TECHNICAL ADVANCE

Development and Use of an Efficient DNA-Based Viral Gene Silencing Vector for Soybean

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Virus-induced gene silencing (VIGS) is increasingly being used as a reverse genetics tool to study functions of specific plant genes. It is especially useful for plants, such as soybean, that are recalcitrant to transformation. Previously, *Bean pod mottle virus* (BPMV) was shown to be an effective VIGS vector for soybean. However, the reported BPMV vector requires in vitro RNA transcription and inoculation, which is not reliable or amenable to high-throughput applications. To increase the efficiency of the BPMV vector for soybean functional genomics, a DNA-based version was developed. Reported here is the construction of a *Cauliflower mosaic virus* 35S promoter-driven BPMV vector that is efficient for the study of soybean gene function. The selection of a mild rather than a severe BPMV strain greatly reduced the symptom interference caused by virus infection. The DNA-based BPMV vector was used to silence soybean homologues of genes involved in plant defense, translation, and the cytoskeleton in shoots and in roots. VIGS of the *Actin* gene resulted in reduced numbers of *Soybean mosaic virus* infection foci. The results demonstrate the utility of this new vector as an efficient tool for a wide range of applications for soybean functional genomics.

Additional keywords: Comovirus, Potyvirus

Forward genetics has successfully allowed the study of gene function in many plant species. Characteristically, it is associated with the cloning of a gene responsible for a mutant phenotype. Recently, the availability of whole genome sequences or large numbers of sequenced cDNA clones has provided new approaches to identify genes involved in particular biological functions. One of the most important new approaches is reverse genetics, in which the expression of a known gene or sequence is altered and the phenotype resulting from the altered gene expression is investigated. Recently, virus-induced gene silencing (VIGS), a transient reverse genetics tool for characterizing gene functions, has been widely used in many plants (Burch-Smith et al. 2004; Senthil-Kumar et al. 2008).

VIGS downregulates gene expression through RNA silencing that involves sequence-specific RNA degradation at the post-transcriptional level in plants. The transient nature of VIGS provides several advantages over other methods for associating genes with traits. VIGS overcomes the requirement for stable plant transformation which is time consuming and difficult for many plant species (Burch-Smith et al. 2004; Senthil-Kumar et

al. 2008). VIGS can also be used to target genes that, when stably mutated or silenced, cause lethal effects on plant development (Burch-Smith et al. 2006; Lu et al. 2003; Turnage et al. 2002). The increasing availability of genome databases enables design of VIGS to downregulate gene expression of a single gene or paralogous genes. Due to these inherent advantages, many plant viral gene-silencing vectors have recently been developed (Burch-Smith et al. 2004; Constantin et al. 2004; Ding et al. 2006; Senthil-Kumar et al. 2008). Among the many VIGS vectors that have been developed, some are designed to use prokaryotic promoters for in vitro RNA transcription to produce infectious viral genomic RNA for inoculation. Others utilize eukaryotic promoters that facilitate direct introduction of infectious cDNA clones into plants for inoculation. A key aspect of the design that affects efficiency is placement of the VIGS elements under control of a *Cauliflower mosaic virus* (CaMV) 35S promoter and Nos terminator. DNA-based vectors provide distinct advantages over RNA-based vectors because cost and time are reduced and they are easier to manipulate and introduce into plants. The bypassing of RNA transcription and the DNA-based inoculation provides increased throughput. The latter strategy was employed for the *Tobacco rattle virus* (TRV) and *Potato virus X* (PVX) vectors that have been successfully used for high-throughput screening for the study of gene function (Liu et al. 2002; Lu et al. 2003).

Soybean is a major oilseed crop and the main food protein source in the world. Because of its moderate genome size and rapidly accumulating genomic sequences, the United States Department of Agriculture and United States Department of Energy jointly initiated efforts to sequence the soybean genome (Jackson et al. 2006). VIGS was identified as a necessary functional genomics tool for soybean and high-throughput VIGS was regarded as an ideal reverse-genetics tool for large-scale soybean functional genomics aimed at translating the genomic information into functional knowledge (Jackson et al. 2006; Stacey et al. 2004).

Bean pod mottle virus (BPMV, genus *Comovirus*) has a bipartite positive-strand RNA genome consisting of RNA-1 (approximately 6.0 kb) and RNA-2 (approximately 3.6 kb). Previously, an in vitro RNA transcription-based BPMV VIGS vector was developed and shown to be useful for soybean gene silencing (Zhang and Ghabrial 2006). However, the inoculation efficiency of RNA transcripts limits the use of mild BPMV strains for soybean VIGS study; instead, a severe strain has been used for further study (Kachroo et al. 2008). In the work reported here, a DNA-based vector was developed with high infection efficiency and enhanced throughput by placing BPMV genomic elements under the control of a CaMV 35S promoter and Nos terminator. This enables use of

a mild BPMV strain for VIGS in soybean that can significantly reduce interference caused by severe leaf symptoms resulting from infection by the virus. The DNA-based BPMV vector was used to silence soybean homologues of genes involved in plant defense, translation, and the cytoskeleton. The potential for study of gene function was demonstrated by association of repressed expression of an *Actin* gene, a key component of the cytoskeleton, with a reduction in *Soybean mosaic virus* (SMV, genus *Potyvirus*) infection foci. In addition, the BPMV vector was also used to silence genes in the root. Thus, the results demonstrate the utility of this new vector as an efficient tool for a wide range of applications in soybean functional genomics.

RESULTS

Infectivity of RNA-based BPMV VIGS vectors.

