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Development of *Bean pod mottle virus*-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean

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Abstract

Plant virus-based vectors provide valuable tools for expression of foreign proteins in plants and for gene function studies. None of the presently available virus vectors is suitable for use in soybean. In the present study, we produced *Bean pod mottle virus* (BPMV)-based vectors that are appropriate for gene expression and virus-induced gene silencing (VIGS) in soybean. The genes of interest were inserted into the RNA2-encoded polyprotein open reading frame between the movement protein (MP) and the large coat protein (L-CP) coding regions. Additional proteinase cleavage sites were created to flank the foreign protein by duplicating the MP/L-CP cleavage site. To minimize the chances of homologous recombination and thus insert instability, we took advantage of the genetic code degeneracy and altered the nucleotide sequence of the duplicated regions without affecting amino acid sequences. The recombinant BPMV constructs were stable following several serial passages in soybean and relatively high levels of protein expression were attained. Successful expression of several proteins with different biological activities was demonstrated from the BPMV vector. These included the reporter proteins GFP and DsRed, phosphinothricin acetyltransferase (encoded by the herbicide resistance *bar* gene), and the RNA silencing suppressors encoded by *Tomato bushy stunt virus*, *Turnip crinkle virus*, *Tobacco etch virus*, and *Soybean mosaic virus*. The possible use of BPMV as a VIGS vector to study gene function in soybean was also demonstrated with the phytoene desaturase gene. Our results suggest that the BPMV-based vectors are suitable for expression of foreign proteins in soybean and for functional genomics applications.

Keywords: Soybeans; Comovirus; Suppressors of RNA silencing; Virus-induced gene silencing

Introduction

Plant virus-based vectors for expressing heterologous proteins in plants present promising biotechnological tools to supplement conventional breeding and transgenic technology. Considering the speed with which a virus infection becomes established throughout the plant and the high yield of viral-encoded proteins that accumulate in plants, the use of viral vectors provides an attractive and cost effective means for the overproduction of valuable proteins in plants and for rapid evaluation of new traits.

Several different types of positive sense RNA plant viruses have been developed as vectors for production of recombinant proteins and peptides (Pogue et al., 2002; Scholthof et al., 1996). Depending on the structure of the viruses involved and their genome replication and expression strategies, a number of approaches including gene replacement, gene insertion, epitope presentation, and complementation have been utilized. Plant viral vectors are presently available for recombinant protein expression in a wide range of host plants including *Nicotiana benthamiana*, tobacco, squash, cucumber, wheat, barley, cowpea, *Nicotiana clevelandii*, *Chenopodium quinoa*, and *Arabidopsis* (Allison et al., 1988; Brisson et al., 1984; Choi et al., 2000; Constantin et al., 2004; Dolja et al., 1992; Fernandez-Fernandez et al., 2001; French et al., 1986; Gopinath et al., 2000; Hagiwara et al., 1999; Haupt et al., 2001; Lacomme et al., 2003; Turnage et al., 2002). Even with these advances, there are only a limited number of plant viral vectors that are suitable for systemic expression of foreign proteins in major crops like soybean. Soybean is a main source of oil and high-quality protein worldwide, and there is critical need for tools that allow for rapid evaluation of new traits

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involving expression of valuable proteins that confer disease/pest resistance and/or those that enhance the commercial value of soybean. Here, we describe the development of *Bean pod mottle virus* (BPMV) as a gene vector capable of systemic expression of foreign genes in soybean.

Another important application of plant viral vector systems is in studies on host gene function. With more plant genomic information available, a high throughput tool is required. Virus-induced gene silencing (VIGS) is an exceptional reverse genetics tool that can be employed to generate mutant phenotypes for conveying function to unknown genes. VIGS has many advantages over other methods; it is quick and does not require plant transformation (Burch-Smith et al., 2004). In VIGS systems, viruses are designed to carry partial sequence of known

