The Development of an Efficient Multipurpose Bean Pod Mottle Virus Viral Vector Set for Foreign Gene Expression and RNA Silencing[1][C]

Chunquan Zhang*, Jeffrey D. Bradshaw[2], Steven A. Whitham, and John H. Hill

Department of Plant Pathology, Iowa State University, Ames, Iowa 50011

Plant viral vectors are valuable tools for heterologous gene expression, and because of virus-induced gene silencing (VIGS), they also have important applications as reverse genetics tools for gene function studies. Viral vectors are especially useful for plants such as soybean (*Glycine max*) that are recalcitrant to transformation. Previously, two generations of bean pod mottle virus (BPMV; genus *Comovirus*) vectors have been developed for overexpressing and silencing genes in soybean. However, the design of the previous vectors imposes constraints that limit their utility. For example, VIGS target sequences must be expressed as fusion proteins in the same reading frame as the viral polyprotein. This requirement limits the design of VIGS target sequences to open reading frames. Furthermore, expression of multiple genes or simultaneous silencing of one gene and expression of another was not possible. To overcome these and other issues, a new BPMV-based vector system was developed to facilitate a variety of applications for gene function studies in soybean as well as in common bean (*Phaseolus vulgaris*). These vectors are designed for simultaneous expression of multiple foreign genes, insertion of noncoding/antisense sequences, and simultaneous expression and silencing. The simultaneous expression of green fluorescent protein and silencing of phytoene desaturase shows that marker gene-assisted silencing is feasible. These results demonstrate the utility of this BPMV vector set for a wide range of applications in soybean and common bean, and they have implications for improvement of other plant virus-based vector systems.

Plant virus-based vectors have been recently developed to express heterologous proteins in plants for the study of gene function, production of pharmaceuticals, analysis of plant-microbe interactions, fungicide and insecticide screening, metabolic engineering, and nutrient improvement. Plant viral gene expression vectors have many advantages over conventional transgenic technology for protein expression. They are fast, low cost, high yield, and can be used in a variety of genetic backgrounds. Plant viral vectors also have applications as virus-induced gene silencing (VIGS) tools for reverse genetic studies of gene function (Burch-Smith et al., 2004). VIGS can specifically down-regulate a single gene, members of a gene family, or sets of distinct genes (Peele et al., 2001; Turnage et al., 2002; Lu et al., 2003). Due to these advantages, many positive sense RNA plant viruses have been developed as vectors for production of recombinant proteins or as VIGS vectors for many plant species (Pogue et al., 2002; Burch-Smith et al., 2004; Constantin et al., 2004; Ding et al., 2006; Grønlund et al., 2008; Igarashi et al., 2009; Meng et al., 2009; Zhang et al., 2009). With the rapid increase in genomic information, VIGS vectors have substantial potential to advance gene function studies in both monocot and dicot plants.

Bean pod mottle virus (BPMV; genus *Comovirus*) has a bipartite positive RNA genome consisting of RNA1 (approximately 6 kb) and RNA2 (approximately 3.6 kb), which is further divided into two subgroups, subgroup I and subgroup II, as reported by Zhang et al. (2007). BPMV RNA1 and RNA2 are expressed as single polyprotein precursors, and subsequent proteolysis yields mature viral gene products. BPMV was developed as a vector for foreign gene expression and VIGS in soybean (*Glycine max*) based on a strategy in which the foreign sequences were inserted between the movement protein (MP) and large coat protein subunit (L-CP) in the RNA2 polyprotein (Zhang and Ghabrial, 2006). This vector requires the synthesis of in vitro RNA transcripts for inoculation onto soybean. Recently, a DNA-based BPMV VIGS vector was reported in which the BPMV RNA1 and RNA2 were placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (Nos) terminator. This modification enabled the vector DNA to be directly inoculated onto soybean plants,

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[2] Present address: Department of Entomology, Panhandle Research and Extension Center, University of Nebraska, Scottsbluff, NE 69361.

* Corresponding author; e-mail czhan2@iastate.edu.

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which enhanced the utility of BPMV for large-scale functional genomics experiments (Zhang et al., 2009). Both of these vectors have been used successfully in reverse genetic studies to identify resistance genes and resistance gene signaling components in soybean (Kachroo et al., 2008; Fu et al., 2009; Meyer et al., 2009). However, insertion of foreign sequences into both vectors is constrained by the fact that all inserts must be cloned in frame between the MP and the L-CP encoded by the RNA2 polyprotein. In the case of VIGS, the translated peptide from the foreign sequence may result in unanticipated phenotypes. Additionally, VIGS target sequences are limited to open reading frames (ORFs).

To further enhance the potential of BPMV as a viral vector for functional genomics, we describe here a set of DNA-based BPMV vectors that employ new designs for gene expression and VIGS. VIGS target sequences were inserted after the translation stop codon of RNA2 to overcome the necessity of cloning foreign sequences in the same reading as the RNA2 polyprotein. This modification also allows insertion of antisense and noncoding sequences into the BPMV VIGS vector. Thus, the BPMV VIGS vector can be used for applications such as cDNA library screening, promoter silencing, and silencing of untranslated regions (UTRs) of mRNAs. Furthermore, RNA silencing and foreign gene expression can be achieved in a single BPMV construct, making marker gene-assisted silencing possible. These vectors also enable two foreign genes to be inserted into the BPMV genomic RNA2 for simultaneous expression in soybean and common bean (*Phaseolus vulgaris*) plants. Successful infections were also consistently achieved when soybean plants were mechanically inoculated with plasmid DNA of BPMV constructs carrying foreign gene inserts or endogenous target gene fragments. Bypassing the requirement for in vitro transcription, biolistic delivery, and agroinoculation procedures makes the new BPMV functional genomics tool an ideal "one-step" viral vector system for high-throughput applications. These results demonstrate that the BPMV vector set has a wide range of applications in legume functional genomics, and they have implications for the improvement of other plant viral vectors, especially those employing polyprotein expression strategies.

RESULTS

Construction of BPMV Vectors with Mild and Moderate Symptom Phenotypes

The BPMV isolate IA-Di1 was used for the development of the new set of BPMV vectors. IA-Di1 induces mild symptoms, making it suitable as a vector, because there is concern that a severe viral phenotype will interfere with VIGS and overexpression phenotypes. RNA1 and RNA2 of the IA-Di1 isolate were placed under the control of the CaMV 35S promoter and the Nos terminator to construct the infectious cDNA clones pBPMV-IA-R1 and pBPMV-IA-R2 (Fig. 1A). These infectious clones were mixed in a 1:1 molar ratio, and biolistic inoculation was used to infect 10-d-old primary leaves of soybean plants. Consistent with the parental virus, infection by pBPMV-IA-R1 with pBPMV-IA-R2 produced mild symptoms (Fig. 2B). The mild infection was confirmed by mechanical inoculation to a new set of soybean seedlings followed by ELISA (data not shown).

Previously, the helicase protein encoded on BPMV RNA1 was shown to be a pathogenicity factor, and it was suggested that amino acid position 359 of the helicase may be critical for severe symptoms induced by strain K-Ho1 (Gu and Ghabrial, 2005). As a result of amino acid sequence comparison of the C-terminal regions of BPMV helicases, the amino acids at posi-

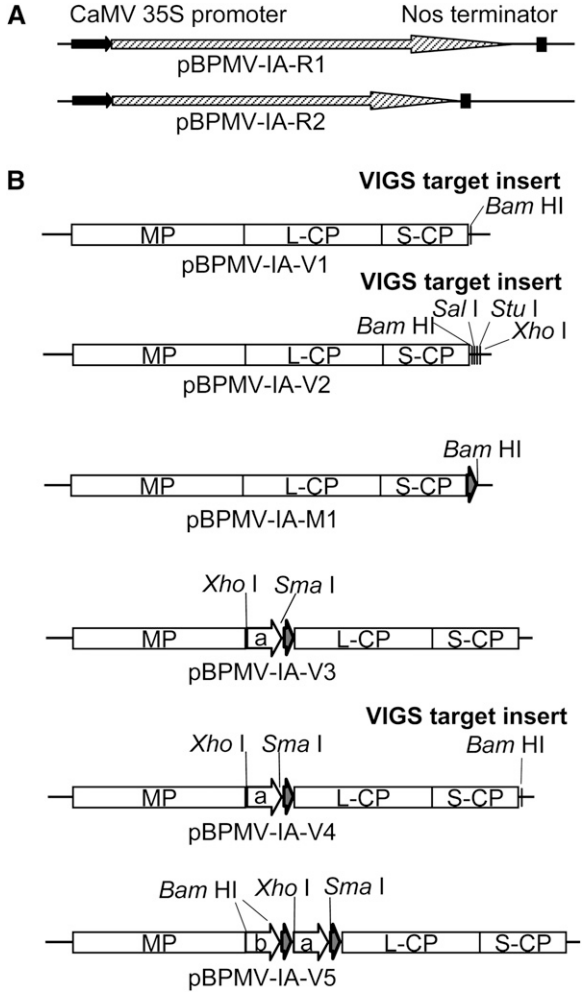

Figure 1. Schematic representation of the vector set derived from BPMV isolate IA-Di1. A, BPMV genomic RNA1 and RNA2 are expressed under the control of the CaMV 35S promoter and Nos terminator. B, Cloning site for insertion of foreign sequences into BPMV RNA2 derivatives. Arrows a and b indicate the foreign gene inserts. S-CP, Small coat protein subunit.