Previously, soybean phytoene desaturase (PDS) and stearoyl-acyl carrier protein-desaturase were silenced using the RNA-based BPMV vector that requires in vitro RNA transcription and mechanical RNA inoculation (Kachroo et al. 2008; Zhang and Ghabrial 2006). In order to determine infectivity of the RNA-based VIGS vector, we selected soybean homologues of a variety of genes, including those involved in plant defense, translation, and the cytoskeleton, for VIGS. Seven single-gene VIGS constructs were developed using the RNA-based BPMV vector pGG7R2-V from BPMV strain K-G7 (Tables 1 and 2). When soybean cvs. Williams and Essex were inoculated with transcribed RNAs from the VIGS clones together with mild RNA1 transcripts from strain K-G7 (Zhang and Ghabrial 2006), the number of plants infected was very low (Table 1). Because most work published by Zhang and Ghabrial (2006) was done using the severe BPMV RNA1 from strain K-Ho1 on soybean cv. Essex, we compared the infection of BPMV K-G7 RNA2 and RNA1 from either K-Ho1 or K-G7 on cvs. Williams and Essex. There was no major difference in infection of the empty vector with the severe K-Ho1 RNA1 on either cultivar (Table 1). However, there was a major reduction in the number of plants infected using the mild K-G7 RNA1

compared with the severe K-Ho1 RNA1 (Table 1). Because the phenotype of the K-Ho1 RNA1 was severe leaf symptoms and necrosis (Gu and Ghabrial 2005), we preferred to use the mild K-G7 RNA1 to obviate interference of symptoms induced by BPMV with those associated with the VIGS phenotype. Therefore, because of the low infection and the requirement of in vitro transcription, we developed an alternative and more efficient DNA-based BPMV VIGS vector technology.

Infectivity of DNA-based BPMV VIGS vectors.

To facilitate high-throughput VIGS studies, the cDNAs of BPMV genomic RNA1 and RNA2 were placed under control of the CaMV 35S promoter (Fig. 1A) to circumvent the need for making in vitro RNA transcripts. In addition, the cloning site in RNA2 was modified to allow multiple host gene sequences to be concatenated for co-silencing (Fig. 1B and C). The use of the CaMV 35S promoter allowed, through biolistic delivery, direct introduction of the plasmid DNA of the BPMV VIGS constructs into soybean plants. Both the severe and mild BPMV RNA1s were adapted for use as DNA-based vectors. Three independent tests with empty vectors showed that the DNA-based vector was highly infectious, with close to 100% of plants infected on either cultivar (Table 1). This contrasts with the low infection by the RNA-based vector, especially for the mild K-G7 RNA1. The high infection makes it feasible to use it as a VIGS vector with the mild K-G7 RNA1 to reduce interference by the severe phenotype associated with strain K-Ho1 RNA1. Interestingly, we do see a decrease in the number of plants infected with the VIGS constructs compared with the empty vector (Table 1). Nevertheless, satisfactory infection can be readily achieved with the DNA-based vector containing the mild K-G7 RNA1 for which the RNA-based infection was not successful. Additionally, the construct for co-silencing *Sgt1A+Sgt1B* was as infectious as each of the constructs for silencing *Sgt1A* or *Sgt1B* alone, demonstrating that the vector can be used to co-silence at least two genes with no loss in infection efficiency. We observed little difference among the two soybean cultivars in infection by the different VIGS constructs.

Table 1. Comparison of soybean inoculation with RNA- and DNA-based *Bean pod mottle virus* (BPMV) virus-induced gene silencing (VIGS) vectors^a

Cultivar	RNA1 type	RNA2 type	Target genes	RNA-based inoculation	DNA-based inoculation
Williams	K-Ho1	K-G7	Empty vector	8/16	15/15
Essex	K-Ho1	K-G7	Empty vector	9/16	14/15
Williams	K-G7	K-G7	Empty vector	2/16	13/15
Essex	K-G7	K-G7	Empty vector	2/16	14/15
Williams	K-G7	K-G7	Actin	1/16	10/15
Essex	K-G7	K-G7	Actin	0/8	3/5
Williams	K-G7	K-G7	<i>Rps6A</i>	0/16	10/15
Essex	K-G7	K-G7	<i>Rps6A</i>	0/8	3/5
Williams	K-G7	K-G7	<i>Rps13</i>	0/16	9/15
Essex	K-G7	K-G7	<i>Rps13</i>	0/8	4/5
Williams	K-G7	K-G7	<i>Mpk4A</i>	1/16	10/15
Essex	K-G7	K-G7	<i>Mpk4A</i>	1/8	3/5
Williams	K-G7	K-G7	<i>Mpk4B</i>	0/16	9/15
Essex	K-G7	K-G7	<i>Mpk4B</i>	0/8	2/5
Williams	K-G7	K-G7	<i>Sgt1A</i>	0/16	9/15
Essex	K-G7	K-G7	<i>Sgt1A</i>	1/8	3/5
Williams	K-G7	K-G7	<i>Sgt1B</i>	0/16	8/15
Essex	K-G7	K-G7	<i>Sgt1B</i>	0/8	2/5
Williams	K-G7	K-G7	<i>Sgt1A+Sgt1B</i>	–	8/15
Essex	K-G7	K-G7	<i>Sgt1A+Sgt1B</i>	–	2/5

^a BPMV RNA1 plasmids for RNA-based inoculation are pGG7R1 for mild strain K-G7 and pGHO1 for severe strain K-Ho1. BPMV RNA1 plasmids for DNA-based inoculation are pG35S-G7R1D for mild strain K-G7 and pG35S-Ho1 for severe strain K-Ho1. The empty RNA2 vector used for RNA-based inoculation is pGG7R2 and for DNA-based inoculation is pG35S-G7R2. A total of 200 µl of RNA1 and RNA2 transcripts was used to inoculate eight soybean plants. RNA-based inoculation was repeated once on soybean cv. Williams. One preparation for DNA-based vectors was used to inoculate five soybean plants. Three repetitions were performed on Williams. Both RNA-based and DNA-based inoculations were done when soybean primary leaves were fully expanded (stage VC). BPMV infection was confirmed by double-antibody sandwich enzyme-linked immunosorbent assay. Numbers above and below the diagonal for each cultivar are the numbers of plants infected and the total plants tested, respectively; – = not tested.