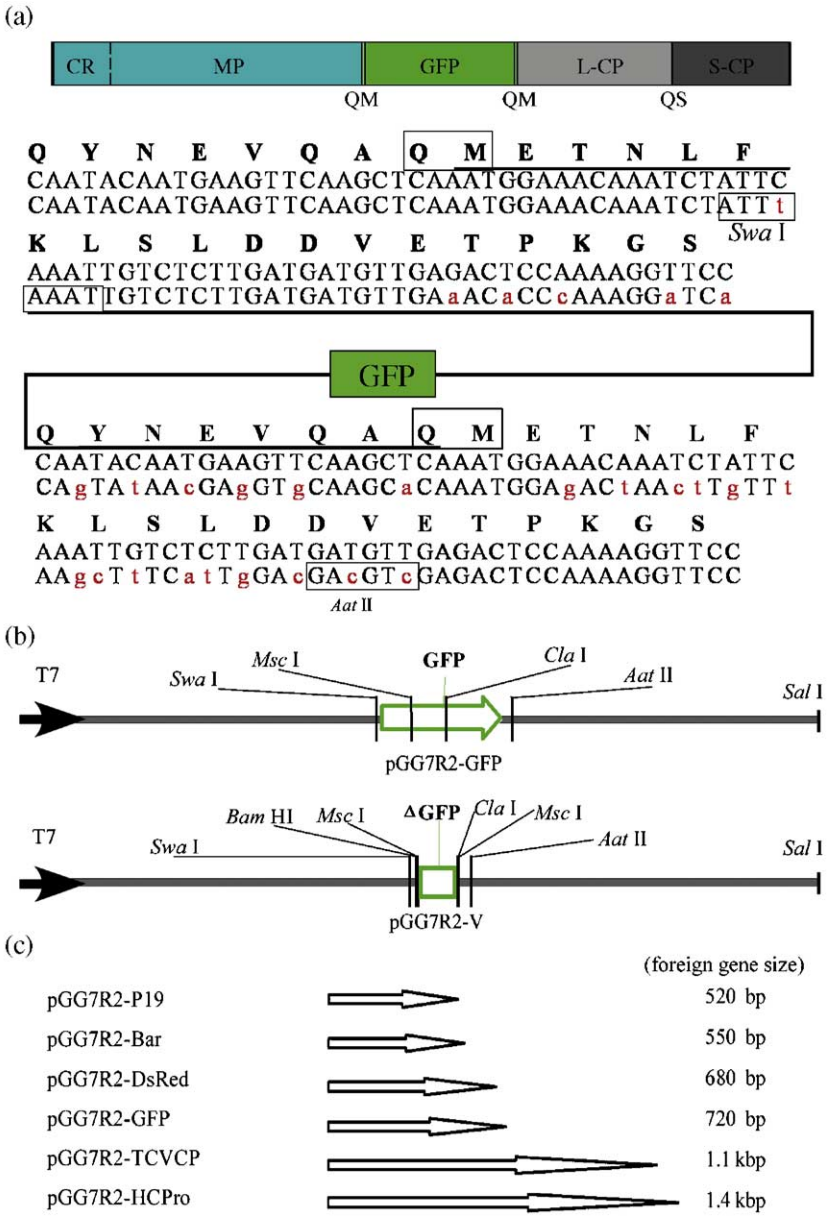

Fig. 1. Schematic representation of BPMV RNA2 vector constructs. (a) Genome organization of BPMV RNA2 and vector construction strategy. RNA2 is translated into two overlapping carboxy coterminal polyproteins. CR, RNA2 replication cofactor; MP, movement protein; L-CP, large coat protein; S-CP, small coat protein. A foreign gene (*GFP*) is inserted between MP and L-CP coding sequences. The cleavage site (QM; boxed) is duplicated with the 8 C-terminal amino acids of the MP and the 19 N-terminal amino acids of the L-CP included for efficient processing. Amino acids, in the one-letter code, are indicated above the nucleotide sequences. Altered nucleotides are printed in red in lowercase. The *GFP* gene is shown as a green box. The introduced restriction sites, *Swa*I and *Aat*II, are boxed. (b) Schematic presentation of BPMV RNA2 vector constructs. (Upper) construct pGG7R2-GFP with GFP inserted between two artificial proteolytic cleavage sites; the designation G7R2 indicates that RNA2 was derived from BPMV strain G7. (Lower) construct pGG7R2-V, which is a modified version of construct pGG7R2-GFP, contains additional restriction sites for cloning of foreign genes. A foreign gene can be cloned as a *Bam*HI–*Msc*I fragment in the pGG7R2-V vector after the vector is digested with same two enzymes. Alternatively, the foreign gene can be blunt-end ligated into *Msc*I-digested pGG7R2-V vector. (c) A diagrammatic representation of the proteins expressed from the BPMV RNA2 vector listed in increasing order of their sizes: P19, *Tomato bushy stunt virus* P19 protein; Bar, phosphinothricin acetyltransferase; DsRed, DsRed red fluorescent protein; GFP, green fluorescent protein; TCVCP, *Turnip crinkle virus* coat protein; HCPro, potyvirus helper component-protease protein.

or candidate genes in order to link their function to the mutant phenotype. Replication of the recombinant virus and generation of dsRNA intermediates trigger the RNA-mediated host defense system resulting in degradation of RNA with sequence identity to the recombinant virus including mRNA of the gene of interest. The targets of VIGS can be a single gene, several members of a gene family, or several distinct genes (Lu et al., 2003a; Peele et al., 2001; Turnage et al., 2002). Currently, many model host plants including *N. benthamiana*, tomato, tobacco, *Arabidopsis*, and cassava have been explored (Burch-Smith et al., 2004). With the current abundance of genomic information on soybean and model legume species (Stacey et al., 2004), it is timely to apply VIGS to soybean to enhance our knowledge of gene function in such a major legume crop. Here, we also successfully show that BPMV can be used as a VIGS vector for studies on gene function in soybean.

BPMV is a member of the genus *Comovirus* in the family *Comoviridae* (Lomonossoff and Ghabrial, 2001). BPMV has a bipartite positive-strand RNA genome consisting of RNA1 (approximately 6.0 kb) and RNA2 (approximately 3.6 kb) that are separately encapsidated in isometric particles 28 nm in diameter. Two distinct subgroups of BPMV strains, designated subgroups I and II, have been previously isolated and extensively characterized (Gu et al., 2002; Gu and Ghabrial, 2005). The BPMV genome is expressed via the synthesis and subsequent proteolytic processing of polyprotein precursors. BPMV RNA-1 codes for five mature proteins required for replication, whereas RNA-2 codes for a putative cell-to-cell movement protein (MP) and the two coat proteins (L-CP and S-CP). In this report, we demonstrate that stable BPMV-based vectors can be generated by inserting the gene of interest into the RNA2-encoded polyprotein open reading frame, between the MP and L-CP coding regions, and constructing additional proteinase cleavage sites to flank the foreign protein.

Results

Construction of BPMV RNA2 vectors

For development of BPMV as a viral vector for expression of heterologous proteins in soybean, the gene of interest was inserted into the RNA2-encoded polyprotein ORF between the MP and the L-CP coding regions. Additional proteinase cleavage sites were created to flank the foreign protein by duplicating the MP-LCP cleavage site (as exemplified by the GFP gene construct in Fig. 1a). The coding sequences for the 8 C-terminal amino acids of the MP and the 19 N-terminal amino acids of the L-CP were included for efficient processing. To minimize the chances of homologous recombination, thus instability, we took advantage of codon degeneracy by changing the third nucleotide in each codon (in accordance with BPMV codon usage) so that the encoded amino acid residues remain unchanged (Fig. 1a). Initially, BPMV recombinant vectors expressing GFP or DsRed were constructed and shown to be infectious and stable. Under greenhouse conditions, the GFP construct was passaged 4 times without any apparent reduction in fluorescence intensity (Fig. 2).

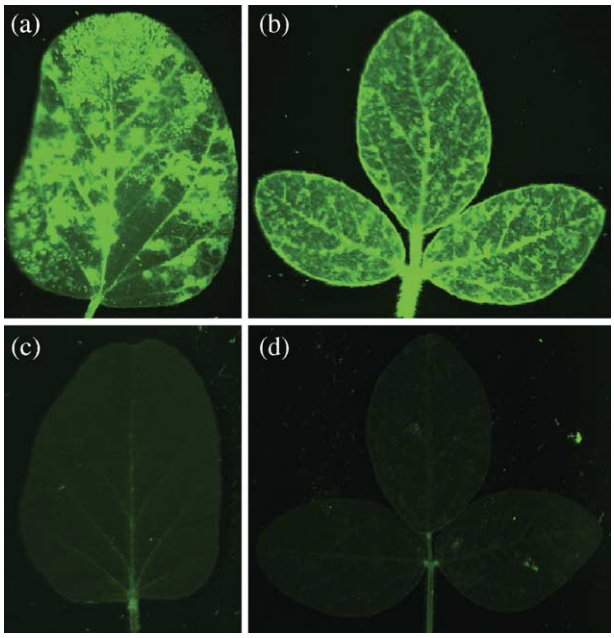

Fig. 2. Green fluorescence on inoculated and systemic leaves of soybean plants. Soybean seedlings were inoculated on their primary leaves with leaf extracts prepared from plants infected with the BPMV-GFP construct after four serial passages in soybean. Alternatively, the primary leaves were inoculated with the wild-type K-Ho1 isolate or mock-inoculated with buffer only. (a) The primary leaf and (b) second trifoliolate leaf from a soybean plant, previously inoculated with the BPMV-GFP construct, showed intense green fluorescence under UV light. No fluorescence was detected on the mock-inoculated primary leaf (c) or on the second trifoliolate of K-Ho1-infected plants (d). Leaves in panels a, b, and d showed symptoms typical of isolate K-Ho1; mosaic and necrosis on inoculated leaves and mottling on systemic leaves. All leaves were photographed under UV light 11 days postinoculation.