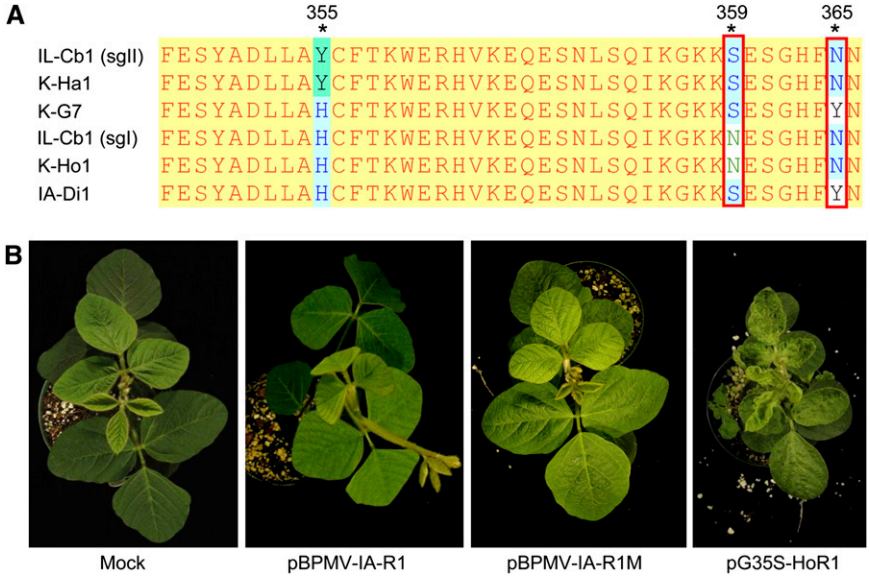

Figure 2. Strategy for modulating vector symptoms by modification of BPMV IA-Di1 RNA1. A, Alignment of the helicase gene amino acid of selected BPMV strains and isolates. The three differences are highlighted with asterisks, and relative positions are indicated above the asterisks. The two boxed differences were selected for IA-Di1 RNA1 mutation. sgI and sgII indicate subgroup I and subgroup II of BPMV. The GenBank accession numbers for the BPMV RNA1s are as follows: IL-Cb1 (sgII), AY744932; K-Ha1, AF394606; K-G7, U70866; IL-Cb1 (sgI), AY744931; K-Ho1, AF394608; IA-Di1, GU562879. B, Shoot phenotypes on the Williams soybean cultivar induced by different BPMV infectious RNA1 clones. Plants were photographed at 3 weeks post inoculation. Mock indicates the mock-inoculated control. Infectious BPMV RNA2 clone pBPMV-IA-R2 was used as the RNA2 component for all infections. The infectious RNA1 clones are pBPMV-IA-R1 (wild-type IA-Di1 sequence), pBPMV-IA-R1M (IA-Di1 sequence with S359N and Y365N mutations), and pG35S-HoR1 (Zhang et al. 2009). All plants were maintained in a growth chamber at 20°C with a 16-h photoperiod. [See online article for color version of this figure.]

tions 359 and 365 of IA-Di1 RNA1 (pBPMV-IA-R1) were each mutated to Asn (N), as they are in strain K-Ho1 (Fig. 2A). This modified clone is referred to as pBPMV-IA-R1M. Unlike the wild-type RNA1 clone pBPMV-IA-R1, the mutant pBPMV-IA-R1M induced obvious moderate symptoms upon inoculation with wild-type RNA2 clone pBPMV-IA-R2 (Fig. 2B). The moderate infection phenotype of pBPMV-IA-R1M was confirmed by mechanical inoculation to a new set of soybean seedlings followed by ELISA. Interestingly, the enhanced symptoms are not as severe as those of strain K-Ho1. Because the infection by pBPMV-IA-R1 only induced very mild visual symptoms that are almost indistinguishable from the uninfected mock control, it is necessary to confirm the positive infection by ELISA. Instead, infection by pBPMV-IA-R1M is evident by the moderate visual symptoms, rendering the ELISA confirmation step unnecessary; hence, it was used as the RNA1 component for all the following experiments.

BPMV VIGS Using Sense or Antisense Target Sequence Insertions

A series of modifications were made to pBPMV-IA-R2 to evaluate different strategies for foreign gene expression and VIGS (Fig. 1B). First, a *Bam*HI restriction site was introduced after the stop codon of the RNA2 ORF to allow insertion of target gene fragments for silencing (pBPMV-IA-V1; Fig. 1B). Additionally, a multiple cloning site to facilitate directional insertion was introduced into the *Bam*HI site to generate the VIGS vector pBPMV-IA-V2 (Fig. 1B). The soybean phytoene desaturase (PDS) gene was selected to test VIGS efficiency. Four sets of primers were designed to amplify soybean *PDS* sequences from the 5′ UTR, 5′ end of the ORF, 3′ end of the ORF, and the 3′ UTR. Because each of the four regions was inserted into the pBPMV-IA-V1 *Bam*HI site, clones could be selected in order to test whether the sense or antisense orientation affects the efficiency of *PDS* gene silencing. In three independent experiments, we found that the antisense insertion of the 3′ ORF induced the best silencing (PDS-3R; Fig. 3B). The third and fourth trifoliolates of soybean plants infected by pBPMV-PDS-3R were almost totally bleached, while the same sequence inserted in the sense orientation only induced mosaic bleaching. In a comparison between the 5′ and 3′ ORFs, the 3′ end insertion was better for *PDS* VIGS. This positional effect was more pronounced for the antisense orientation than the sense orientation. While the antisense 3′ UTR caused mosaic-type bleaching, the sense insertion of the 3′ UTR of *PDS* showed little photobleaching. It is interesting that the 5′ UTR in either the sense or antisense orientation showed relatively little photobleaching under the same conditions (Fig. 3B).

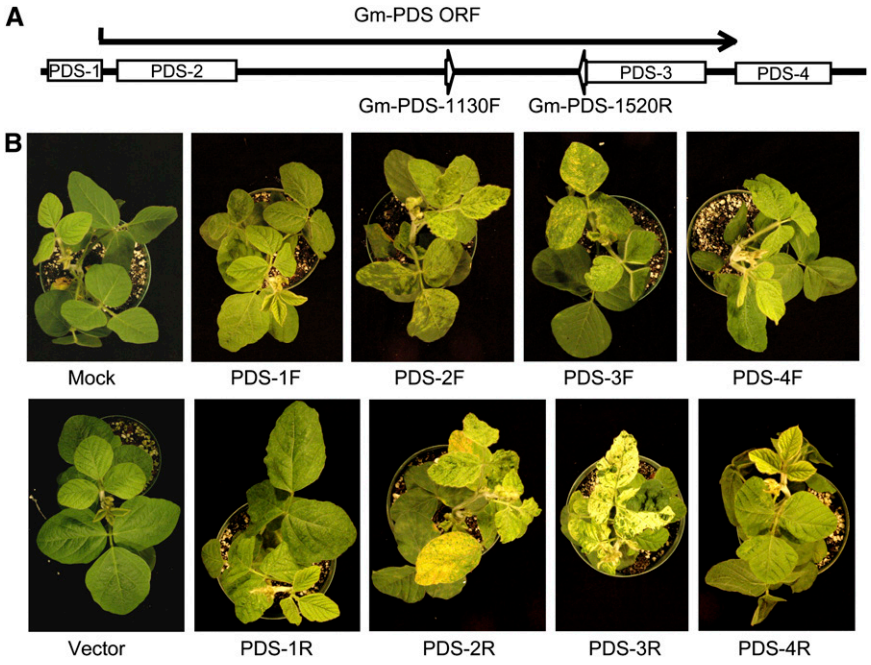

Figure 3. Soybean *PDS* gene silencing is affected by target position and insertion orientation. A, Diagram of soybean *PDS* mRNA organization. The four regions that were targeted by VIGS are indicated by boxes. The ORF is illustrated above the mRNA. The middle region between primers Gm-PDS-1130F and Gm-PDS-1520R, as indicated by hollow arrows, was used as a probe for northern-blot hybridization. B, Soybean *PDS* gene silencing induced by different VIGS constructs. Plants were photographed at 3 weeks post inoculation. pBPMV-IA-R1M was used as the RNA1 clone for all inoculations. PDS-1F, PDS-2F, PDS-3F, and PDS-4F represent the four sense insertion VIGS constructs pBPMV-PDS-1F, pBPMV-PDS-2F, pBPMV-PDS-3F, and pBPMV-PDS-4F, respectively. PDS-1R, PDS-2R, PDS-3R, and PDS-4R represent the four antisense insertion VIGS constructs pBPMV-PDS-1R, pBPMV-PDS-2R, pBPMV-PDS-3R, and pBPMV-PDS-4R, respectively. Mock indicates the mock-inoculated control, and Vector indicates the empty vector control inoculated with pBPMV-IA-R2. PDS-3R induced the strongest *PDS* photobleaching phenotype. All plants were maintained in a growth chamber at 20°C with a photoperiod of 16 h. [See online article for color version of this figure.]