Diverse shoot and root symptoms induced by VIGS constructs.

One advantage of VIGS is that essential genes that are critically required for plant metabolism can be studied whereas stable mutation may be lethal. With the new DNA-based VIGS vector, we tested the efficacy of silencing soybean homologues of essential genes involved in translation and the cytoskeleton, which would be expected to have severe phenotypes when silenced (Table 1). Silencing two ribosomal proteins, Rps6 and Rps13, induced very severe foliar symptoms (Fig. 2). Silencing of an *Actin* gene homologue, which is required for the plant cytoskeleton, resulted in severe mosaic, leaf deformation, and stunting (Fig. 2).

The extensive soybean expressed sequence tag (EST) sequence database (Schlueter et al. 2004) allows identification of soybean homologues of *Arabidopsis* genes previously reported to be involved in defense signaling (Hammond-Kosack and Parker 2003). Two soybean *Mpk4* homologues (*Mpk4A* and *Mpk4B*), which share 57.4% nucleotide identity and 70.4% amino acid similarity (data not shown), were identified in soybean in contrast to one in *Arabidopsis* (Peterson et al. 2000). MPK4 is a negative regulator of *Arabidopsis* defenses, and loss-of-function mutations result in severely stunted phenotypes. Unlike the mild symptoms induced by silencing *Mpk4B* (Fig. 2), silencing *Mpk4A* caused severe symptoms, including severe stunting comparable with that on *Arabidopsis Mpk4* mutants (Peterson et al. 2000).

Other defense genes are expected to have no phenotype with respect to plant growth and development when silenced. For this purpose, two *Arabidopsis Sgt1* gene homologues, also involved in defense signaling (Austin et al. 2002; Tör et al. 2002), were cloned from soybean and inserted into the VIGS vector; after inoculation, infected plants showed mild symptoms similar to the empty vector infected control. To test multiple-gene silencing in soybean, the two *Sgt1* homologues,

Sgt1A and *Sgt1B* were concatenated into one single construct. Symptoms at 21 days after inoculation showed that the *Sgt1A/Sgt1B* construct induced mild symptoms similar to those induced by *Sgt1A* or *Sgt1B* single infection (Fig. 2).

To investigate the effect of the BPMV VIGS vector on silencing endogenous genes in soybean roots, root length of plants infected by the above constructs was measured. One week after

Fig. 1. Schematic representation of the *Cauliflower mosaic virus* (CaMV) 35S promoter driven *Bean pod mottle virus* (BPMV) virus-induced gene silencing (VIGS) vector. **A**, BPMV genomic RNA1 and RNA2 are expressed under control of the CaMV 35S promoter and Nos terminator. pBPMV-35S RNA2 is used for insertion of foreign gene sequences. **B**, Cloning site for insertion of foreign sequences into BPMV RNA2. **C**, Cloning strategies for inserting single and multiple genes into the RNA2 VIGS vector. *MscI* and *PmlI* are compatible and *MscI/PmlI* sites destroyed in construct c.

Table 2. Soybean gene fragments cloned into the *Bean pod mottle virus* vector and resulting phenotype in infected plants

<i>Arabidopsis</i> gene type ^a	Accession number ^b	Primer sequences ^c	Phenotype ^d
<i>Actin</i> (312 bp)	TC204141	VIGS-F: CGCGGATCCACGTGGCTGGATTGCTGGTGATGA VIGS-R: ACCATCGATTTCATGGCAGGCACATTGAAGGT Probe-F: CGCGGATCCGCCAGCTATCGTTTCATCGGA Probe-R: CGCGGATCCCAATCAGAACCTACAAAGTTCCA	S
<i>Rps6A</i> (297 bp)	TC205856	VIGS-F: CGCGGATCCACGTGGCGAATCCACCACTGGA VIGS-R: ACCATCGATTGCACCCACGAACAGACTTTC Probe-F: CGCGGATCCCCTCTCTGTTTGAACCTGGT Probe-R: CGCGGATCCCCTCCGATTGGCCTTGCCA	S
<i>Rps13</i> (333 bp)	TC225449	VIGS-F: CGCGGATCCACGTGCGTATGCACAGCCGCGGT VIGS-R: ACCATCGATTGGAGTCTTGTCTTCCTGT Probe-F: CGCGGATCCGAGAGCAGAATCCACCGACT Probe-R: CGCGGATCCCCCAGTCAAACCATAGTTCT	S
<i>Mpk4A</i> (297 bp)	TC226687	VIGS-F: CGCGGATCCACGTGGCTGCTGTAATGCAGAGACAGGT VIGS-R: ACCATCGATTTCGAGCCCTCGTAACAATTGATA Probe-F: CGCGGATCCCTCGTGACTTGAAACCTGGGA Probe-R: CGCGGATCCGATAGGAGCTCTGGTGCCCTA	S
<i>Mpk4B</i> (306 bp)	TC216864	VIGS-F: CGCGGATCCACGTGGAGAAGGTTGCCATCAAGAAGAT VIGS-R: ACCATCGATTAAAGATGTTAGCGGAGTGAAGAT Probe-F: CGCGGATCCCTCGTGACTTGAAACCTGGGA Probe-R: CGCGGATCCGATAGGAGCTCTGGTGCCCTA	M
<i>Sgt1A</i> (306 bp)	TC203662	VIGS-F: CGCGGATCCACGTGGTTCCTGGCCAAGATGCCTAT VIGS-R: ACCATCGATTAGCAGCATCACCATCAAGCTTT Probe-F: CGCGGATCCTACCAAAATGCAGATGAGGACA Probe-R: CGCGGATCCCCACTTCCACAGCAAAAGCA	M
<i>Sgt1B</i> (306 bp)	TC206509	VIGS-F: CGCGGATCCACGTGTCCGATCTCGAAGCAAAAGCCA VIGS-R: ACCATCGATTGTCTGGAGACAATGATGCACCCA Probe-F: CGCGGATCCAGGTTTGCTACTTTGATCAAAGA Probe-R: CGCGGATCCCACGAAGTCAACAGTAATGCT	M

^a Name of gene used for virus-induced gene silencing (VIGS) constructs. The target gene fragment size in base pairs (bp) is given in parentheses.