The BPMV vector was further modified to include additional cloning sites (Fig. 1b); foreign genes can be cloned by digesting the vector pGG7R2-V with *Bam*HI and *Msc*I (for directional cloning) or by digestion with *Msc*I (for blunt end cloning). Two sets of BPMV RNA2 vectors corresponding to BPMV RNA2 subgroups I and II were constructed.

Several different genes that varied in size and biological activity were cloned into the BPMV RNA2 vectors utilizing the *Bam*HI and *Msc*I restriction sites in the modified vector (Fig. 1b). In all cases, the foreign protein was placed between two artificial cleavage sites with duplication of 27 virus-derived amino acids, for efficient processing, as described for the GFP constructs. These genes ranged in size from 520 bp to 1400 bp (Fig. 1c) and included the herbicide resistance *bar* gene (coding for phosphinothricin acetyltransferase) and several viral-encoded suppressors of host-mediated RNA silencing. These included the P-19 and coat protein (CP) encoded by *Tomato bushy stunt virus* (TBSV) and *Turnip crinkle virus* (TCV), respectively, and the helper component-protease (HC-Pro) encoded by *Soybean mosaic virus* (SMV) and *Tobacco etch virus* (TEV).

Expression levels of foreign genes from BPMV vectors

The recombinant BPMV-GFP constructs were used to evaluate foreign gene expression levels in soybean. The

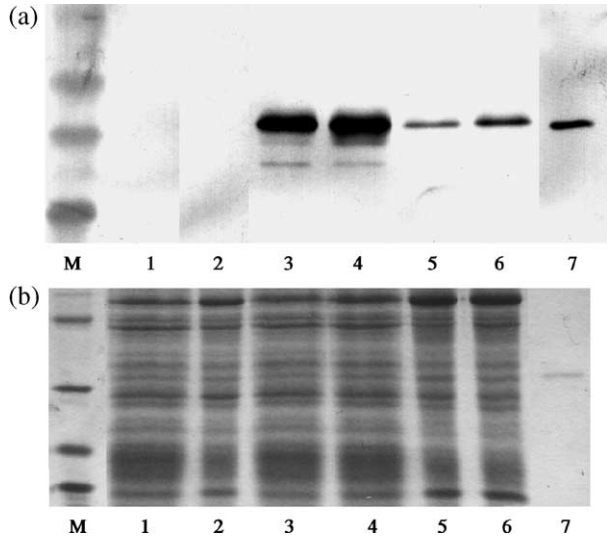

Fig. 3. Immunoblot analysis of total proteins from soybean plants infected with GFP constructs. (a) Western blot analysis using an anti-GFP antiserum. Samples of total proteins (15 μg) extracted from soybean plants subjected to the following treatments were used: mock-inoculated (1st trifoliolate; lane 1), wild-type BPMV K-G7-infected (1st trifoliolate; lane 2), pGG7R2-GFP-infected (1st and 2nd trifoliolate leaves; lanes 3 and 4, respectively), and pGHoR2-GFP-infected (1st and 2nd trifoliolate leaves; lanes 5 and 6, respectively). Purified His_6-tagged GFP protein (50 ng) was included in lane 7. Lane M contains low molecular weight protein markers. (b) Levels of protein loading were assessed by SDS-PAGE analysis and Coomassie blue staining of the proteins tested in panel a.

primary leaves of 7- to 10-day-old soybean seedlings were inoculated with the BPMV-GFP constructs derived from either subgroup I or subgroup II BPMV RNA2. Three weeks postinoculation, total soluble proteins were extracted from the first and second trifoliolate leaves and subjected to Western blot analysis (Fig. 3). Affinity-purified His-tagged GFP, which was expressed in *E. coli*, was used as a control (Fig. 3, lane 7). Interestingly, the expression level provided by subgroup I RNA2 vectors was higher than that obtained with subgroup II RNA2 vectors in both the first and second trifoliolate leaves (compare lanes 3 and 5, Fig. 3). To assess the GFP expression level, the Western blot was scanned, and the generated images of band intensity were analyzed by the ImageQuant v5.2 program (Amersham). The results indicated that GFP expression level can account for as much as approximately 1% of total proteins in soybean.

Stability of the foreign gene expressed from BPMV RNA2 vectors

To assess the stability of inserted foreign genes during serial plant passages, virions were purified from soybean plants previously infected with the BPMV-GFP or BPMV-DsRed constructs. Following three passages of the recombinant BPMV vector, viral RNA was isolated from purified virions and subjected to Northern hybridization analysis (Fig. 4). Only a single band of the predicted size of the recombinant RNA2 containing the coding sequences for GFP or DsRed was resolved. No wild-type RNA2 was detected even following extended overexposure of the blots. Furthermore, fluorescence due to expression of GFP or DsRed was readily detected in the seed coats from immature seeds (data now shown), suggesting that the foreign genes were stably expressed at a later developmental stage during pod formation.

Biological activity of gene products expressed from BPMV RNA2 vectors

Plants infected with the BPMV-bar construct were resistant to ammonium glufosinate when applied as a 0.1% solution (w/v) in deionized water (Fig. 5a). In contrast, the noninoculated control, BPMV K-G7-infected plants, and plants infected with the BPMV-GFP construct were killed within 3 weeks after herbicide treatment (Fig. 5). Furthermore, plants infected with BPMV-bar construct were found to withstand ammonium glufosinate treatment at a concentration of 1% (w/v) in deionized water with little or no damage (data not shown).