Northern-blot analysis was used to evaluate *PDS* mRNA levels in leaves infected by these VIGS constructs. Total RNA was extracted from the third and fourth trifoliolates at 3 weeks post inoculation. Twenty-five micrograms of total RNAs was loaded for each treatment, and equal loading was confirmed by ethidium bromide staining (Fig. 4A). To examine the accumulation of BPMV RNA2, a portion of IA-Di1 RNA2 was PCR amplified with primer pair R2-1548F and R2-2688R (Table I), and this PCR product was radiolabeled for use as a probe to detect BPMV RNA2 accumulation (Fig. 4B). A major band was observed for each PDS VIGS construct, as opposed to multiple bands, demonstrating that there was no significant loss of the *PDS* inserts (Fig. 4B). However, a lower band was observed for all constructs, including even the wild-type vector control. Therefore, reverse transcription (RT)-PCR was performed with forward primer R2-3195F and each reverse primer corresponding to the 3′ end of the *PDS* target sequence of each VIGS construct to examine the integrity of the BPMV RNA2 *PDS* insertion. While the vector control produced no PCR products due to the lack of *PDS* primer sequence on BPMV RNA2, a single band with the size expected observed for each BPMV RNA2 carrying corresponding *PDS* inserts demonstrated that the *PDS* fragments were stably maintained during the virus infection (Fig. 4D). Interestingly, all antisense insertions resulted in a significant reduction of BPMV RNA2. For the sense orientation insertions, only the 5′ UTR caused a similar reduction. The remainder of the sense insertions had minimal impact on BPMV RNA2 accumulation as compared with the wild-type empty vector (Fig. 4, B and E). The 3′ ORF and 3′ UTR in the antisense orientation resulted in the lowest RNA2 abundance, which was about 5- to 7-fold lower than the empty vector control.

A central region of *PDS* that was not targeted for silencing in any of the VIGS constructs was amplified using primer pair Gm-PDS-1130F and Gm-PDS-1520R (Table III). This region was used as a probe to detect the *PDS* mRNA accumulation in plants infected with each of the *PDS* VIGS constructs or the empty vector control. Based upon band intensity relative to the empty vector control, mRNA levels ranged from 12.6% to 28.0% of the empty vector control for the antisense constructs but 50.3% to 116.1% for the sense constructs. Significantly, the levels of RNA2 and *PDS* mRNA accumulation were well correlated (Fig. 4, E and F). The antisense orientation caused the greatest reduc-

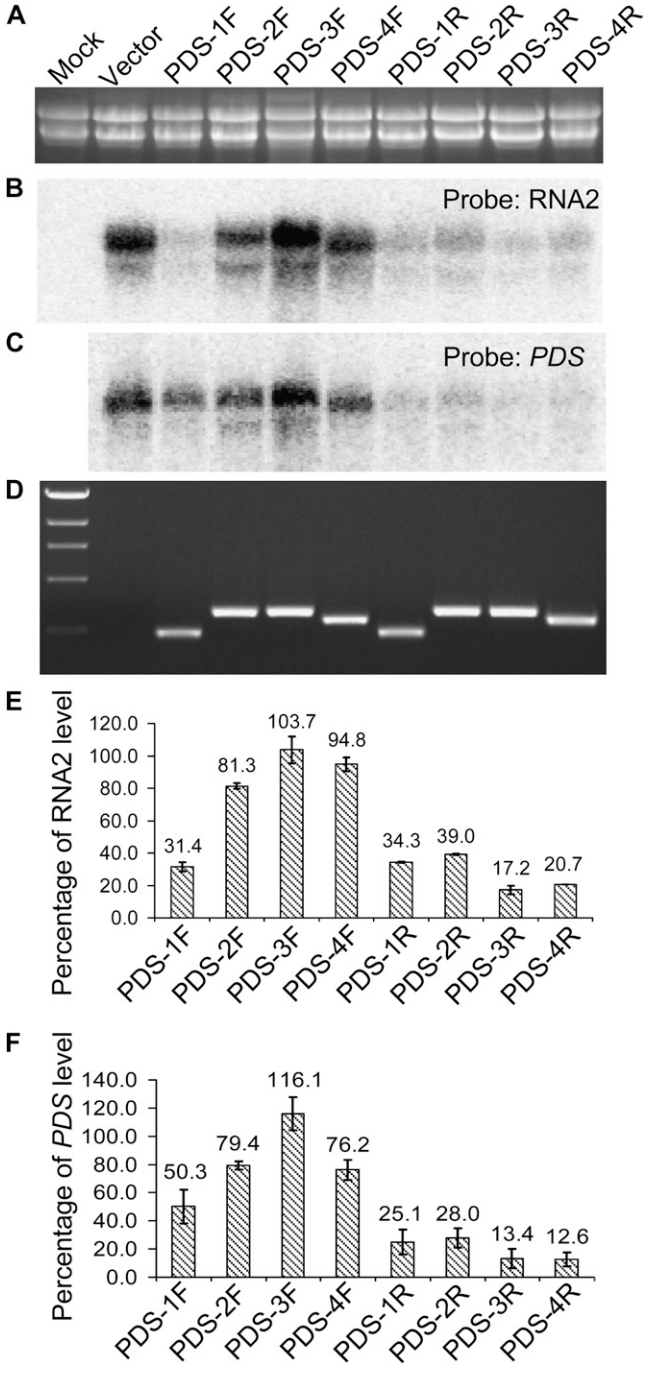

Figure 4. BPMV RNA2 and *PDS* mRNA accumulation in plants infected with sense and antisense *PDS* VIGS constructs. Northern-blot and RT-PCR analyses were used to assess the *PDS* mRNA levels and VIGS insertion of BPMV RNA2 in soybean plants infected with different soybean *PDS* gene-silencing constructs targeting different regions in sense and antisense orientations. A, Equivalent RNA loading was assessed by ethidium bromide staining of total RNA extracted from the third and fourth trifoliolates of infected plants by the BPMV VIGS constructs shown in Figure 3. B, Northern-blot analysis of BPMV RNA2 accumulation using probes prepared from the PCR products using primer pair R2-1548F and R2-2688R (Table I). C, Northern-blot analysis of *PDS* mRNA levels induced by different VIGS constructs. The *PDS* probe was prepared from the middle region using primers Gm-PDS-1130F and Gm-PDS-1520R (Fig. 3A). D, RT-PCR analysis performed with forward primer R2-3195F and each reverse primer corresponding to the 3′ end of PDS target sequence in each VIGS construct to confirm the PDS fragment insertion. The first lane is the 1-kb marker, and the sizes are 3, 2, 1.5, 1, and 0.5 kb from top to bottom. Note that the vector control is negative due to the lack of a PDS primer region. E, Percentage of the BPMV RNA2 accumulation level of each VIGS construct compared with the levels in vector control plants. The relative levels of the BPMV RNA2 accumulation were calculated by dividing the radioactivity signal of the VIGS treatments by the signal for the vector control. F, Percentage of the *PDS* mRNA levels caused by each VIGS construct compared with the levels in vector control plants. The relative levels of the *PDS* mRNA accumulation were calculated by dividing the radioactivity signal of the VIGS treatments by the signal for the vector control. Data are averages of two independent biological replicates, and SD values (bars) are indicated.

tion of *PDS* mRNA accumulation in two independent biological replicates.

Multiple Gene Expression and Marker Gene-Assisted Silencing

Cowpea mosaic virus (CPMV) was previously shown to express a foreign gene that was inserted after the foot-and-mouth disease virus (FMDV) 2A proteinase peptide fused to the C terminus of the small coat protein (Gopinath et al., 2000). Following this strategy, we synthesized the FMDV-2A proteinase coding region and placed it immediately after the C terminus of the small coat protein gene of BPMV. A *Bam*HI restriction site was introduced after FMDV-2A to facilitate gene insertion (pBPMV-IA-M1; Fig. 1B). To test this strategy, the GFP gene was introduced into pBPMV-IA-M1 to generate pBPMV-M1-GFP. After three repetitions with controls, infection was not achieved with either pBPMV-IA-M1 or pBPMV-M1-GFP when biolistically inoculated with either pBPMV-IA-R1 or pBPMV-IA-R1M (data not shown). We ruled out the possibility of faulty inoculation, because the wild-type control pBPMV-IA-R2 was nearly 100% infectious and it was handled concurrently with pBPMV-IA-M1 and pBPMV-M1-GFP. Furthermore, pBPMV-IA-M1 and pBPMV-M1-GFP were fully resequenced and no mutations were found.

The previous BPMV gene expression vectors (Zhang and Ghabrial, 2006; Zhang et al., 2009) have a duplicated protease cleavage site between the C terminus of MP and the N terminus of L-CP. Instead of duplicating the cleavage site, we introduced a FMDV-2A proteinase peptide between the MP and L-CP cistrons (pBPMV-IA-V3; Fig. 1B). The vector is designed for gene insertion before the FMDV-2A proteinase peptide. The FMDV-2A proteinase peptide has autocleavage activity at the penultimate amino acid residue (Gopinath et al., 2000), leaving the mature L-CP with one additional Pro at the N terminus. This strategy for gene expression was tested by inserting genes including GFP, BAR (herbicide resistance gene encoding phosphinothricin acetyltransferase), and *Tomato bushy*

