g of leaf tissue and used as primary BPMV inoculum.

4.3 | RNA sequencing to confirm the presence of target gene-specific sRNAs in HIGS-treated soybean leaves

Fourteen days after *P. pachyrhizi* inoculation, leaves with ASR symptoms from greenhouse-grown plants that had been previously rub-inoculated with modified BPMV viruses were collected and frozen in liquid nitrogen before being stored at −80 °C until RNA extraction. Total RNAs extracted from five independent biological replicates of leaf samples containing EV only or BPMV with one of the three *P. pachyrhizi* genes (*ATC*, *GCS_H* or *RP_S16*) were checked for quality using NanoDrop and 1.5% agarose gel before being pooled together for sRNA library construction using a TruSeq Small RNA Library Preparation kit according to the manufacturer's instructions (Illumina). The single-end 50 cycle sequencing was performed using an Illumina HiSeq 4,000 platform at Genome Sequencing Core at UC Davis (Davis, California, USA), which produces 50 single-end reads. The bioinformatics pipeline used in the sRNA analysis was modified from Tian *et al.* (2017). After low-quality and adapter sequences were removed by cutadapt (Martin, 2011), sRNA reads ranging from 18 to 28 nt were used for further analyses. Bowtie v. 1.2.0 (Langmead *et al.*, 2009) was used to align sRNA reads, allowing non-mismatch against sequences inserted into HIGS constructs. Linux command lines were used to extract sRNA specific to targeted *P. pachyrhizi* genes from alignment result files. R (R Development Core Team, 2013) was used to generate sRNA mapping figures.

4.4 | HIGS study of target genes using whole plants in the greenhouse

To get even virus symptom and silencing efficiency, 20 primary leaves of 2-week-old Williams 82 seedlings grown in a growth chamber for each HIGS construct were dusted with carborundum and subsequently rub-inoculated with primary inoculum corresponding to each HIGS construct. BPMV-inoculated plants were maintained in the growth chamber at 20 °C with a 16-hr photoperiod. Three days following BPMV infection, plants were transferred to the greenhouse. Three weeks after BPMV rubbing inoculation, the plants were inoculated with urediniospores (10^5/ml) of *P. pachyrhizi* resuspended in sterile water containing 0.01% Tween 20 and then placed in a dew chamber for 48 hr. Plants were then moved back to the greenhouse. In addition to the HIGS vector-inoculated plants, three control treatments were included: the mock control (mock-inoculation: the same experimental conditions as the HIGS-treated plants but rub-inoculated with inoculation buffer only), empty vector (EV) control (plants that were inoculated with a BPMV vector lacking an insert), and healthy control (plants that were not treated). All of these control plants were inoculated with *P. pachyrhizi* urediniospores as described for the experimental plants. For each construct/treatment, five plants (replicates) were usually used for data collection and final analysis.

4.5 | HIGS constructs confirmation, target gene silencing efficiency, and fungal growth assessment

Changes in soybean resistance to *P. pachyrhizi*, such as pustule density, were evaluated using soybean leaves collected at 2 weeks post-inoculation with *P. pachyrhizi*. After being photographed to allow counting of pustule density, soybean leaves were ground in liquid nitrogen, and total RNA was isolated and first-strand cDNA was synthesized as described above. Fungal biomass was assessed by quantifying the constitutively expressed pathogen *α-tubulin* gene (van de Mortel *et al.*, 2007; Meyer *et al.*, 2009). Expression data of fungal target genes were normalized to the soybean *ubiquitin-3* gene (GenBank accession number D28123.1), which showed stable expression following *P. pachyrhizi* infection (van de Mortel *et al.*, 2007). The expression of *P. pachyrhizi* target genes was determined by RT-qPCR with *P. pachyrhizi* in HIGS and EV-treated plants at 14 days post-inoculation (dpi) and was normalized to the *P. pachyrhizi CytB* gene (Table 3). Real-time PCRs in a final volume of 20 µl containing 2 µl of cDNA and 0.3 µM of each primer were run on a CFX96 Real-Time-PCR System (Bio-Rad) under standard conditions with melting curve analysis at the end of cycles. The $\Delta\Delta C_t$ method was used to calculate the target gene relative expression level (Schmittgen and Livak, 2008). The data presented here are the mean values ± *SD* from five biological replicates.

4.6 | HIGS study using detached soybean leaves

Five trifoliate leaves from greenhouse-grown soybean plants that had been previously rub-inoculated with various HIGS vectors were collected 2 weeks later for each construct and then inoculated with *P. pachyrhizi* urediniospores (10^5/ml) resuspended in sterile water

containing 0.01% Tween 20. Following urediniospore inoculation, the leaves were placed in transparent square boxes (230 × 230 × 17 mm) with wetted paper towel and maintained under continuous light at 25 °C. Leaves were then evaluated for pustule number per cm^2 2 weeks after inoculation with *P. pachyrhizi*. After evaluation, leaf tissues were ground in liquid nitrogen and total RNAs were isolated for quantification of target gene suppression and fungal biomass as described above.

4.7 | Synthesis of dsRNA in vitro and spray application

Gene-specific primer pairs with T7 promoter sequence at the 5′ end (Table 3) were used for in vitro dsRNA synthesis using MEGAscript High Yield Transcription Kit following the manufacturer's instructions (Ambion). Synthesized dsRNA was quantified using NanoDrop and stored at −80 °C. For spray application, dsRNA was diluted to a final concentration of 20 µg/ml with diethyl pyrocarbonate-treated water. Each box containing six detached individual leaflets was evenly sprayed with 1 ml of diluted dsRNA (20 µg dsRNA). After spraying, boxes were kept open until the surface of each individual leaflet was dry (approximately 1.5 hr). Leaves were then spray-inoculated with 1 ml of 10^5 urediniospores/ml 2 hr after the dsRNA spray. Boxes were closed and incubated for 2 weeks at approximately 25 °C on the laboratory bench with continuous lights Two controls were included in this study: one was soybean leaflets that were sprayed with H_2O and the other was sprayed with dsRNA targeting the soybean *PDS* gene. The latter was used to determine whether dsRNA can enter soybean leaves. No significant differences in pustule numbers per cm^2, *P. pachyrhizi* tubulin level, and target gene expressions were observed between the water-treated and PDS dsRNA-treated soybean leaves, therefore only the data for the water-treated control are presented here.

4.8 | Statistical analysis

All experiments were repeated and yielded reproducible results. The most representative data are shown in this paper. A two-tailed Student's *t* test was performed with data gained from greenhouse inoculation, detached leaf inoculation, dsRNA spray assays, and RT-qPCR studies. Data are presented as means ± *SD* of the mean. *p* values <.05 were considered significant.

5 | COMPETING INTERESTS

The authors declare no competing financial or non-financial interests.

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AUTHOR CONTRIBUTIONS

Z-Y.C. and D.H. designed the study and wrote the manuscript. D.H. conducted the experiments and M.G. identified the *ATC*. C.Z. provided the BPMV constructs and advised the virus inoculation. D.H. performed the bioinformatics analysis of sRNA data. D.H. and Z-Y.C. analysed all data and drafted the figures.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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