It is known that certain RNA silencing suppressors encoded by plant viruses may enhance symptom severity induced by heterologous viruses (Pruss et al., 1997; Yang and Ravelonandro, 2002). Three different viral RNA silencing suppressors (TBSV-P19, TCV-CP, SMV-HC-Pro) with apparently dissimilar underlying mechanisms (Roth et al., 2004) were cloned into the BPMV vector and tested for their activity in soybean. The resulting constructs were infectious, and the infected plants

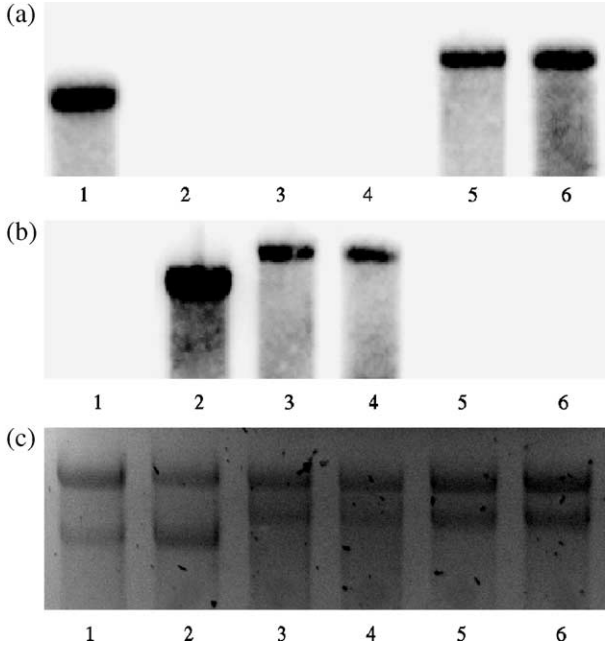

Fig. 4. Stability of the *GFP* and *DsRed* genes expressed from the BPMV vectors. (a and b) Northern blot hybridization analysis to assess the stability of foreign gene inserts. RNA extracted from purified virions from soybean plants previously inoculated with the following virus isolates or transcripts were used: 1, wild-type strain K-Ho1; 2, wild-type strain K-G7; 3, pGHoR1 + pGG7R2-GFP transcripts; 4, pGHoR1 + pGG7R2-DsRed transcripts; 5, pGHoR1 + pGHoR2-GFP transcripts; and 6, pGHoR1 + pGHoR2-DsRed transcripts. In panel a, a probe specific for K-Ho1 RNA2 (type II) was used. In panel b, a probe specific for K-G7 RNA2 (type I) was used. Note that the recombinant RNA2 constructs containing GFP or DsRed (lanes 3–6) are larger in size than those of the wild-type RNA2 (lanes 1 and 2). (c) Levels of RNA loading were assessed by ethidium bromide staining of viral RNA.

Fig. 5. Herbicide resistance in soybean conferred by infection with the BPMV vector expressing the *bar* gene. Soybean seedlings were inoculated onto the primary leaves with either wild-type virus, transcripts from the BPMV-bar construct, transcripts from the BPMV-GFP construct, or mock-inoculated with buffer alone. The herbicide treatment (0.1% amino glufosinate in deionized water) was applied to all plants when the second trifoliolate leaves were fully expanded. Photographs were taken 20 days after the herbicide treatment. Soybean plants infected with: BPMV-bar construct (a); mock-inoculated control (b); wild-type BPMV strain K-G7 (c); and BPMV-GFP construct (d) are shown.

showed very severe symptoms including extensive stunting, leaf deformation, blistering, and veinal necrosis compared with the relatively mild mottling symptoms induced by infections involving wild-type BPMV RNA2 (Fig. 6). The severe symptoms induced by these constructs are reminiscent of the top necrosis syndrome induced by double infection of soybean plants with BPMV and SMV (Anjos et al., 1992).

Silencing of phytoene desaturase in soybean

Virus-induced gene silencing (VIGS) is an attractive tool for studies of gene function. To determine whether a VIGS vector based on BPMV could be useful in silencing of endogenous soybean genes, a 318 bp fragment of the phytoene desaturase (*PDS*) gene, which is necessary for production of carotenoid pigment production, was inserted into the BPMV vector, and the resulting construct was used to infect soybean. Soybean plants inoculated with the recombinant BPMV-PDS developed typical photobleached leaves 2 weeks postinoculation, indicating that the *PDS* gene had been silenced (Fig. 7a). The BPMV-PDS vector was stable when leaf extracts were prepared from the upper leaves and used to inoculate a second set of healthy soybean seedlings. Representative upper leaves harvested at 21 dpi from the second passage plants are shown in Fig. 7. VIGS of the *PDS* gene was clearly evident regardless of the source of RNA1 (whether from the mild strain K-Ha1 or the severe strain K-Ho1) or the soybean cultivar used, Essex, Clark, Williams, or York (data not shown).

Discussion

This study represents the first report to demonstrate that BPMV-based vectors are suitable for efficient expression of heterologous proteins in soybean. The BPMV-RNA2 vector is indeed the only available plant-virus-based vector that is appropriate for expression of foreign proteins in soybean. Although the CPMV-RNA2 vector (Gopinath et al., 2000) could potentially be used as an expression vector in soybean, it is unstable, and CPMV infection induces severe symptoms on soybean (Anjos et al., 1992). Furthermore, soybean is not a natural host for CPMV, and the virus is not believed to be present in the United States (Lomonossoff and Shanks, 1999). Thus, CPMV-based vectors cannot be released in the field for practical applications. The instability of the CPMV-RNA2 vector appears to be related to homologous recombination, which may occur as a consequence of duplication of the cleavage sites that border the inserted foreign protein. In engineering the BPMV-RNA2 vector, we took advantage of the degeneracy of the genetic code and altered the nucleotide sequence of the duplicated regions without affecting amino acid sequence in order to minimize the chances of homologous recombination.

We demonstrated that the BPMV-GFP vector was stable after four serial passages in soybean, and no traces of wild-type virus were detected by Northern hybridization analysis (Fig. 4). The finding that the bright green fluorescence was maintained throughout the soybean plant including the seed coats of immature seeds provides further evidence for the endured stability of the GFP construct. The BPMV-GFP vector was also stable after three serial passages in *Phaseolus vulgaris* cv. Black Velvet (data not shown). In addition to soybean and a few cultivars of bean, the host range of BPMV is very limited and includes only some leguminous weeds (Giesler et al., 2002). Because BPMV does not infect *N. benthamiana* or tobacco, which are known to support the amplification and foreign gene expression of most established plant viral vectors, it is not possible to compare the BPMV-based vectors with others in regard to insert instability. It is known that host factors affect viral RNA replication and recombination and may thus contribute to reported differences in the frequencies of viral RNA recombination among diverse host species (Ahlquist et al., 2003; Desvoyes and Scholthof, 2002; Dzianott and Bujarski, 2004). It is possible that host factors in soybean play a role in the stability of the BPMV-based vectors by suppressing viral RNA recombination.