Table I. *Primers for constructing and sequencing infectious BPMV VIGS vectors*

Name	Sequence (5′ → 3′)
BP-5endF	TATTAAAATTTTCATAAGATTTGAAATTTTG
R1-235F	ATATAGGACTTCGTGTCAGATT
R1-688F	TGCATATCATTTTCAGCATTTTGT
R1-1208F	TGTGCTACCATTGCAGTTTCTA
R1-2245R	AAGTTTGGTCTACAACATAATGA
R1-2797R	TCCCATTCCACACAAAATTGCT
R1-3344R	TCAGGATCATACACATGCCA
R1-3824R	ACTCCCTCTTGACTATCAAC
R1-3746F	GCTTCATTAATACCATATGTTGA
R1-4707R	CCACCACAAAGACTGTTTATCA
R1-5238R	ACAAGATAAGCTTCTTGCATTT
R1-5708F	CAATAAGAAAATTCGGACAGCGCTT
R1-5759R	GGAAAAGGATCAACTCTAGT
R1-3Cla	CCATCGATTTTTTTTTTTTTTTTTTTTATATTTA AACAC
R2-454F	ACTTGGGCATTGGTGCAAATGT
R2-948F	ACTTCTTACTGATGGGAAGTTGTA
R2-1548F	CAAGAGAAAGATTTATTGGAGGGA
R2-1786F	AAGCTCAAATGGAAACAAATCT
R2-2133F	TGGAATCCTGCTTGTACAAAAGCA
R2-2688R	TGTGAGAAACTCCTCTTGTGA
R2-2746F	TGGCTGATGGGTGCCCATATT
R2-3195F	CCTCATTGGTACAAGTGTTT
R2-3303F	ATGGTTTCGAAATGTGGAGTTCTGA
R2-3Cla	CCATCGATTTTTTTTTTTTTTTTTTTTAAAATAA CACAC
R1-Modi-F	AAAAATGAAAGTGGTCATTTTAATAAT
R1-Modi-R	ATTATTAAAATGACCACTTTCATTTTT
35S-Seq	ACGCACAATCCCACTATC
Nos-Rev	AGACCGGCAACAGGATTCA

stunt virus (TBSV) P19, which is a potent RNA-silencing suppressor (Voinnet et al., 1999). Three weeks post inoculation, typical mosaic symptoms were seen on pBPMV-GFP2- and pBPMV-BAR-inoculated plants. However, severe symptoms including necrosis were induced by pBPMV-P19, which expresses the TBSV P19 RNA-silencing suppressor (Fig. 5).

To facilitate double gene expression, a second FMDV-2A proteinase peptide was introduced before the first FMDV-2A sequence (pBPMV-IA-V5; Fig. 1B). To reduce instability of the introduced genetic elements, the sequence identity of the second FMDV 2A site was altered by taking advantage of codon degeneracy. The *Xho*I and *Sma*I restriction sites were used for insertion of the first gene, and *Bam*HI was used for insertion of the second gene (pBPMV-IA-V5; Fig. 1B). The double gene expression construct pBPMV-GFP-BAR induced mosaic symptoms (Fig. 5). The activities of GFP and BAR were tested by examining green fluorescence and herbicide resistance (Figs. 6 and 7). The pBPMV-BAR and pBPMV-GFP-BAR constructs conferred herbicide resistance, whereas the mock- and empty vector-infected soybean plants were killed by herbicide treatment. This result demonstrated that the BAR protein was sufficiently active to protect plants from herbicide damage when expressed alone or in tandem with GFP (Fig. 6). Examination of plants under UV light showed that expression of GFP alone or in tandem with BAR was sufficient to readily observe GFP fluorescence (Fig. 7). These results show that GFP and BAR were biologically active when coexpressed from the pBPMV-GFP-BAR construct; thus, double gene expression is feasible.

Another BPMV vector was created to test the possibility of expressing a foreign gene and at the same time silencing a target gene (pBPMV-IA-V4; Fig. 1B). pBPMV-P19-PDS, which expresses TBSV P19, a potent RNA-silencing suppressor, induced severe symptoms similar to pBPMV-P19. There was no visible photobleaching phenotype typical of *PDS* silencing (Fig. 5). However, pBPMV-BAR-PDS and pBPMV-GFP-PDS both induced visible photobleaching on soybean plants, as expected if *PDS* was silenced (Fig. 5). The construct pBPMV-GFP-PDS was examined for GFP expression and *PDS* silencing more closely. Interestingly, the visible phenotypic expression of photobleaching due to *PDS* gene silencing overlaps with visible fluorescence resulting from GFP expression (Fig. 7). This result shows that marker gene-assisted silencing is feasible in this system. Because double gene silencing was previously achieved by combining two VIGS target sequences into a single construct (Zhang et al., 2009), pBPMV-IA-V2 was created for concatemerizing multiple target sequences for simultaneous silencing (Fig. 1B).

Gene Expression in Shoots and Roots of *P. vulgaris*

BPMV is known to infect other legumes, including *P. vulgaris* (common bean; Wickizer and Gergerich, 2007), but it has not been formally tested as a vector in this plant species. To investigate the possibility of foreign gene expression by the BPMV vector in *P. vulgaris*, plants were infected with the single gene expression construct pBPMV-GFP2 or the double gene expression construct pBPMV-GFP-BAR. At 3 weeks post inoculation, infected plants showed extensive green fluorescence on the upper systemic leaves when exposed to UV light. It is interesting that both veinal and interveinal areas have strong fluorescence (Fig. 8A). Roots from the same plants were rinsed and photographed (Fig. 8B). Green fluorescence due to GFP expression from both pBPMV-GFP2 and pBPMV-GFP-BAR was readily visualized, while the mock and the empty vector controls produced no fluorescence. However, we observed that the lateral roots rather than the primary roots generally had the most intense fluorescence. The single gene expression construct pBPMV-GFP2 gave consistent green fluorescence, whereas the double gene expression construct pBPMV-GFP-BAR produced sporadic fluorescence (Fig. 8B). This difference is likely due to the fact that pBPMV-GFP-BAR is larger than pBPMV-GFP2; thus, we do not expect the double gene expression construct to replicate and move as efficiently and quickly as the smaller single gene expression construct.

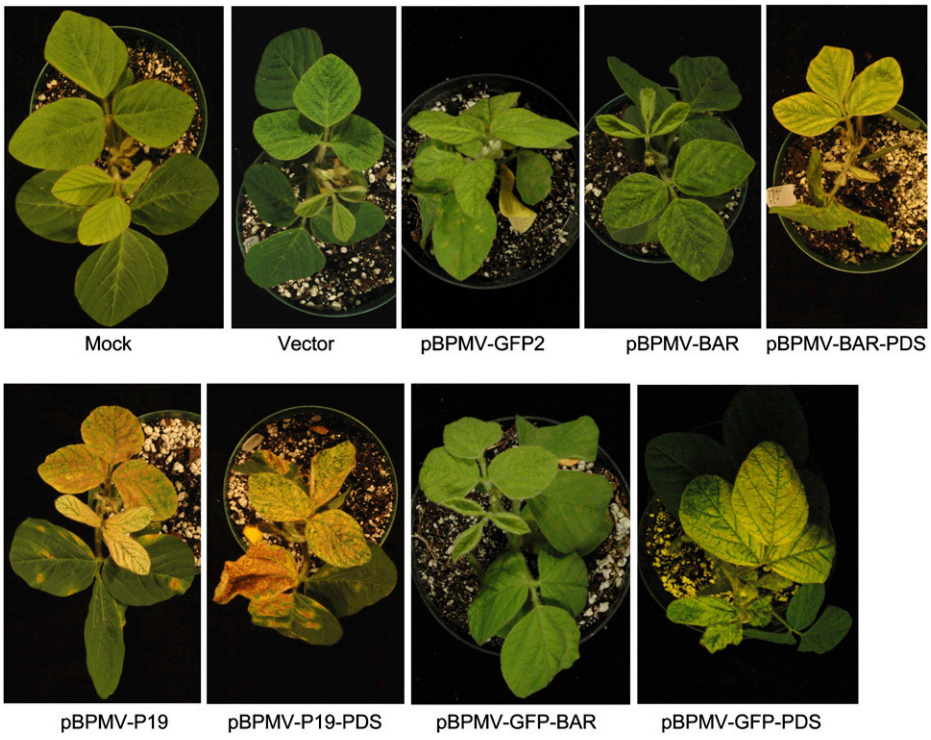

Figure 5. Diverse symptoms induced by BPMV vector recombinants expressing foreign genes. pBPMV-IA-R1M was used as the RNA1 clone for all inoculations. Plants were photographed at 3 weeks post inoculation. Mock and vector treatments are described in Figure 3. Plants were maintained in a growth chamber at 20°C with a photoperiod of 16 h. [See online article for color version of this figure.]

BPMV Infection through Direct DNA-Rubbing Inoculation

While the biolistic inoculation is highly efficient, inoculation by direct DNA rubbing may make use of the BPMV vectors even more facile, as was reported for *Soybean mosaic virus* (SMV; Seo et al., 2009) in soybean and *Turnip yellow mosaic virus* (TYMV; Pflieger et al., 2008) in Arabidopsis (*Arabidopsis thaliana*). We evaluated direct DNA rubbing of soybean seedlings for two constructs: pBPMV-P19, which expresses the heterologous TBSV P19 RNA-silencing suppressor; and the RNA-silencing construct pBPMV-PDS-3R, which contains an endogenous *PDS* gene 3′ ORF antisense insertion in the BPMV RNA2. Results showed that direct DNA rubbing onto primary leaves of soybean seedlings grown under normal conditions resulted in low frequency or no infection. However, if plants were placed in the dark for 24 h preceding rub inoculation, about 50% infection was achieved for both constructs (Table II). The DNA rub inoculations resulted in necrotic and photobleaching phenotypes for the pBPMV-P19 and pBPMV-PDS-3R constructs, respectively (Fig. 9), as was observed for biolistic inoculation in Figures 3 and 5.