^b Accession numbers are the The Institute for Genomic Research tentative contig (TC) numbers of genes selected for VIGS.

^c Primer sequences used for polymerase chain reaction amplification of gene fragments are designated as VIGS-F and VIGS-R for forward and for reverse primers, respectively. Forward or reverse primers used for preparing probes are designated as Probe-F and Probe-R, respectively.

^d Soybean phenotype associated with silencing of designated soybean genes on soybean cv. Williams. Abbreviations: M = mild symptoms similar to empty-vector control and S = severe symptoms relative to empty-vector control.

biolistic inoculation of the primary leaves of soybean plants (stage VC), soybean roots were trimmed to 1 in. and maintained in deeper pots in the growth chamber under standard growth conditions. Four weeks after inoculation, soybean roots from each of five plants per construct were rinsed and photographed (Fig. 3A). Because the severe symptoms induced by *Mpk4A* caused death of the plants at 4 weeks postinoculation, they were not included. As shown (Fig. 3), there was no significant root length difference between the Mock, Vector-alone, and plants infected by the four constructs *Mpk4B*, *Sgt1A*, *Sgt1B*, and *Sgt1A/B*. However, significant root length differences were observed between the empty-vector-infected plants and plants infected by the three constructs *Rps6*, *Rps13*, and *Actin* that are involved in translation and cytoskeleton (Fig. 3) (*t* test, $P < 0.001$). Interestingly, the three constructs that induced severely stunted root growth also induced severe foliar symptoms (Fig. 2), indicating the essential role of these genes in protein translation and the cytoskeleton in root and leaf cells.

Quantification of gene expression.

To confirm that the observed soybean phenotypes associated with various VIGS constructs involve silencing, expression of the target genes was evaluated both at the mRNA level and protein level. Initially, potential changes in the mRNA levels of target genes were examined by Northern blot analysis of total RNAs extracted from leaves of infected plants. Six sets of

primers (Table 2, Probe-F and Probe-R) were used to amplify a region of the targeted genes for hybridization that was different from the target gene fragments carried in the VIGS constructs. Total RNAs (15 μ g each) were loaded in each lane and equal loading was confirmed by ethidium bromide staining (Fig. 4A). Based upon band intensity relative to the empty vector control (pG35SG7R2) in each blot, reduction in gene expression ranged from 42 to 72%. Symptom severity was not associated with reduction in gene expression because constructs exhibiting a severe phenotype ranged from 42% for *Rps6* to 64 and 67% for *Rps13* and *Actin*, respectively. Among VIGS constructs exhibiting a mild phenotype, reduction was 62, 69, and 72% for *Sgt1A*, *Sgt1B*, and *Mpk4B*, respectively. Multiple gene silencing, demonstrated by the concatamer construct *Sgt1A/B*, reduced gene expression of *Sgt1A* and *Sgt1B* by 51 and 57%, respectively, which was lower than the reduction of gene expression levels of 62 and 69% induced by the single gene constructs of the same genes (Fig. 4).

Ribosomal protein S6 (*Rps6*) is a highly conserved component of cytosolic ribosomes (Barakat et al. 2001). Soybean RPS6 has 90% amino acid identity with *Arabidopsis* RPS6 (GenBank accession number Y14052) and 92% with maize RPS6 (GenBank accession number NM_001112074). We used antisera of maize RPS6 for a Western blot analysis of RPS6 accumulation in leaves from empty BPMV vector-infected plants or *Rps6*-silenced plants. For comparison, 15 μ g of total protein was loaded per lane and equal loading was confirmed by Coomassie blue staining (Fig. 5). By quantifying the band intensity of the blot, RPS6 was reduced 40% compared with the

Fig. 2. Diverse shoot phenotypes on the Williams soybean cultivar induced by *Bean pod mottle virus* (BPMV) virus-induced gene silencing (VIGS) of soybean genes. Plants were photographed at 3 weeks postinoculation. Silencing of *Mpk4A* induced very severe symptoms, including stunting and deformation. Severe phenotypes were also induced by silencing of two translation components, *Rps6* and *Rps13*, as well as the cytoskeleton component *Actin*. Mock = mock-inoculated control. Vector = empty-vector control inoculated with pBPMV-35SG7R1D and pBPMV-35SG7R2. All plants were maintained in a growth chamber at 20°C with a photoperiod of 16 h.

Fig. 3. Root phenotypes on the Williams soybean cultivar induced by *Bean pod mottle virus* (BPMV) virus-induced gene silencing (VIGS) of soybean genes. **A**, Effect of silencing essential genes on root growth. Roots were photographed at 4 weeks postinoculation. The names of targeted genes were labeled. Note the difference in root length for plants infected with the three VIGS constructs silencing translation components and cytoskeleton component *Actin* compared with the other treatments. **B**, Mean value of root length with standard deviation (bars) for each treatment; * indicates that significant differences (*t* test, $P < 0.001$) were found between the treatments compared with the Mock and Vector controls.

empty-vector-infected control (Fig. 5). This protein level reduction is consistent with the mRNA transcript reduction of 42%.

Identification of host genes involved in SMV infection in compatible interaction.