The level of foreign gene expression, as exemplified by the BPMV-GFP vector, was estimated to account for 1% of total

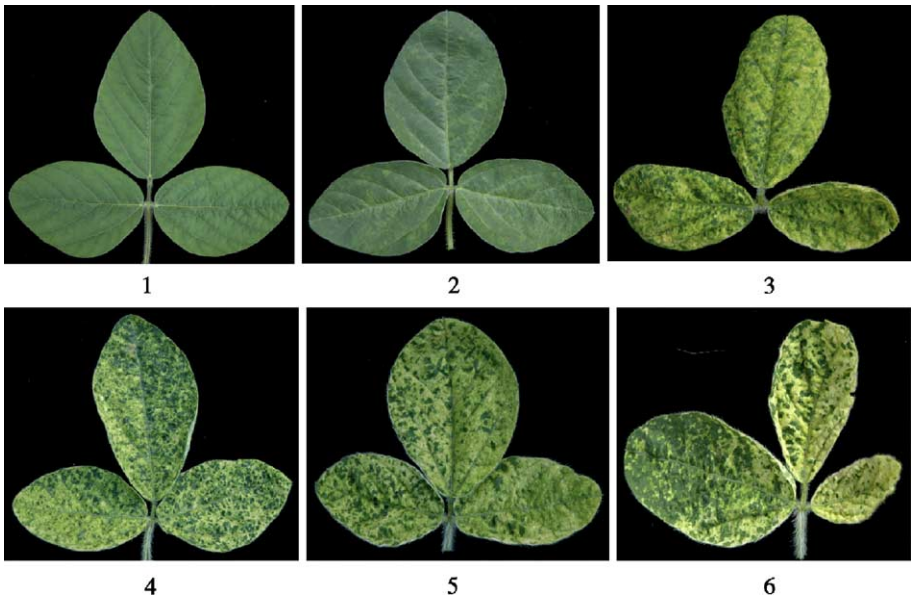

Fig. 6. Enhancement of symptom severity in soybean plants infected with the BPMV vector carrying known viral suppressors of RNA silencing. Photographs of first trifoliolate leaves from soybean plants inoculated with leaf extracts from plants infected with transcripts from pGHoR1 plus transcripts from: pGG7R2 (panel 2); pGG7R2-P19 (panel 3); pGG7R2-TCVCP (panel 4); pGG7R2-HCPro(S7) (panel 5); or pGG7R2-HCPro(T) (panel 6) are shown. A mock-inoculated control plant is shown in panel 1. Note enhanced symptom severity including necrosis on soybean plants infected with BPMV constructs carrying suppressors of RNA silencing (panels 3–6). The photographs were taken 2 weeks postinoculation.

soluble proteins. This level is comparable to that reported for the PVX-based vectors (Culver, 1996). Expression of RNA silencing suppressors from recombinant BPMV vectors showed significant enhancement in symptom severity (Fig. 6) and in the accumulation of the viral coat proteins, as assessed by immunoblot analysis and ELISA for soybean plants infected

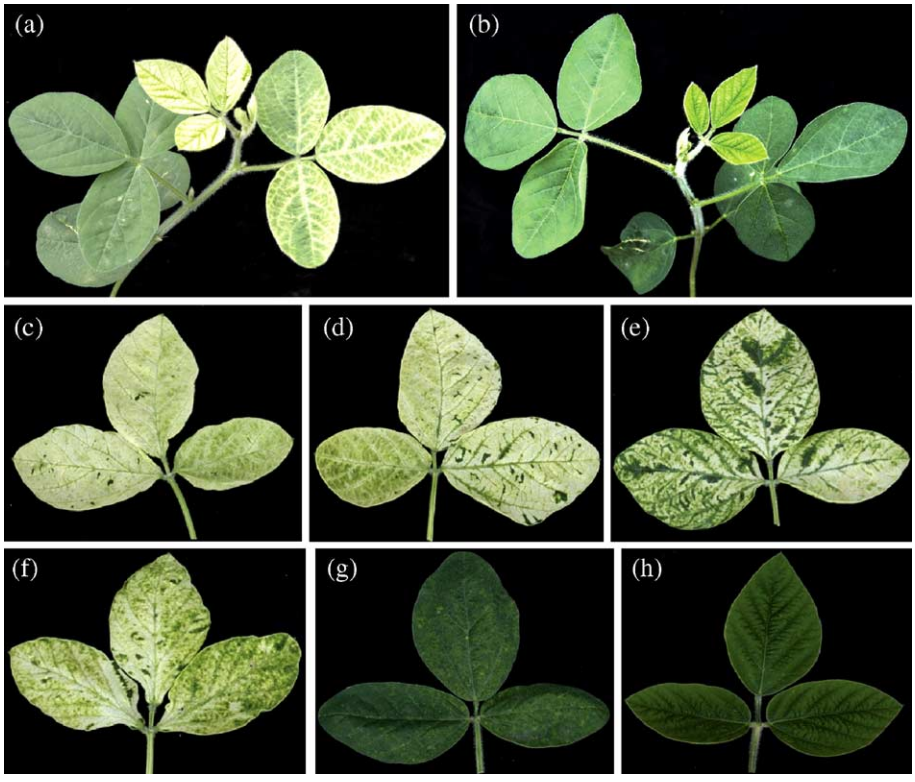

Fig. 7. Virus-induced gene silencing (VIGS) of the soybean *PDS* gene. (a, b) Phenotypes of soybean plants 21 days postinoculation with the BPMV vector carrying a fragment of the soybean *PDS* gene (pGG7R2-PDS) and empty vector control (pGG7R2), respectively. (c–f) Representative 3rd trifoliolate leaves from soybean plants previously inoculated with the pGG7R2-PDS vector showing different degrees of photobleaching are shown. (g) A soybean plant previously inoculated with the vector control pGG7R2 showing typical mottling symptoms and no bleaching. (h) A mock-inoculated soybean plant. The photographs were taken 21 days postinoculation.

with the BPMV-HC-Pro construct (Zhang and Ghabrial, unpublished). The enhancement in symptom severity and BPMV accumulation in soybean plants infected with BPMV-HC-Pro construct is reminiscent of dual infection of soybean with BPMV and SMV (Anjos et al., 1992) and is consistent with the idea that BPMV does not effectively suppress RNA silencing (Gu and Ghabrial, 2005). Expression of RNA silencing suppressors in combination with recombinant BPMV vectors may be useful for enhancing foreign protein expression levels (Mallory et al., 2002; Voinnet et al., 2003). Although expression of more than one product (suppressor plus the proteins of interest) from the same BPMV vector is theoretically feasible (upper limit for insert size is 2.4 kbp), stability might be a concern because of the additional duplicated cleavage sites. Thus, expression of RNA silencing suppressors from co-infecting recombinant BPMV vector is probably a better approach.

Soybean is the top oilseed crop in the world and provides an extremely valuable, multi-billion dollar, source of high quality protein. It is highly desirable to increase the level of soybean resistance to environmental stress, targeted pests, and diseases in commercial varieties. The availability of the BPMV expression vector will allow rapid evaluation of candidate proteins with antifungal or insecticidal activities as well as other valuable proteins that may enhance the commercial value of soybean. The potential advantages that make BPMV an attractive vector system are that the virus (including mild strains) multiplies to high levels in soybean (20–50 mg virus from 100 g leaf tissue; Ghabrial, unpublished) and that it is stable and easily purified. For inoculation purposes under greenhouse conditions, we successfully used purified recombinant BPMV virions or extracts from fresh or dried leaves from plants previously infected with the recombinant vector. Although agroinoculation via leaf infiltration is known to provide the most efficient means for introducing cDNA-derived viral RNA into the leaves of some plant species (Lu et al., 2003b), soybean leaves are difficult to infiltrate, and no alternative conventional methods are presently available for soybean agroinoculation.