DISCUSSION

Two distinct subgroups of BPMV RNA1 and RNA2 have been described (Zhang et al., 2007). Infectious clones were developed previously for BPMV isolates of both subgroups using either T7 promoter-driven in vitro RNA transcription and inoculation or DNA-based direct biolistic inoculation (Zhang and Ghabrial, 2006; Zhang et al., 2009). The IA-Di1 BPMV isolate has subgroup I RNA1 and subgroup II RNA2 (J. Bradshaw, C. Zhang, J. Hill, and M. Rice, unpublished data), for which infectious clones were developed in this report. The infectious IA-Di1 clones induced very mild symptoms indistinguishable from the phenotype of the mock control under the experimental conditions. While the mild symptoms are amenable for VIGS study, a disadvantage is that the infection has to be confirmed by either ELISA assay or RT-PCR. Gu and Ghabrial (2005) previously developed infectious clones for two subgroup I BPMV RNA1s, K-Ho1 and K-G7. BPMV K-Ho1 RNA1 induces severe symptoms and K-G7 RNA1 causes mild symptoms on the soybean cv Williams. The helicase cistron of subgroup I RNA1 encodes the pathogenicity determinant for foliar symptom severity. Based on amino acid sequence comparison of the C-terminal region of the helicase gene, two amino acids (positions 359 and 365) of IA-Di1 RNA1 were mutated to that of the severe RNA1 of BPMV strain K-Ho1 (Fig. 2A). Unlike the wild-type RNA1 clone pBPMV-IA-R1, the mutant pBPMV-IA-R1M, upon inoculation with wild-type RNA2 clone pBPMV-IA-R2, produced visible moderate symptoms on the soybean cv Williams (Fig. 2B). pBPMV-IA-R1M was subsequently used in this study for gene expression as well as for VIGS and consistently gave visible symptoms that were not as severe as those caused by strain K-Ho1. The moderate symptoms make pBPMV-IA-R1M an optimal vector, because infections are easily confirmed by visual inspection and the moderate symptoms are less likely to interfere with phenotypic tests

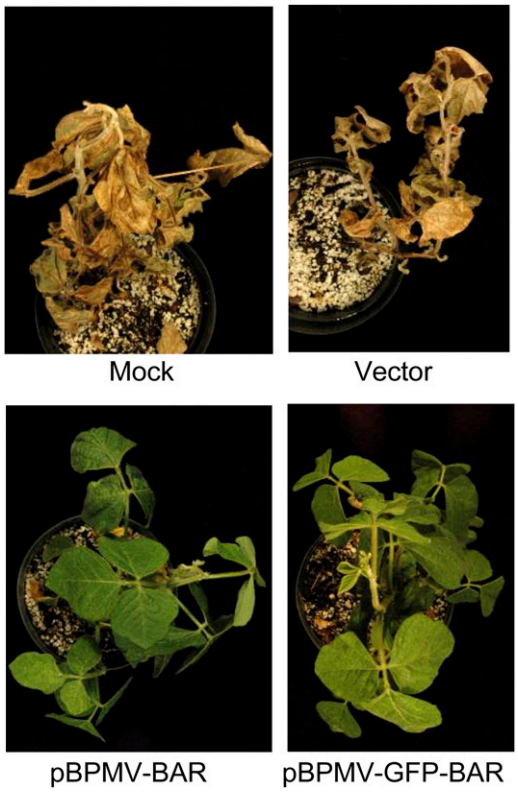

Figure 6. Herbicide resistance is conferred by expression of the BAR gene alone or in tandem with GFP. pBPMV-IA-R1M was used as the RNA1 clone for all inoculations. Mock and vector treatments are described in Figure 3. All plants were maintained in a greenhouse room. Soybean seedlings were inoculated when the primary leaves were fully expanded. Three weeks post inoculation, plants were treated with the herbicide (0.05% ammonium-glufosinate in deionized water). Photographs were taken 3 weeks after the herbicide treatment. pBPMV-BAR and pBPMV-GFP-BAR conferred herbicide resistance, while the mock- and vector-treated plants were killed. [See online article for color version of this figure.]

than a severe isolate. In addition, the ability to modulate symptom severity by selecting the appropriate RNA1 clone provides an opportunity to optimize BPMV symptoms to suit the phenotypic assay conditions.

Soybean is a major oilseed crop and an important source of vegetable oil and protein world-wide. The availability of the soybean genome sequence, microarray data sets, and an extensive EST collection provides various lists of candidate gene sequences to test for function in association with important soybean traits. The VIGS approach is an important component of the soybean functional genomics tool box (Stacey et al., 2004; Jackson et al., 2006). Five viruses, *Clover yellow vein virus* (ClYVV), *Apple latent spherical virus* (ALSV), *Cucumber mosaic virus*, SMV, and BPMV, have been developed as soybean functional genomics tools (Masuta et al., 2000; Wang et al., 2006; Zhang and Ghabrial, 2006; Nagamatsu et al., 2007; Igarashi et al., 2009). Fusion protein expression was used for both ALSV and BPMV gene expression and VIGS studies. ClYVV and SMV are potyvirus-based vectors that also employ fusion protein expression strategies, but it has not been shown that a potyvirus can be an effective VIGS vector because potyviruses encode helper component-proteinase gene HC-Pro, which is a potent RNA-silencing suppressor (Anandalakshmi et al., 1998).

In the previous BPMV VIGS vectors (Zhang and Ghabrial, 2006; Zhang et al., 2009), foreign sequences were inserted between the MP and L-CP in the RNA2 ORF. Similarly, foreign sequences are inserted in frame between the MP and the Vp25 coat protein subunit on ALSV RNA2. This design strategy restricts the utility of these vectors for RNA silencing, because they cannot be used to target genes with very short reading frames or noncoding sequences such as promoter regions and UTRs. In addition, it is not possible to discern if RNA polarity influences the efficiency of target gene silencing, and there is always a short peptide expressed that may confound the interpretation of the silencing result. To overcome these shortcomings, foreign sequences were inserted after the stop codon of the BPMV RNA2 ORF. The soybean *PDS* gene was selected to test this strategy because of the obvious photobleaching phenotype as well as the opportunity to compare with previous results (Nagamatsu et al., 2007; Igarashi et al., 2009; Zhang et al., 2009). *PDS* inserts in the sense orientation generally were the least effective for inducing *PDS* silencing. These results are consistent with those using ALSV in soybean, where the 5′ end *PDS* sense insertion had little *PDS*-silencing effect (Igarashi et al., 2009). Similar results were also reported in Arabidopsis, where sense insertion in TYMV had minimal effect on GUS transgene and *PDS* silencing (Pflieger et al., 2008). Here, we found that the antisense orientation generally resulted in stronger silencing phenotypes. Two antisense *PDS* VIGS constructs, pBPMV-PDS-3R and pBPMV-PDS-4R, resulted in 7- to 8-fold reductions of *PDS* mRNA. This contrasts with the 2- to 3-fold reductions we observed when testing other genes using the sense orientation (Zhang et al., 2009). Similar to the ALSV results, we found that the middle to 3′ end of the *PDS* gene coding region resulted in stronger silencing, particularly in the antisense orientation.

Interestingly, we observed that, in general, greater reduction in *PDS* mRNA is correlated with reduced BPMV RNA2 accumulation (Fig. 4). This is not surprising, because RNA2 carries the inserts for the target gene and thus is targeted by VIGS of PDS. Similarly, when GFP transgenic *Nicotiana benthamiana* was infected by a potato virus X (PVX) construct carrying a partial sequence of GFP, VIGS of GFP was triggered and associated with greatly reduced PVX genomic RNA accumulation (Ruiz et al., 1998). We thus hypothesize that when soybean activates RNA silencing against BPMV viral infection, there is an added effect by VIGS of *PDS* that targets the recombinant BPMV-carrying *PDS* gene fragment. However, demonstration that this correlation is a general feature will require studies targeting a wide range of genes involved in different pathways. In addition, further comparisons

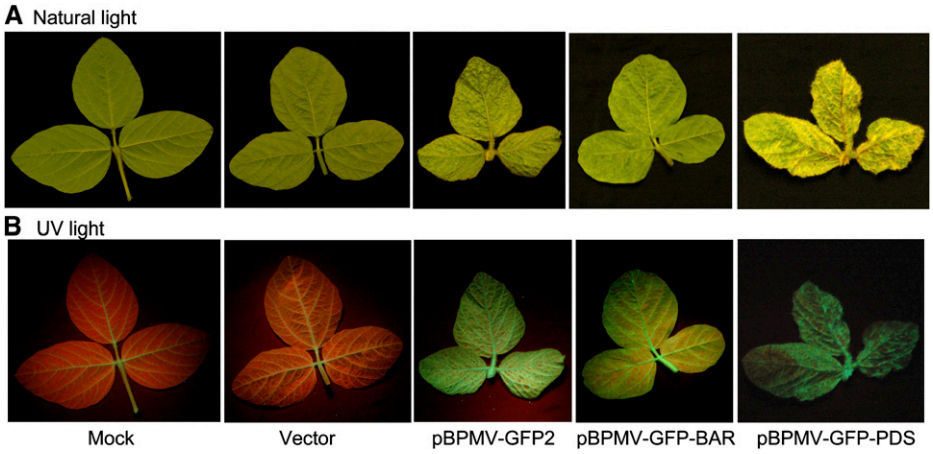

Figure 7. GFP gene expression alone or in tandem with BAR or a *PDS* VIGS insert. Green fluorescence on systemic leaves of soybean plants was examined by epifluorescence photography. pBPMV-IA-R1M was used as the RNA1 clone for all inoculations. Mock and vector treatments are described in Figure 3. All plants were maintained in a growth chamber at 20°C with a photoperiod of 16 h. Soybean seedlings were inoculated when the primary leaves were fully expanded. The third trifoliolates were photographed 3 weeks post inoculation for all treatments except for pBPMV-GFP-PDS, which is the fourth trifoliolate. A, Leaves were photographed under natural light. B, The same leaves in A were photographed under UV excitation for GFP fluorescence. Note the green fluorescence in B for constructs pBPMV-GFP2, pBPMV-GFP-BAR, and pBPMV-GFP-PDS. [See online article for color version of this figure.]

of VIGS using different viral vectors for effects on the viruses as well as on the host genes may yield more information on host defenses against viral infections.