In order to test the effect of silencing on gene function, BPMV VIGS construct-infected Williams plants, susceptible to both BPMV and SMV, were screened for the effect of silencing on subsequent inoculation by a β -glucuronidase (GUS)-tagged SMV infectious clone (SMV-N-GUS) (Wang et al. 2006). Three weeks after BPMV inoculation and confirmation of infection by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), detached leaves from the empty BPMV vector

(pG35SG7R2) or from BPMV VIGS construct-infected plants were biolistically inoculated with SMV-N-GUS DNA. At 5 days post SMV-GUS inoculation, a GUS histochemical assay was performed to test for the presence of SMV infection foci. Data were compiled from at least three independent tests for each construct with a total of at least 15 leaves to evaluate VIGS of the selected soybean genes on SMV infection. The *Mpk4A* construct was not tested because its silencing resulted in severely stunted plants and few leaves were available for the test. For most constructs, including *Mpk4B*, *Sgt1A*, *Sgt1B*, *Rps6*, and *Rps13*, there was no discernable difference in the number of SMV infection foci between the empty vector and the VIGS constructs infected plants (data not shown). However, silencing of the soybean *Actin* gene reduced SMV-N-GUS foci by more than 20-fold when *Actin* was silenced (Fig. 6). Significant differences occurred between both the Mock and *Actin* ($t = 4.55$, $P < 0.0001$) as well as the vector and *Actin* ($t = 2.37$, $P = 0.0221$) treatments. The difference was not significant ($t = 1.73$, $P = 0.0902$) between the Mock and the Vector treatments.

DISCUSSION

The DNA-based BPMV VIGS vector is effective in soybean.

Two RNA transcription and inoculation-based vectors were recently developed for soybean VIGS (Nagamatsu et al. 2007; Zhang and Ghabrial 2006). Following Zhang and Ghabrial's report, the RNA-based BPMV vector was further used to silence two stearoyl-acyl carrier protein-desaturase genes (Kachroo et al. 2008). However, in both BPMV VIGS studies, transcripts from the severe BPMV K-Ho1 RNA1 were used for inoculation because of greater infection efficiency relative to inoculation with transcripts from the mild BPMV K-G7 (Kachroo et al. 2008; Zhang and Ghabrial 2006).

Although BPMV RNA2 was engineered to carry foreign gene sequences, BPMV RNA1 was shown to encode the pathogenicity component that determines foliar symptom severity (Gu and Ghabrial 2005). BPMV strain K-Ho1 RNA1 induces severe symptoms but strain K-G7 RNA1 only causes

Fig. 4. Changes in the mRNA levels of virus-induced gene silencing (VIGS) target genes. Northern blot analysis was used to assess the mRNA transcript level of each target gene. **A**, Equivalent RNA loading was assessed by ethidium bromide staining of total RNA extracted from leaves of infected plants by *Bean pod mottle virus* (BPMV) VIGS constructs. **B**, Northern blot analysis using probes prepared for each construct. The probe used for each blot is indicated below the corresponding autoradiogram. **C**, Percentage reduction of the mRNA transcript levels of each target gene compared with their levels in nonsilenced vector control plants. The relative reduction of the mRNA level of each target gene was calculated by subtracting the radioactivity signal of the VIGS treatments from the signal for the nonsilenced vector control and then dividing the difference by the signal of the nonsilenced vector control. The value of the empty-vector-infected plants is 100% in each gel blot; * indicates the reduction is from the concatemer construct *Sgt1A/B*.

Fig. 5. Immunoblot analysis of RPS6 protein levels in nonsilenced and *Rps6*-silenced soybean plants. **A**, Levels of protein loading were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie blue staining of the proteins tested. **B**, Western blot analysis using a maize polyclonal RPS6 antiserum. Total proteins (15 μ g) were extracted from soybean plants at 21 days postinoculation with the following constructs: Vector, empty-vector control.

mild symptoms. Even though severe infection by strain K-Ho1 RNA1 enhances foreign gene expression by the BPMV vector, the mild infection by K-G7 RNA1 is ideal for VIGS studies, because there is less potential for viral symptoms to confound the silencing phenotypes.

This study showed that infectivity of K-G7 RNA1 was relatively low compared with transcripts from K-Ho1 RNA1 when combined with RNA2 from K-G7 and used to inoculate two soybean cultivars. Experiments were conducted in parallel using the same reagents and freshly prepared RNA transcripts for inoculation. Further, no difference was observed in integrity of the transcripts on 1% agarose gels (data not shown). Although increased repetition is feasible, it is not cost effective or suitable for studies requiring high-throughput analyses. Consequently, an alternative approach was used to develop a DNA-based BPMV vector. Comparison of the DNA-based vector with the RNA-based vector showed greater infectivity of the DNA-based vector. Further, when the mild K-G7 RNA1 was used with K-G7 RNA2, infectivity was equivalent to use of the severe K-Ho1 RNA1. Therefore, the K-G7 RNA1 was used in experiments in which a variety of soybean gene homo-

logues were inserted into K-G7 RNA2 and used in direct comparison of infectivity of the RNA- and DNA-based vectors. In all cases, infectivity of the DNA-based vector was greater and, in a majority of the trials (*Rps6A*, *Rps13*, *Mpk4B*, and *Sgt1B*), no infectivity was obtained with the RNA-based vector in the number of repetitions attempted. The DNA-based vector, using the mild K-G7 RNA1 with reduced symptom interference, has been convenient to handle; for example, the K-G7 RNA1 plasmid DNAs prepared and stored at -20°C for more than 1 year have shown no reduction in infectivity after biolistic inoculation. This provides convenience that enables large-scale studies and distribution of the vector to multiple collaborators.

The DNA-based BPMV vector was also designed to silence multiple genes using a single construct to make possible simultaneous testing of different combinations of genes or homologues in soybean that have genetic redundancy (Blanc and Wolfe 2004; Schlueter et al. 2004; Shoemaker et al. 1996) or function in parallel signaling pathways (Lawrence and Pikaard 2003). We did not test the possibility of using two RNA2 VIGS constructs for simultaneous silencing of two genes because of the potential complexity of co-infection by two independent constructs in a single plant (Fraser 1998; Roossinck 2005). Instead, the vector described here is designed for multiple-gene silencing with a single construct and is expandable by concatenation (Fig. 1). Because it is necessary to clone the foreign gene insert in frame with the RNA2 polypeptide, the vector was designed so that fusion of foreign inserts from two constructs does not disrupt translation (Fig. 1). This concept was validated by the simultaneous silencing of *Sgt1A/Sgt1B* (Fig. 4). Zhang and Ghabrial (2006) reported that up to 1.4 kb of foreign sequence can be inserted into BPMV RNA2 and, therefore, at least four 300-bp gene fragments can theoretically be inserted into one construct for silencing.