We have also demonstrated that the BPMV-based vector is suitable for use as a VIGS vector to study gene function in soybean. The bleached silencing phenotype of soybean plants inoculated with BPMV vector carrying a fragment of the soybean *PDS* gene was stable overtime as it continued to develop throughout the duration of the experiment (35 dpi). VIGS has proved to provide an impressive means to study gene function and has also been demonstrated to be particularly useful in plants with genetic redundancy like soybean (Lawrence and Pikaard, 2003). The most widely used VIGS vectors are based on PVX or TRV (Liu et al., 2002; Lu et al., 2003a), and their applications have been mainly studied in *N. benthamiana* where VIGS response is generally stronger and more enduring than in other plants (Lu et al., 2003a). Recently, efficient VIGS systems have also been developed for a few additional host plants including barley, tomato, and *Pisum sativum* (Constantin et al., 2004; Holzberg et al., 2002; Liu et al., 2002). There is presently an urgent need for a VIGS vector suitable for use in soybean considering the substantial wealth of available information on soybean genomics. None of the currently established VIGS vectors is appropriate for use in soybean. Although full-length cDNA infectious clones are available for the potyviruses SMV and *Clover yellow vein virus* that can infect soybean (Hajimorad et al., 2003; Masuta et al., 2000), neither has been evaluated as a VIGS vector. Potyviruses are unlikely to provide efficient VIGS vectors because they encode potent suppressors of RNA silencing (HC-Pro proteins). HC-Pro has been shown to suppress both VIGS and transgene-induced RNA silencing (Anandalakshmi et al., 1998; Roth et al., 2004).

Our results with the recombinant BPMV-PDS indicated that the BPMV-based VIGS vector induced efficient and reliable gene silencing in soybean. This represents the first report providing experimental evidence that the RNA silencing machinery is operational in soybean. RNA silencing (also known as posttranscriptional gene silencing or PTGS) is implicated in the synergistic interaction between BPMV and SMV in dually infected soybean plants that results in enhanced symptom severity and accumulation of BPMV (Anjos et al., 1992). This synergy is caused by SMV HC-Pro-mediated suppression of RNA silencing, as was clearly demonstrated by inoculation of soybean with the recombinant BPMV-HC-Pro construct (this study; Zhang and Ghabrial, unpublished). BPMV does not appear to code for any suppressors of RNA silencing. In a recent study, using an *Agrobacterium*-mediated transient expression system, we were unable to demonstrate suppression of GFP-RNA silencing in transgenic *N. benthamiana* (line 16c) infiltrated with any of the recombinant agrobacteria carrying BPMV coding regions for the primary or secondary polyprotein precursors or for any of the individual mature proteins (Gu and Ghabrial, 2005). In apparent contrast, the small coat protein (S-CP) of the related CPMV was reported to function as a weak suppressor of amplicon-induced silencing in *N. benthamiana* (Liu et al., 2004). Similar experiments cannot be carried out with BPMV because it does not infect *N. benthamiana*. Assuming that BPMV S-CP, like that of CPMV, functions as a weak suppressor of RNA silencing, such activity has little or no effect on the RNA silencing system of soybean, as judged by our results of the BPMV-PDS experiments.

There are presently available more than 300,000 expressed sequence tags (ESTs) that are derived from over 80 different cDNA libraries representing a wide range of soybean organs, developmental stages, genotypes, and environmental conditions (Stacey et al., 2004). This soybean EST collection provides a large resource of publicly available genes and gene sequences that can potentially provide valuable insight into structure and function of this model crop legume. VIGS would present an ideal tool for large-scale functional genomics to convert the soybean sequence information into functional information. Our results suggest that the BPMV-based vector is suited for this purpose. A possible disadvantage of VIGS is that symptoms induced by virus infection may obscure the phenotype associated with silencing of the

gene of interest. This should not be a problem with the BPMV-soybean system based on current knowledge of symptom severity determinants in BPMV. We have recently mapped BPMV-induced symptom severity to RNA1 and more specifically to the coding regions of the protease cofactor and the C-terminal half of the putative helicase. Furthermore, we identified the amino acid positions that are responsible for differences in symptom severity between mild and severe strains (Gu and Ghabrial, 2005). Since BPMV RNA2 does not play a direct role in symptom severity and since it is the genomic segment that carries the foreign gene of interest, it is then a simple matter to avoid interference from virus symptoms by using RNA1 derived from a mild strain (as demonstrated with the mild strain K-Ha1; this study) or from a modified RNA1 engineered to cause attenuated symptoms combined with enhanced production of the recombinant RNA2.

Materials and methods

Virus strains

BPMV strains K-Ho1, K-Ha1, and K-G7 have been previously described, and their complete nucleotides sequences have been reported (Gu et al., 2002; Gu and Ghabrial, 2005). The BPMV strains were propagated in the soybean cultivar 'Essex', and infected tissues were used for virion purification as previously described (Ghabrial et al., 1977). SMV strains G6 and G7 were used for amplification of the HC-Pro coding regions. SMV strain designation was based on the differential reactions of soybean cultivars carrying resistance genes to SMV (Cho and Goodman, 1979; Gunduz et al., 2004).

RNA extraction and Northern hybridization analysis

Viral RNA was isolated from purified virions by the SDS-phenol method (Peden and Symons, 1973). Total RNA was extracted from plant tissue using a hot phenol method (Verwoerd et al., 1989). For Northern blot hybridization analysis, the RNA samples were denatured in the presence of glyoxal and dimethyl sulfoxide and separated by electrophoresis on a 0.8% agarose gel in 10 mM sodium phosphate buffer, pH 6.3 (Sambrook and Russell, 2001). RNA was transferred onto Hybond-N^+ membranes (Amersham, Piscataway, NJ), according to the manufacturer's instructions. The membranes were then prehybridized, hybridized, and air-dried as previously described (Gu et al., 2002). Full-length RNA1 and RNA2 cDNA clones of strain K-G7 (strain subgroup I) or K-Ha1 (strain subgroup II) were used as templates for probe preparation by the Rediprime II random prime labeling system (Amersham, Piscataway, NJ) according to the manufacturer's instructions. The Northern blots were exposed to a phosphorimager screen, and the images were visualized with a PhosphorImager 445 SI system and analyzed with the ImageQuant 4.1 software program (Amersham).