To evaluate the potential of BPMV for double gene expression, we first tested inserting the second foreign gene after the small coat protein cistron, since previously it was shown that a single foreign gene can be expressed between the MP and L-CP cistrons (Zhang and Ghabrial, 2006). To our surprise, with repeated attempts, the constructs pBPMV-IA-M1 and its derivative pBPMV-M1-GFP did not infect soybean systemically. BPMV belongs to the viral genus *Comovirus* of which CPMV is the type member, and a similar strategy for CPMV was successful. However, RNA transcripts for CPMV were first introduced into cowpea (*Vigna unguiculata*) protoplasts, whereas the BPMV constructs were directly inoculated to soybean plants (Gopinath et al., 2000). The difference in infection could be due to the methods of inoculation, differences in the abilities of these virus species to tolerate protein fusion to the 3′ end of the small subunit of the coat protein, or potential trace amounts of wild-type revertants of the CPMV GFP expression construct when it was passed from protoplasts to cowpea plants.

To attain double gene expression in BPMV, two foreign genes were inserted between the MP and L-CP cistrons. After inserting the FMDV-2A autocleavage peptide, biological functions of several genes, including GFP, BAR, and TBSV-P19, were demonstrated. Subsequently, a second nonidentical FMDV-2A peptide sequence was inserted for double gene expression. Both GFP and BAR were biologically active in leaves and roots when expressed from pBPMV-GFP-BAR. The FMDV-2A peptide used in this study belongs to a family termed CHYSEL (for cis-acting hydrolase element). Typical CHYSELs have about 20 to 30 amino acids and show direct cotranslational cleavage of the nascent polypeptide chain. Other FMDV-2A-like CHYSEL peptides are encoded by some picornaviruses and a number of other single- and double-stranded RNA viruses (Halpin et al., 1999; Felipe, 2004; Doronina et al., 2008). If multiple gene expression is required, other members of the CHYSEL peptide family can be selected to disrupt sequence homology to help maintain foreign gene insert stability.

It was previously shown that the SMV HC-Pro gene (1.4 kb) can be efficiently expressed by BPMV (Zhang and Ghabrial, 2006). The bacterial GUS marker gene (1.8 kb) was inserted into pBPMV-IA-V3 to generate pBPMV-GUS for GUS expression. However, no infection was achieved for pBPMV-GUS in three replications, even though the control was infectious (data not shown). Similar to the noninfectious clone pBPMV-M1-GFP, pBPMV-GUS was thoroughly sequenced and no mutation was identified. From the above, we conclude that the BPMV RNA2 vector capacity for foreign gene insertion size is between 1.4 and 1.8 kb.

Because the silencing or expression phenotypes can have mosaic distributions in this system as well as others (Fig. 7; Ding et al., 2006; Igarashi et al., 2009; Meng et al., 2009), marker genes such as GFP may be helpful in determining more precisely where silencing of the target gene is occurring in a given plant. *PDS* is frequently used as a positive control to confirm that the growth conditions were adequate for silencing. However, because the PDS-silenced plants are separate, they do not provide a precise indicator of where silencing is occurring within the experimental plants.

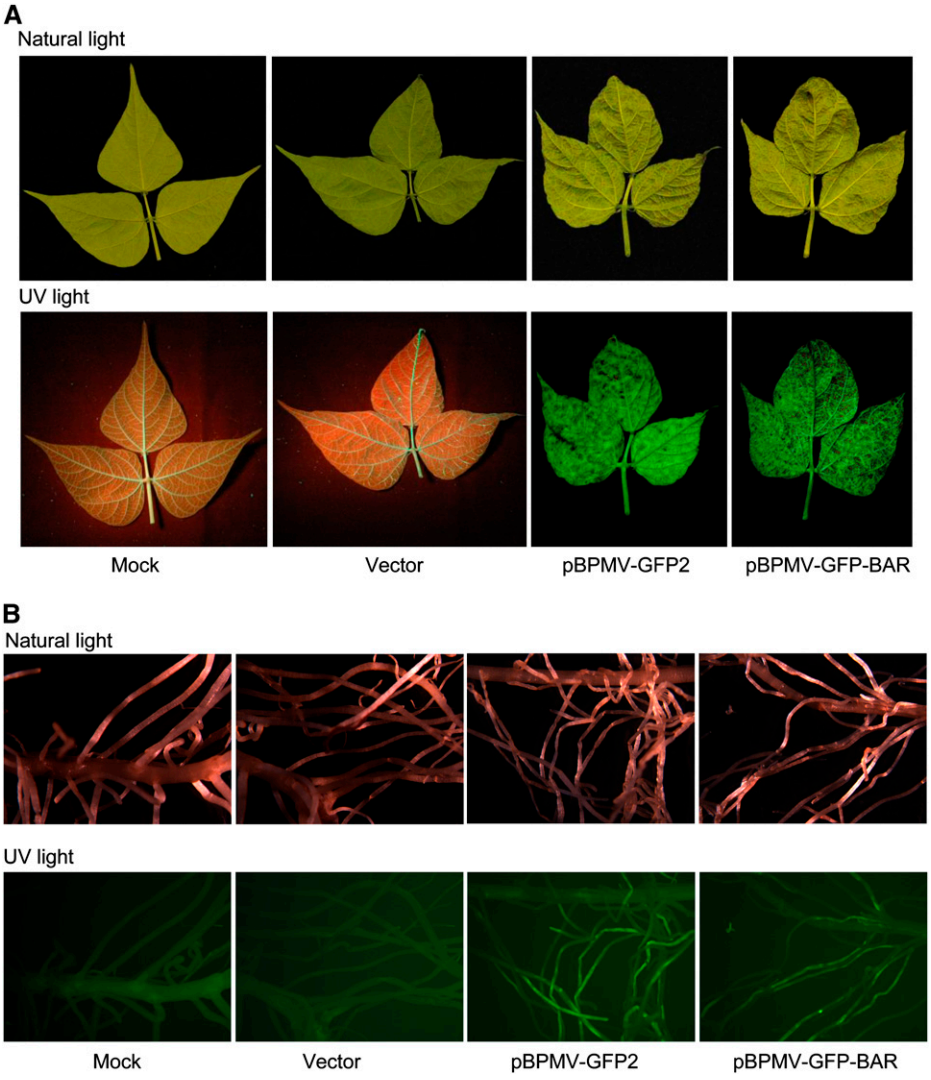

Figure 8. GFP gene expression in shoots and roots of *P. vulgaris*. Green fluorescence in both bean shoots and roots was examined by epifluorescence photography. pBPMV-IA-R1M was used as the RNA1 clone for all inoculations. Mock and vector treatments are described in Figure 3. All plants were maintained in a growth chamber at 20°C with a photoperiod of 16 h. Bean plants were inoculated when the primary leaves were fully expanded. Three weeks post inoculation, bean leaves were photographed as described in Figure 7. Bean roots were rinsed with water and photographed under either natural light (top rows) or UV excitation for GFP fluorescence (bottom rows). Note that green fluorescence was observed in leaves and roots inoculated with either pBPMV-GFP2 or pBPMV-GFP-BAR. [See online article for color version of this figure.]

An approach that couples expression of GFP with the simultaneous VIGS of an endogenous plant gene can provide a nondestructive and presumably benign marker for the tissues that are virus infected and most likely also undergoing VIGS of the target gene. The extensive overlap of GFP expression and *PDS* silencing induced by the pBPMV-GFP-PDS construct demonstrated the feasibility of marker gene-assisted silencing in soybean (Fig. 7).

In a further enhancement of the BPMV vector system, we demonstrated that efficient infection is possible by rub inoculating the BPMV DNA constructs onto soybean plants. The Arabidopsis plants used for one-step DNA rubbing by the TYMV *PDS*-silencing construct were placed in the dark for 24 h before inoculation (Pflieger et al., 2008). Similar dark treatment of soybean plants resulted in efficient infection (50%; Table II). There was no significant difference for BPMV constructs either expressing a heterologous gene or silencing an endogenous gene. While efficient infection was achieved, factors such as soybean cultivar, temperature, and plasmid concentration may be evaluated to further enhance DNA-rubbing infection efficiency. Other than TYMV and BPMV, other plant viral vectors have been developed by insertion of the viral genomes under control of the CaMV 35S promoter and Nos terminator, such as ALSV (Igarashi et al., 2009) and *Barley stripe mosaic virus* (Meng et al., 2009). It remains to be tested if direct DNA rubbing of similarly dark-treated host plants will also lead to efficient infection. Successful direct DNA-rubbing infection greatly reduces cost and dependence on specialized equipment necessary for biolistic delivery.

In summary, this new one-step BPMV-derived vector set is a high-throughput functional genomics tool that enables efficient, cost-effective, and simplified gene functional screening for important legume plants. The modifications reported here can be applied or adapted to other plant viral vectors, especially those that use a polyprotein gene expression strategy. The continued improvement of viral vectors and their wide adoption in crop plants will substantially advance our ability to associate specific genes with traits.

Table II. *Inoculation of soybean plants by direct plasmid DNA rubbing*

Soybean plants were maintained in a growth chamber at 20°C. BPMV RNA1 plasmid was pBPMV-IA-R1M. A 20-μL plasmid DNA mix containing 1 μg of RNA1 and RNA2 each was used to inoculate one primary leaf of soybean seedlings. Numbers represent the number of infected plants/total number of plants inoculated.