A potential rate-limiting step in the use of VIGS for high-throughput analyses of genes is the necessity to clone and insert multiple gene fragments. Recently, we modified the DNA-based BPMV vector with topoisomerase for high-throughput cloning, and infectivity tests showed that there is no observable difference in infection efficiency between the topoisomerase-modified vector and the parent vector (*unpublished data*).

BPMV as an effective gene-silencing vector in soybean shoot and root systems.

The BPMV vector was used to examine gene silencing in both soybean shoots and roots. As expected, very mild leaf symptoms occurred when K-G7 RNA1 was used with the empty vector RNA2. Similar mild leaf phenotypes also occurred with several gene inserts; however, enhanced foliar phenotypes did occur with soybean homologues of genes associated with plant defense, the cytoskeleton, and translation. For example, a severe phenotype was induced by silencing soybean *Mpk4A* and this phenotype is similar to that associated with *Mpk4* loss-of-function in *Arabidopsis* (Peterson et al. 2000). Interestingly, BLAST search of the translated soybean EST database, using the *Arabidopsis Mpk4* protein sequence, identified two soybean homologues, *Mpk4A* and *Mpk4B*, that share 57.4% nucleotide identity. *Mpk4B* is expressed in soybean (Fig. 4). Silencing *Mpk4B* induced a mild phenotype. The different phenotypes associated with silencing *Mpk4A* and *Mpk4B* suggested that similar functions were shared between *Arabidopsis Mpk4* and soybean *Mpk4A* but not soybean *Mpk4B*, demonstrating the usefulness of the new DNA-based VIGS vector for soybean gene function validation.

Soybean homologues of *Arabidopsis* defense genes *Sgt1A* and *Sgt1B* (Austin et al. 2002; Tör et al. 2002) induced mild phenotypes in soybean upon silencing. The concatamer construct *Sgt1A/B* did not significantly enhance the phenotype al-

Fig. 6. Bean pod mottle virus (BPMV) virus-induced gene silencing (VIGS) of an *Actin* gene reduced *Soybean mosaic virus* (SMV) virus accumulation. **A**, Scanned leaves of the Williams soybean cultivar with β -glucuronidase (GUS)-tagged SMV infectious clone (SMV-N-GUS) infection foci. Plants were first inoculated with the BPMV VIGS constructs indicated above each picture. Three weeks postinoculation with VIGS constructs and confirmation by double-antibody sandwich enzyme-linked immunosorbent assay, upper soybean leaves were detached and biolistically inoculated with SMV-N-GUS. Following SMV inoculation, the detached leaves were kept in a growth chamber for 5 days before staining and scanning. Mock = soybean plant was first inoculated with leaf sap from healthy plants and then inoculated with SMV-N-GUS. Vector = soybean plant was first inoculated with empty BPMV vector pBPMV-35SG7R2 and then challenged with SMV-N-GUS. Mock and Vector treatments showed typical SMV-GUS infection foci. *Actin* = BPMV construct silencing cytoskeleton actin component reduced SMV-N-GUS infection foci. **B**, Mean number of foci/leaflet plus standard deviation (bars) for each treatment; * = significant differences ($P < 0.05$, t test) were found between Mock and *Actin* treatments as well as between Vector and *Actin* treatments. Differences in foci number between Mock and Vector treatments were not significant at $P < 0.05$.

though both genes were successfully silenced. Thus, the results support the suggestion that repression of gene expression and plant phenotype are not necessarily closely associated in soybeans.

As in shoots, the application of a reverse genetics tool will be important for potential study of water and nutrient uptake as well as microbial- and nematode-root interactions (Constantin et al. 2008; Rosso et al. 2005; Ryu et al. 2004). Therefore, the effect of silencing the array of soybean genes on root development was measured. The silencing of three essential genes was associated with stunted root development, demonstrating the usefulness of the vector for the study of gene function in soybean roots. Because the BPMV vector is inherently designed for foreign gene expression (Zhang and Ghabrial 2006), the new high efficiency vector developed here may also be used for foreign gene expression to test gene function in a variety of applications in soybean roots as well as shoots.

Reverse-transcription polymerase chain reaction (RT-PCR) has been successfully used to demonstrate silencing of target genes in numerous plant species (Burch-Smith et al. 2004; Liu et al. 2002; Oh et al. 2008; Xu et al. 2007). However, RT-PCR did not consistently reveal reduced gene expression in soybean (data not shown). Alternatively, similar to a *Cucumber mosaic virus* (CMV) VIGS study in soybean (Nagamatsu et al. 2007), a RNA Northern blot analysis was used to evaluate soybean gene expression level. A consistent reduction of mRNA transcript levels corresponding to all the genes targeted by VIGS was observed. The reduction in gene expression, ranging from 42 to 72%, is close to the 53% reduction reported for a soybean chalcone synthase gene using a CMV VIGS vector (Nagamatsu et al. 2007). The mosaic bleaching phenotype resulting from silencing PDS as reported by Zhang and Ghabrial (2006) is also suggestive of incomplete silencing. However, the previous study did not examine the potential that a differential level of silencing occurred in the bleached and non-bleached areas of soybean leaves. Further, our tests showed that reduction in gene expression is independent of phenotypes we observed (Figs. 2 and 4).

Because *Rps6* is highly conserved among soybean and maize, antisera against maize *Rps6* was used to confirm reduced gene expression at the protein level. Northern blots revealed a 42% reduction in the *Rps6* mRNA and a corresponding 40% reduction in RPS6 protein level by Western blot. Recently, Brodersen and associates (2008) reported that siRNA, the key molecule for VIGS, has a widespread translational repression effect on gene expression. Interestingly, we did not observe a further reduction in RPS6 protein based on the comparison of the Northern and Western blots.