Construction of BPMV RNA2 vectors

Full-length infectious BPMV RNA2 cDNA clones (pGG7R2 and pGHoR2), derived from subgroup I and II strains, respectively, were used for construction of the BPMV RNA2 vectors. Unless otherwise specified, transcripts derived from plasmid pGHoR1 containing a full-length infectious RNA1 cDNA (type I, RNA1) were used along with transcripts from recombinant plasmids derived from pGG7R2 or pGHoR2 in all

Table 1
List of primers used to construct BPMV vectors

Name	Sequence
F1	TAATACGACTCACTATAGTATTAAAATTTTCATAAGATTTGAAATTTTGATAAACCG
R1	TTCCGCGGCCGCTATGGCCGACGTCGACTTTTTTTTTTTTTTTTT
*Aat*II-For-R2	GGACGTCGAGACTCCAAAAGGTTCCAT
*Swa*I-Rev-R2	AATTTAAATAGATTTGTTTCCATTTGAGC
GFP-For	AATTTAAATTGTCTCTTGATGATGTTGAAACACCCAAAGGATCAATGAGTAAAGGAGAAGAACTTTTCACT
GFP-Rev	GGACGTCGTCCAATGAAAGCTTAAACAAGTTAGTCTCCATTTGTGCTTGCACCTCGTTATATTGTTTGTATAGTTCATCCATGCCATGTG
RFP-For	ATTTAAATTGTCTCTTGATGATGTTGAAACACCCAAAGGATCAATGGCATCCTCTGAAGATGTTATCAAG
RFP-Rev	GACGTCGTCCAATGAAAGCTTAAACAAGTTAGTCTCCATTTGTGCTTGCACCTCGTTATATTGGGCGCCGGTGGAGTGG
VecModi-For1	AATTTAAATTGTCTCTTGATGATGTTGAAACACCC
VecModi-Rev1	TTGGCCAGGATCCTTTGGGTGTTTCAACATCATC
VecModi-For2	ATCGATGGCCACAATATAACGAGGTGCAAGCCCAAATGGAGACC
VecModi-Rev2	GACGTCGTCCAATGAAAGCTTAAACAAGTTGGTCTCCATTTGGG
SMV-Af	GGATCCTCCCAAAATCCTGAAGCTCAGTT
SMV-Ar	ACTGTCAAAGATCCAAAAGAGTC
SMV-Bf	GACTCTTTTGGATCTTTGACAGT
SMV-Br	TCATCCTCTGTTGCACGATATCACCAACTCT
TEV-P2-For	GGATCCAGCGACAAATCAATCTCTGAGGCA
TEV-P2-Rev	GATATCTCCAACATTGTAAGTTTTCATTTCGGA
TBSV-P19-For	CGCGGATCCATGGAACGAGCTATACAAGGA
TBSV-P19-Rev	TGTGTTGGCCACTCGCTTTCTTTTTCGAAGGT
TCV-CP-For	CGCGGATCCATGGAAAATGATCCTAGAGTC
TCV-CP-Rev	ATTGGATATCAATCCTGAGTGCTTGCCATTTTCC
PDS-For	CCGCGGATCCGCCGCTTGTGGCTATATATCT
PDS-Rev	CACAGATATCTCCTGCACCGGCAATAACGAT

inoculations. Plasmids pGHoR1, pGG7R2, and pGHoR2 were described previously (Gu and Ghabrial, 2005).

GFP constructs

The 5′-half of BPMV RNA2 cDNA in plasmids pGG7R2 or pGHoR2 (1830 bp) was amplified by PCR using the primer pair F1 and *Swa*I-Rev-R2 (Table 1), and the PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI). The resultant clones were digested with *Swa*I and *Nco*I, and two clones, pGG7R2-1 and pGHoR2-1, were selected following verification by restriction enzyme digestion and nucleotide sequencing. Clones pGG7R2-1 and pGHoR2-1 were digested with *Aat*II, blunt-ended, and self-ligated to remove the *Aat*II restriction site in the vector and to create the new constructs pGG7R2-2 and pGHoR2-2. The *GFP5* gene was amplified using plasmid pZGFP (Soldevila and Ghabrial, 2000) as a template and the primer pair GFP-For and GFP-Rev (Table 1). The PCR product was cloned into the pGEM-T easy vector, and the resultant clone (pGGFP-1) was verified by sequencing. The pGG7R2-2 and pGHoR2-2 constructs were digested with *Swa*I and *Sal*I and ligated into similarly digested pGGFP-1 to generate constructs pGG7R2-3 and pGHoR2-3, respectively. The 3′-half of BPMV RNA2 cDNA in plasmids pGG7R2 or pGHoR2 (1841 bp) was amplified by PCR using the primer pair *Aat*II-For-R2 and R1 (Table 1), and the PCR products were cloned into the pGEM-T easy vector to generate clones pGG7R2-4 and pGHoR2-4, which were verified by sequencing. Clones pGG7R2-4 and pGHoR2-4 were digested with *Sac*I and *Pst*I, blunt-ended, and self-ligated to remove the vector *Sal*I site and to generate clones pGG7R2-5 and pGHoR2-5, respectively. Finally, clones pGG7R2-5 and pGHoR2-5 were digested with *Aat*II and *Sal*I, and the resultant smaller fragments were isolated and ligated into *Aat*II/*Sal*I-digested pGG7R2-3 and pGHoR2-3, respectively, to produce the infectious constructs pGG7R2-GFP and pGHoR2-GFP.

DsRed constructs

The DsRed gene was amplified by PCR using plasmid pDsRed2-C1 (Clontech, Palo Alto. CA), as a template, and primers RFP-For and RFP-Rev (Table 1). The PCR product was cloned into the pGEM-T easy vector to generate clone pGdsRed-1, which was confirmed by sequencing. The DsRed gene was released from pGdsRed-1 by digestion with *Swa*I and *Aat*II, and the resultant fragment was ligated into plasmids pGG7R2-GFP and pGHoR2-GFP, which were *Swa*I/*Aat*II-digested, to replace the *GFP* gene and generate the infectious constructs pGG7R2-DsRed and pGHoR2-DsRed, respectively.

Vector modification

To generate a suitable BPMV-RNA2 vector for cloning and expression of foreign genes, the GFP construct, pGG7R2-GFP (Fig. 1), was modified to remove most of the GFP sequences and to insert two new restriction sites. To introduce a *Bam*HI restriction site into the BPMV RNA2 vector, primers VecModi-For1 and VecModi-Rev1, which partially anneal to each other, were subjected for PCR, and the product was cloned into the pGEM-T easy vector and confirmed by sequencing (pVecModi-1). A similar approach was used to introduce a second *Msc*I restriction site into the BPMV RNA2 vector; primers VecModi-For2 and VecModi-Rev2 (Table 1), which partially anneal to each other, were subjected to PCR, and the product was cloned into the pGEM-T easy vector and confirmed by sequencing (pVecModi-2). Plasmid pGG7R2-GFP was digested with *Swa*I and *Msc*I, and the resultant larger fragment was isolated and ligated into similarly digested pVecModi-2 to generate plasmid pGG7R2-6. The latter was then digested with *Cla*I and *Aat*II, and the resultant larger fragment was isolated and ligated into similarly digested pVecModi-1 to generate the BPMV-RNA2 vector, designated pGG7R2-V (Fig. 1).