RNA2 Plasmid	Regular Maintenance[a]	24-h Dark Treatment[b]
pBPMV-P19	1/12	7/12
pBPMV-PDS-3R	0/12	6/12

[a]Soybean plants were maintained with a photoperiod of 16 h before inoculation. [b]Soybean plants were maintained for 24 h in dark before inoculation.

MATERIALS AND METHODS

Plants, Virus Strains, Inoculation, and BPMV Virus Detection

The BPMV isolate IA-Di1 was isolated in Iowa in 2006 (Bradshaw et al., 2007) and maintained in the soybean (*Glycine max*) cv Williams (Zhang et al., 2009), which was used in all experiments in this study. Common bean (*Phaseolus vulgaris*) cv Black Valentine (Wickizer and Gergerich, 2007) seeds and soybean seeds used in the study were harvested from greenhouse-grown plants previously indexed for the absence of BPMV and SMV. BPMV viral infections were verified by double antibody sandwich-ELISA (Bradshaw et al., 2007). Mechanical inoculation of plants dusted with 600-mesh Carborundum was done using 50 mM potassium phosphate buffer, pH 7.0, for both leaf sap and direct DNA rubbing.

DNA-based BPMV constructs were biolistically introduced into the primary leaves following methods described by Zhang et al. (2009). Following bombardment or mechanical inoculation, plants were maintained in the greenhouse or growth chamber at 20°C with a photoperiod of 16 h.

Infectious BPMV IA-Di1 RNA1 and RNA2 Constructs

Unless otherwise stated, all plasmids were propagated in ElectroMax DH5α-E cells (Invitrogen) and purified using the QiaPrep Spin MiniPrep kit (Qiagen). All PCR was performed using Takara PrimeSTAR HS DNA Polymerase (TaKaRa Bio). Nucleotide sequencing was done using the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems) and the ABI Prism 310 genetic analyzer. Sequence analysis was performed using the Vector NTI program (Invitrogen).

Total RNA was extracted from BPMV-infected soybean leaves by the Trizol method (Invitrogen). First-strand cDNA was synthesized using 0.5 μg of mRNA, 0.5 μg of oligo(dT)$_{20}$ primer, 1 μL of 10 mM deoxyribonucleotide triphosphate, and SuperScript III reverse transcriptase (Invitrogen) to a final volume of 20 μL. A 2-μL aliquot of first-strand cDNA RT product was used as template in a 100-μL PCR for amplification of full-length RNA1 with primer pair BP-5endF and R1-3Cla. The PCR conditions were as follows: (1) 1 min of denaturing at 98°C followed by three cycles of denaturing at 98°C for 10 s, annealing at 40°C for 12 s, and extending at 68°C for 6.5 min; (2) 30 cycles of denaturing at 98°C for 10 s, annealing at 52°C for 12 s, and extending at 68°C for 6.5 min; and (3) an extra 10 min of extending at 68°C. The 6-kb PCR product was gel extracted, treated with T4 DNA kinase, and ligated into *Stu*I-digested and dephosphorylated pGEM-35S-M1 (Zhang et al., 2009) vector to generate construct pBPMV-IA-R1. Clones were screened by PCR with primer pair R1-5708F and Nos-Rev for correct insertion direction. Insertion orientation of pBPMV-IA-R1 was further confirmed by sequencing with primer 35-Seq (Table I), and the entire genomic BPMV RNA1 insertions were sequenced with the primers for RNA1 listed in Table I. The RNA1 full sequence of BPMV isolate IA-Di1 was deposited in GenBank with accession number GU562879.

Following the same procedure as described for RNA1, the full-length BPMV RNA2 was amplified from the above cDNA template using the primer pair BP-5endF and R2-3Cla (Table I). The 3.6-kb PCR product was gel extracted, treated with T4 DNA kinase, and ligated into *Stu*I-digested pGEM-35S-M1 to generate construct pBPMV-IA-R2. Clones were screened by PCR with primer pair R2-3303F and Nos-Rev for correct insertion direction (Table I). Clone pBPMV-IA-R2 was fully sequenced with the primers for RNA2 listed in Table I. Clone pBPMV-IA-R2 was fully sequenced with the primers for RNA2 listed in Table I, and the sequence was deposited in GenBank with accession number GU562880.

Modification of BPMV RNA1 to Provide a Vector with Moderate Symptoms

pBPMV-IA-R1 was selected using overlapping PCR for further BPMV RNA1 modification. The first PCR was performed with pBPMV-IA-R1 as template and primer pair R1-235F and R1-Modi-R. The second PCR was performed with pBPMV-IA-R1 as template and primer pair R1-Modi-F and R1-3344R. The third PCR was performed using PCR products of the previous two reactions as template and primer pair R1-235F and R1-Modi-R. Products of the third PCR were digested with *Swa*I and *Msc*I and ligated into similarly treated pBPMV-IA-R1 to yield pBPMV-IA-R1M. Insertion orientation was confirmed by PCR with primer pair R1-235F and R1-2245R. pBPMV-IA-R1M was sequenced using the primers listed in Table I.

BPMV RNA2 Vector Construction and Modification

BPMV RNA2 was selected for foreign gene insertion. The same reagents were used in BPMV vector modification as were used for generating infectious

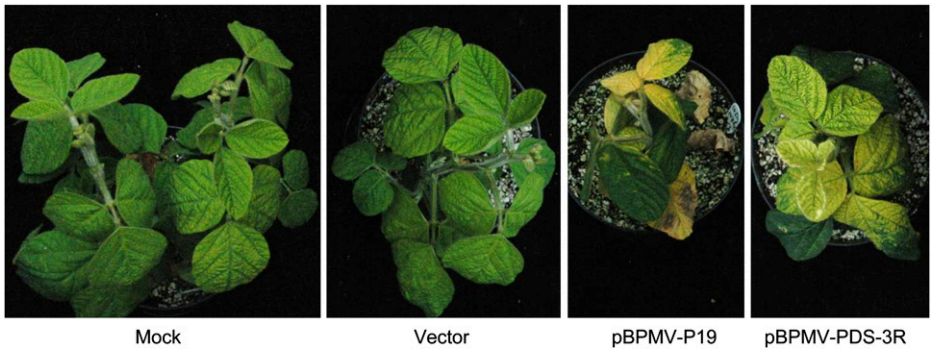

Figure 9. Symptoms induced by direct plasmid DNA rubbing. pBPMV-IA-R1M was used as the RNA1 clone for all inoculations. Plants were photographed at 4 weeks post inoculation. Mock indicates the mock-inoculated control, and Vector indicates the empty vector control inoculated with pBPMV-IA-R2. pBPMV-P19 induced necrosis due to expression of the TBSV P19 gene, a strong RNA-silencing suppressor. pBPMV-PDS-3R induced the PDS photobleaching phenotype. Soybean plants were maintained in the dark for 24 h before DNA rub inoculation. All plants were maintained in a growth chamber at 20°C with a photoperiod of 16 h. [See online article for color version of this figure.]

Table III. *Primers used for vector modification and foreign gene cloning*

Primer Name	Primer Sequence (5′ → 3′)
R2-For1	TGCATGAGGATCCTGATCTGGAATTTGTGT
R2-Rev1	CCAGATCAGGATCCTCATGCAGAGGATTCCGCA
R2-For2	TCTGGATCCGCGTCGACTCCAGGCCTCGA
R2-Rev2	GGAAGATCTCTCGAGGCCTGGAGTCGAC
R2-For3	TTGAGTCCAACCCTGGGCCCGGATCCTGATCTGGAATTTGTGTTT
R2-Rev3	CCAGGGTTGGACTCAACGTCACCTGCTAACTTAAGTAGGTCAAAGT
R2-For4	TCTGCAGCGCCTGCAAAACAGCTCTTAAACTTTGACCTACTTAAG
R2-Rev4	TGCAGGCGCTGCAGAGGATTCCGCATTTT
R2-For5	CCCGTCGACCCCGGGGCCCCAGCTAAGCAATTGCTGAATTTCGAT CTCTTGAAACTGGCTGGAGATGTAGAATCAAATCCAGGCCCGATG GAAACAAATTTGTTTAAATTGT
R2-Rev5	ACAATGAGGTTCAGGCTCAGATGGAGACCAACCTCTTCAAGCTCAGC TTGGACGACGTAGAGACACCAAAGGGAAGCCTCGAGCCCGTCGA CCCCGGG
GFP-Bam-For	CCTTGGATCCATGAGTAAAGGAGAAGAACTTTTCA
GFP-Bam-Rev	TCCGGATCCTTATTTGTATAGTTCATCCATGCCA
GFP-Xho	CCGCTCGAGATGAGTAAAGGAGAAGAACTTT
GFP-Sma	TCCCCCGGGTTTGTATAGTTCATCCATGCCA
TBSV-P19-Xho	CCTCGAGATGGAACGAGCTATACAAGG
TBSV-P19-Sma	TCCCCCGGGCTCGCTTTCTTTTTCGAAGGT
BAR-Xho	CCGCTCGAGAGCCCAGAACGACGCC
BAR-Sma	TCCCCCGGGGATCTCGGTGACGGGCA
GFP-modi-1F	ACGCGTCGACGGATCCATGAGTAAAGGAGAAGAACTTT
GFP-modi-1R	AGGCGCGGATCCTTTGTATAGTTCATCCATGCCA
GFP-modi-2F	CTATACAAAGGATCCGCGCCTGCAAAACAGCTCT
GFP-modi-2R	CCGCTCGAGGGGCCCAGGGTTGGACTCAACGT
Gm-PDS-1F	CGCGGATCCCGTGGTGCTTTCACCACTGCT
Gm-PDS-1R	CGCGGATCCCACTTTGAAAGTAGATTTGGGA
Gm-PDS-2F	CGCGGATCCGCCGCTTGTGGCTATATAT
Gm-PDS-2R	CGCGGATCCTCCTGCACCGGCAATAACGA
Gm-PDS-3F	CGCGGATCCGCAAGGAATATTATAGCCCAAA
Gm-PDS-3R	CGCGGATCCCAGAAAGAACAGCGCCTTCCA
Gm-PDS-4F	CGCGGATCCCAAGAATTGAAAGAGTCATGGT
Gm-PDS-4R	CGCGGATCCCCAATACAAACATTGATCCAGA
Gm-PDS-1130F	CGCGGATCCTGGATGGCAATCCACCCGA
Gm-PDS-1520R	CGCGGATCCTTTGGGCTATAATATTCCTTGC

BPMV RNA1 and RNA2 clones. All PCRs was performed with the primers listed in Tables I and III.