Functional analysis of the soybean *Actin* gene in SMV infection.

Actin is a ubiquitous protein found in eukaryotic cells associated with the plant cytoskeleton which is primarily composed of microtubules and microfilaments that interact and are involved in diverse plant responses (Rodriguez et al. 2003; Wasteneys and Yang 2004). Previously, the association of microtubules with the movement protein of TMV was shown to be important for TMV virus movement (Boevink and Oparka 2005; McLean et al. 1995). Further, silencing of the *Actin* gene reduced virus movement of TMV, a member of *Tobamovirus* (Liu et al. 2005). Our data demonstrate that silencing of the *Actin* gene is also associated with a reduced infection of SMV, a member of *Potyvirus*. This suggests that the *Actin* gene may have a broader role in plant virus infection. In addition, this result demonstrates the utility of the BPMV VIGS vector as an effective tool for identifying soybean genes with roles in host-virus interactions.

MATERIALS AND METHODS

Virus strains, inoculation, and BPMV virus detection.

BPMV strains K-G7 and K-Ho1 and the clones used in this study have been previously described (Zhang and Ghabrial 2006). Seed of the two soybean cvs. Essex and Williams used in this study were harvested from greenhouse-grown plants previously indexed for the absence of BPMV and SMV. All BPMV viral infection was verified by DAS-ELISA (Bradshaw et al. 2007).

Synthesis of in vitro RNA transcripts of BPMV was previously described by Zhang and Ghabrial (2006). DNA-based BPMV constructs were biolistically introduced into the primary leaves (VC stage) of 12-day-old soybean seedlings by a single particle bombardment as described (Sanford et al. 1993) using a Biolistic PDS-1000/He system (Bio-Rad Laboratories, Hercules, CA, U.S.A.), 1.0 μ m gold particles, and 1,100-psi rupture disks at a distance of 6 cm. The leaves were supported by a plastic disk that is notched so it can be centered under leaves without damaging the stem. The leaves were held in place and flat on the disk by a coarse metal mesh. Following bombardment, plants were maintained in the greenhouse or growth chamber at 20°C with a photoperiod of 16 h.

In vitro RNA transcription-based VIGS constructs.

The primer pairs (VIGS-F for forward and VIGS-R for reverse) (Table 2) were used for PCR amplification using oligo(dT)₂₀ cDNA template, and the PCR products were cloned into pGEM-T Easy (Promega Corp., Madison, WI, U.S.A.). Inserted sequences were confirmed by sequencing using M13 forward and reverse primers. For directional insertion of the soybean gene fragments into the BPMV VIGS vector, a *Bam*HI and a *Cl*aI restriction site was included in the forward and reverse primers, respectively (Table 2). An extra restriction site for *P*mlI was included in the forward primer for future concatemer construction. The insert in the pGEM-T Easy vector was digested with *B*amHI and *C*laI sequentially and ligated into similarly treated empty-vector pGG7R2-V. The resulting constructs were sequenced and tested for infectivity with either K-Ho1 or K-G7 RNA1 transcripts by in vitro transcript inoculation as described by Zhang and Ghabrial (2006).

Vector modification and construction of CaMV 35S promoter-driven BPMV RNA1.

Unless otherwise stated, all plasmids were propagated in ElectroMax DH5 α -E cells (Invitrogen, Carlsbad, CA, U.S.A.) and purified using the QiaPrep spin miniprep kit (Qiagen, Valencia, CA, U.S.A.). All nucleotide sequencing was done using the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and the ABI Prism 310 genetic analyzer. Sequence analysis was performed using the Vector NTI program (Invitrogen).

The fragment containing transcription elements from pBR322-35S (Wang et al. 2006) was released by digestion with *E*coRI and *N*otI and put into the same sites in digested pGEM-11zf(+) vector (Promega Corp.) to generate the pGEM-35S vector. pGEM-35S was digested with *M*scI and *E*coRI and then Klenow treated and self ligated to remove *M*scI from the pGEM-11zf(+) vector backbone. The resultant vector was named pGEM-35S-M1.

BPMV RNA1 cDNA clone pGG7R1 (Zhang and Ghabrial 2006) was used as a template to amplify the 5' end of RNA1 with primer pair BP-5endF and BP-G7R1-1064R (Table 3) using Takara PrimeSTAR HS DNA Polymerase following the manufacturer's recommended protocol (TaKaRa Bio Inc., Otsu, Shiga, Japan). The PCR product was treated with T4 DNA

kinase and ligated into *Stu*I-digested pGEM-35S to generate construct pG35S-G7R1A. Insertion was confirmed by sequencing with primers 35S-Seq, BP-R1-235F, BP-R1-688F, and Nos-Rev (Table 3). BPMV RNA1 3' end was amplified from pGG7R1 with primer pair BP-G7R1-5708Cla and BP-R1-3Cla. The 3' end PCR product was digested with *Cla*I and inserted into *Cla*I-digested clone pG35S-G7R1A to generate construct pG35S-G7R1B. Insertion direction and sequence were confirmed by sequencing with primer Nos-Rev. Clone pGG7R1 was digested with *Nhe*I and *Eco*47III and the released 4.3 kb was gel purified using the QIAquick gel extraction kit (Qiagen). This fragment was ligated into pG35S-G7R1B digested with the same enzymes to generate construct pG35S-G7R1C. Clone pGG7R1 was digested with *Swa*I and *Xho*I and ligated into pG35S-G7R1C digested with the same enzymes to generate construct pG35S-G7R1D, which is the full-length infectious clone used as the RNA1 component for all experiments. BPMV RNA1 cDNA clone pGHoR1 (Zhang and Ghabrial 2006) was used as template to amplify K-Ho1 RNA1 with primer pair BP-5endF and BP-R1-3Cla (Table 3). The PCR product was digested with *Swa*I and *Cla*I and the 5.7-kb fragment was ligated into pG35S-G7R1D digested with the same enzymes to yield full-length infectious clone pG35S-HoR1.