Bar constructs

The bar gene (coding for phosphinothricin acetyltransferase) was released from plasmid pBG-GD (Straubinger et al., 1992) by digestion with *Bgl*II, blunt-ended with Klenow large fragment DNA polymerase (Invitrogen, Carlsbad, CA, USA), and then digested with *Bam*HI. The DNA fragment containing the *bar* gene was gel-purified and ligated into pGG7R2-V, previously digested *Msc*I and *Bam*HI, to produce pGG7R2-Bar.

Constructs of RNA silencing suppressors

TBSV P19 gene was amplified from plasmid PZP-TBSVp19 (Qu et al., 2003) using the primer pair TBSV-P19-For and TBSV-P19-Rev (Table 1), and the resulting PCR product was cloned into the pGEM-T easy vector. Clones in the correct orientation were selected and digested with *Bam*HI and *Msc*I, and the released P19 gene was cloned into *Bam*HI/*Msc*I-digested pGG7R2-V to produce pGG7R2-P19. TCV coat protein (CP) gene was amplified from plasmid PZP-TCVCP (Qu et al., 2003) using primers TCV-CP-For and TCV-CP-Rev (Table 1), and the resultant PCR product was cloned into the pGEM-T easy vector. Clones in the correct orientation were selected and digested with *Bam*HI and *Eco*RV, and the released CP gene was cloned into *Bam*HI/*Msc*I-digested pGG7R2-V to produce pGG7R2-TCP. The coding region of TEV HC-Pro was amplified by PCR using plasmid pTEV7D, which contains a full-length cDNA of TEV-RNA (Dolja et al., 1992), as a template along with primers TEV-P2-For and TEV-P2-Rev (Table 1). The resultant PCR product was cloned into the pGEM-T easy vector, and clones in the correct orientation were digested with *Bam*HI and *Eco*RV. The released HC-Pro gene was then cloned into *Bam*HI/*Msc*I-digested pGG7R2-V to produce pGG7R2-HCPro(T). An RT-PCR approach was used to clone SMV HC-Pro coding region. A reverse primer (SMV-Br; Table 1) was used for first strand cDNA synthesis with total RNA from soybean leaves infected with SMV strains G6 or G7 and a Superscript II reverse transcription kit (Invitrogen). To eliminate a *Bam*HI site in the SMV-HC-Pro coding region without changing the amino acid sequence, a two-step PCR method was used. In the first step, two overlapping cDNA fragments containing the entire HC-Pro sequence (fragments A and B covering the 5′ and 3′ halves, respectively) were PCR-amplified in separate reactions using first strand cDNA as a template and two pairs of primers (SMV-Ar and SMV-Af and

SMV-Br and SMV-Bf). The reverse primer of fragment A (SMV-Ar; 23 nucleotides in length) is complementary to the forward primer of fragment B (SMV-Bf). Equimolar amounts of each fragment along with primers SMV-Br and SMV-Af were used for the second step PCR. The final PCR product was cloned into the pGEM-T easy vector, and clones in the correct orientations were confirmed by sequencing. The inserted HC-Pro genes from strains G6 and G7 were digested with *Bam*HI and *Eco*RV and ligated into *BamH*I/*Msc*I-digested pGG7R2-V to produce pGG7R2-HCPro(S6) and pGG7R2-HCPro(S7), respectively.

PDS constructs

Soybean genomic DNA was extracted from leaves of the soybean cultivar 'Essex', as previously described (Srinivasa et al., 2001). A 318 bp *PDS* fragment was PCR-amplified using the primer pair PDS-sen5-For and PDS-sen5-Rev (Table 1). The PCR product was digested with *Bam*HI and *Eco*RV and ligated into *Bam*HI/*Msc*I-digested pGG7R2-V to generate construct pGG7R2-PDS.

Nucleotide sequencing

All sequencing was done using the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 310 genetic analyzer. Sequence analysis was performed using the DNA strider (CEA, France) and Vector NTI programs (Informax Inc., Frederick, MD, USA).

In vitro transcription and inoculation

Plasmids pGHoR1 (containing full-length cDNA clone to type I RNA1, from strain K-Ho1) and pCRHaR1 (containing full-length cDNA to type II RNA1, from strain K-Ha1) were used as templates for in vitro transcription as previously described (Gu and Ghabrial, 2005). After transcription, 5-μl samples of the reaction mixture were analyzed on a 1% agarose to assess yield and quality of the transcripts. RNA transcripts (a mixture of RNA1 and RNA2 transcripts) were used to inoculate fully expanded primary soybean leaves by rub inoculation.

Protein expression and Western blot analysis

Total protein extraction from soybean leaves was performed as described by Osherov and May (1998). Protein concentration was estimated by the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA, USA). A known amount of purified, bacterially expressed, GFP was used as a standard in assays to assess expression levels of recombinant GFP. For this purpose, the wild-type GFP gene was released from plasmid pIVEX2.3 (Roche Applied Science, Indianapolis, IN, USA) by digestion with *Xba*I and *Bam*HI and cloned into pET21d vector (EMD Biosciences, San Diego, CA, USA). The resulting clone was transformed into *E. coli* strain BL21 (DE3), and GFP expression was induced and purified according to manufacturer's instructions (EMD Biosciences). Western blot analysis was carried out as previously described (Srinivasa et al., 2001) using antisera to BPMV CP and GFP (Chemicon international Inc., Temecula, CA, USA). GFP expression level was assessed using ImageQuant v5.2 (Amersham).

Fluorescence detection

Whole leaf green fluorescence images were acquired using BioChemi-V cooler camera mounted on Epi Chemi II Darkroom (UVP company, Upland, CA, USA). The settings were overhead excitation light 365 nm and filter set as SYBR Green (Hoechst Blue). The Labworks Ver 4.0.0.8. software was used for acquiring images, which were exported as TIFF files.

Herbicide treatment

One-week-old soybean seedlings were inoculated with the recombinant BPMV-bar construct. Two weeks later, the infected soybean plants were sprayed with the herbicide Liberty, which contains glufosinate-ammonium (GA) as the active ingredient (Aventis CropScience, Research Triangle PK, NC, USA), at a concentration of 0.1% GA (w/v) in deionized water. The soybean plants were photographed 3 weeks after herbicide treatment.

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