A set of overlapping PCRs was used to introduce the *Bam*HI restriction site after the stop codon of BPMV RNA2 polyprotein coding sequence. PCR A was performed with pBPMV-IA-R2 as template and primer pair R2-2133F and R2-Rev1. PCR B was performed using pBPMV-IA-R2 as template and overlapping primer pair R2-For1 and Nos-Rev. PCR C was performed with PCR products of A and B as template and primer pair R2-2133F and Nos-Rev. The product of PCR C was digested with *Bsr*GI and *Cla*I and ligated into similarly digested pBPMV-IA-R2 to yield pBPMV-IA-V1 (Fig. 1B). Self-annealing primer pair R2-For2 and R2-Rev2 was used for PCR without template, and the PCR product was digested with *Bgl*II and *Bam*HI. The digested PCR product was gel purified and ligated to *Bam*HI-digested and dephosphorylated pBPMV-IA-V1. Insertion orientation was confirmed by PCR with R2-For2 and Nos-Rev, and the clone is designated as pBPMV-IA-V2 (Fig. 1B).

A set of overlapping PCRs was used to introduce the FMDV 2A proteinase sequence after the small coat protein subunit gene for foreign gene expression. PCR D was performed with pBPMV-IA-R2 as template and primer pair R2-For3 and Nos-Rev. PCR E was performed with overlapping primer pair R2-For4 and R2-Rev3. PCR F was performed with pBPMV-IA-R2 as template and primer pair R2-2133F and R2-Rev4. PCR G was performed with PCR products of D and E as template and primer pair R2-For4 and Nos-Rev. PCR H was performed with PCR products of F and G as template and primer pair R2-2133F and Nos-Rev. The product of PCR H was digested with *Bsr*GI and *Cla*I and ligated into similarly digested pBPMV-IA-R2 to yield pBPMV-IA-M1 (Fig. 1B). A FMDV 2A proteinase site was introduced between the MP and L-CP genes using a similar overlapping PCR method. PCR I was performed with pBPMV-IA-M1 as template and primer pair R2-1548F and R2-Rev5. PCR J was performed with pBPMV-IA-M1 as template and primer pair R2-For5 and R2-2688R. PCR K was performed with PCR products of I and J as template and primer pair R2-1548F and R2-2688R. The product of PCR K was digested with *Xba*I and *Bsr*GI and ligated into similarly digested pBPMV-IA-R2 to yield pBPMV-IA-V3 (Fig. 1B). The product of PCR K was digested with *Xba*I and *Bsr*GI and ligated into similarly digested pBPMV-IA-V1 to yield pBPMV-IA-V4 (Fig. 1B).

CaMV 35S Promoter-Driven BPMV Viral Gene Expression and VIGS Constructs

SMV-N-GFP (Wang et al., 2006) was used as template with primer pair GFP-BamF and GFP-BamR for a PCR. The PCR product was digested with *Bam*HI and inserted into *Bam*HI-digested and dephosphorylated pBPMV-IA-M1 to yield construct pBPMV-M1-GFP. Insertion orientation was confirmed by sequencing with primer R2-3303F (Table I). pBPMV-M1-GFP was used as template with primer pairs GFP-Xho and GFP-Sma for PCR. The products were digested with *Xho*I and *Sma*I and inserted into similarly treated pBPMV-IA-V3 to create constructs pBPMV-GFP1 for GFP expression. The digested full GFP gene PCR product was also put into *Xho*I- and *Sma*I-digested pBPMV-IA-V4 to create pBPMV-GFP2 for GFP expression with insertion site for silencing. TBSV P19 gene was amplified from plasmid PZP-TBSVp19 (Qu et al., 2003) using primer pair TBSV-P19-Xho and TBSV-P19-Sma (Table III), and the PCR product was digested with *Xho*I and *Sma*I and inserted into similarly digested pBPMV-IA-V4 to create pBPMV-P19 for TBSV P19 gene expression with

insertion site for silencing. The herbicide resistance gene BAR was amplified from plasmid pCB302 (Xiang et al., 1999) using primer pair BAR-Xho and BAR-Sma (Table III), and the PCR product was digested with *Xho*I and *Sma*I and inserted into similarly digested pBPMV-IA-V4 to create pBPMV-BAR for BAR gene expression with insertion site for silencing.

For the second FMDV-2A insertion into the BPMV RNA2 for two gene expression, overlapping PCR was used to generate double gene expression. PCR L was performed using pBPMV-M1-GFP as template with primer pair GFP-modi-1F and GFP-modi-1R. PCR M was performed using pBPMV-IA-M1 as template with primer pair GFP-modi-2F and GFP-modi-2R. Overlapping PCR N was performed using PCR products L and M as template with primer pair GFP-modi-1F and GFP-modi-2R. The product of PCR N was digested with *Xho*I and *Sal*I and inserted into *Xho*I-digested pBPMV-BAR to create pBPMV-GFP-BAR. Insertion orientation was confirmed by sequencing with primer R2-1548F. The construct pBPMV-GFP-BAR has the features illustrated for pBPMV-IA-V5 (Fig. 1B).

Total RNA extraction and cDNA synthesis followed the previously described method for generating infectious BPMV clones. The cDNA was used for PCR with primers described below to amplify corresponding soybean *PDS* gene fragments (Table III; Fig. 3A), and the PCR products were digested with *Bam*HI and inserted into similarly digested and dephosphorylated pBPMV-IA-V1 to generate the *PDS* VIGS constructs. The primers Gm-PDS-1F and Gm-PDS-1R were used for generating VIGS constructs pBPMV-PDS-1F (sense insertion) and pBPMV-PDS-1R (antisense insertion). The primers Gm-PDS-2F and Gm-PDS-2R were used for generating VIGS constructs pBPMV-PDS-2F (sense insertion) and pBPMV-PDS-2R (antisense insertion). The primers Gm-PDS-3F and Gm-PDS-3R were used for generating VIGS constructs pBPMV-PDS-3F (sense insertion) and pBPMV-PDS-3R (antisense insertion). The primers Gm-PDS-4F and Gm-PDS-4R were used for generating VIGS constructs pBPMV-PDS-4F (sense insertion) and pBPMV-PDS-4R (antisense insertion). The primers Gm-PDS-1130F and Gm-PDS-1520R were used for generating pBPMV-PDS-5, which was used as template for *PDS* gene probe preparation. All *PDS* gene fragment insertions were confirmed by sequencing with primer R2-3303F. The *PDS* gene fragment insert in construct pBPMV-PDS-3R was released by *Bam*HI digestion and inserted into *Bam*HI-digested and dephosphorylated constructs pBPMV-P19, pBPMV-BAR, and pBPMV-GFP2 to yield pBPMV-P19-PDS, pBPMV-BAR-PDS, and pBPMV-GFP-PDS. Insertion orientation was confirmed by sequencing with primer R2-3303F.

RNA Extraction and Northern Hybridization Analysis

The third and fourth trifoliolates from five plants of each treatment were harvested and pooled together for RNA extraction. Total RNA extraction and reverse transcription were done following methods described by Zhang et al. (2009). For northern hybridization analysis, following RNA extraction, 30 μg of total RNA was resolved on a 1% denaturing agarose gel, and hybridization was performed according to the description by Zhang and Ghabrial (2006). The probe for *PDS* was prepared by PCR with primer pair Gm-PDS-1130F and Gm-PDS-1520R using pBPMV-PDS-5 as template. Probes were labeled using the Prime-a-Gene labeling system (Promega). Northern blot was assessed using ImageQuant version 5.2 (Amersham).

Herbicide Treatment and GFP Imaging

The fully expanded primary leaves of soybean seedlings were inoculated with the BPMV viral gene expression or VIGS constructs. Three weeks later, the infected soybean plants were sprayed with the herbicide Liberty, which contains glufosinate-ammonium as the active ingredient (Aventis CropScience), at a concentration of 0.05% glufosinate-ammonium (w/v) in deionized water. The soybean plants were photographed 3 weeks after herbicide treatment. Foliar GFP expression was examined by visualizing with UV illumination (100-W Blak-Ray longwave UV lamp; UVP), and photographs were taken using a Nikon D70 digital camera fitted with a yellow filter. For root GFP observation, 3 weeks after inoculation, bean roots from each treatment were rinsed and photographed using a Zeiss Stemi SV11 stereoscope (Zeiss). GFP expression was monitored with a Piston GFP filter set (Chroma). Photographs were taken with a Zeiss Axiocam MRc5 digital camera and processed with Zeiss Axiovision software (Zeiss).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GU562879 and GU562880.

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