CaMV 35S promoter driven BPMV RNA2 VIGS constructs.

pGG7R2 and pGG7R2-V (Zhang and Ghabrial 2006) were used as template with primer pair BP-5end-F and BP-R2-3Cla (Table 3) for PCR amplification. The PCR products were treated with T4 DNA kinase and digested with *Cla*I and ligated into *Stu*I and *Cla*I digested pGEM-35S-M1 vector to generate clones pG35S-G7R2 and pG35S-G7R2V1. Insert orientation was confirmed by sequencing with primer 35-Seq (Table 3) and the entire genomic BPMV G7 RNA2 insertion was sequenced with primers listed in Table 3. Infectivity of pG35S-G7R2 was tested with pG35S-G7R1D or pG35S-HoR1. pG35S-G7R2V1 was partially digested with *Cla*I and treated with Klenow polymerase large fragment and self ligated to remove the 3' end *Cla*I site to yield pG35S-G7R2V2. Previously described pGEM-T Easy vector clones containing soybean gene inserts were digested sequentially with *Bam*HI and *Cla*I. The *Bam*HI site is in frame with the viral polypeptide, and it is italicized and underlined in the forward primers for each gene listed in Table 2. The gel-purified inserts were ligated into similarly treated pG35S-G7R2V2, and the resulting constructs were sequenced and tested for infectivity by particle bombardment with pG35S-G7R1D or pG35S-HoR1.

pG35S-Sgt1A was digested with *Not*I and *Pml*I (Fig. 1C, construct a) and the 5-kb fragment was gel recovered. pG35S-Sgt1B was digested with *Not*I and *Msc*I (Fig. 1C, construct b)

and the 3-kb fragment was gel recovered and ligated with the 5-kb pG35S-Sgt1A fragment to yield pG35S-Sgt1A/B (Fig. 1C, construct c).

RNA extraction and Northern hybridization analysis.

Total RNA was extracted from soybean leaves by the Trizol method (Invitrogen). First-strand cDNA was synthesized using 2 µg of total RNA, 0.5 µg of oligo(dT)₂₀ primer, 1 µl of 10 mM dNTP and Superscript III reverse transcriptase (Invitrogen) to a final volume of 20 µl.

For Northern hybridization analysis, RNA was extracted from soybean leaf samples. Following RNA extraction, hybridization was performed according to the description by Zhang and Ghabrial (2006). The probe for each VIGS target gene was selected from a region of the gene fragment using primers (Probe-F for forward and Probe-R for reverse) listed in Table 2. Probe sequences were amplified with the primer pairs and oligo(dT)₂₀ cDNA as template and the PCR products were digested with *Bam*HI and cloned into *Bam*HI-digested and dephosphorylated pGEM-11zf(+) vector. The resultant clones were sequenced with M13 primer to confirm the identity of the inserted sequence and used as template with corresponding Probe-F and Probe-R primer pairs for PCR amplification to obtain template DNA for probe preparation. Probes were labeled using the Prime-a-Gene labeling system (Promega Corp.). Northern blot was assessed using ImageQuant v5.2 (Amersham, Piscataway, NJ, U.S.A.).

Protein extraction and Western blot analysis.

Three weeks after inoculation, fresh leaf (or root) tissues were collected and protein was extracted as described (Moffett et al. 2002). Protein concentration was estimated by the Bio-Rad protein assay method (Bio-Rad Laboratories). Proteins were separated by polyacrylamide gel electrophoresis (PAGE) using 12% stacking and 15% resolving polyacrylamide with sodium dodecyl sulfate (SDS) running buffer (25 mM Tris, pH 8.3; 192 mM glycine; and 1% SDS), and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). Equal protein loading was estimated by staining a parallel gel with Coomassie blue. The PVDF membranes were incubated in 5% nonfat dry milk/phosphate-buffered saline-Tween blocking buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH to 7.4 with HCl, 0.1% Tween-20) for 3 h at room temperature, and then incubated overnight at 4°C with antiserum to maize RPS6 diluted 1:1,250 (Williams et al. 2003). This was followed by incubation with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:10,000 dilution; Amersham) for 1 h at room temperature. The blot images were developed using an enhanced chemilu-

Table 3. Primers for constructing and sequencing DNA-based *Bean pod mottle virus* virus-induced gene silencing vectors

Name	Sequence (5' → 3')
BP-5endF	TATTAAATTTTCATAAGATTGAAATTTTG
BP-R1-3Cla	CCATCGATTTTTTTTTTTTTTTTTTATATTTAAACAC
BP-R1-235F	ATATAGGACTTCGTGTCAGATT
BP-R1-688F	TGCATATCATTTTCAGCATTTTGT
BP-G7R1-1064R	TCCTAGAGCACAGGAGCTAGCCA
BP-G7R1-5708Cla	CCCATCGATCAATAAGAAAATTCGGACAGCGCTT
BP-R2-3Cla	CCATCGATTTTTTTTTTTTTTTTTTAAATAACACAC
BP-G7R2-454F	ACTTGGGCATTGGTGCAATGT
BP-G7R2-948F	ACTCTTACTGATGGGAAGTTGTA
BP-G7R2-1548F	CAAGAGAAAGATTTATTGGAGGGA
BP-G7R2-1786F	AAGCTCAAATGGAACAAATCT
BP-G7R2-2133F	TGGAATCCTGCTTGACAAAAGCA
BP-G7R2-2746F	TGGCTGATGGGTGCCCATATT
BP-G7R2-3303F	ATGGTTTCGAAATGTGGAGTTCTGA
35S-Seq	ACGCACAATCCCACTATC
Nos-Rev	AGACCGGCAACAGGATTCA

minescence system (Amersham) according to the manufacturer's instructions. Western blot was assessed using ImageQuant v5.2.

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