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Short communication

Temporal and spatial *Bean pod mottle virus*-induced gene silencing in soybean

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SUMMARY

Virus-induced gene silencing (VIGS) is a powerful reverse genetics tool in plant science. In this study, we investigated the temporal and spatial silencing patterns achieved by *Bean pod mottle virus* (BPMV)-based VIGS in soybean using virus constructs targeting green fluorescence protein (GFP). Silencing GFP enabled an in-depth analysis of silencing in soybean tissues over time in a transgenic line constitutively expressing GFP. We discovered evidence for variable GFP silencing based on insert orientation and targeted region in the coding sequence. A 3′ sequence in reverse orientation produced the strongest silencing phenotypes. Furthermore, we documented that BPMV VIGS can achieve widespread silencing in a broad range of tissues, including leaves, stems, flowers and roots. Near-complete silencing was attained in leaves and flowers. Although weaker than in shoots, the observed gene silencing in soybean roots will also allow reverse genetics studies in this tissue. When GFP fluorescence was assayed in cross-sections of stems and leaf petioles, near-complete and uniform silencing was observed in all cell types. Silencing was observed from as early as 2 weeks post-virus inoculation in leaves to 7 weeks post-virus inoculation in flowers, suggesting that this system can induce and maintain silencing for significant durations.

Continued technical advances are making large sequencing projects significantly easier. With the help of such technologies, scientists have successfully sequenced the genomes of crop plants including soybean (Schmutz *et al*., 2010). Subsequent data analysis has resulted in the identification of large numbers of genes. Although the availability of these data offers an unprecedented opportunity for scientists to conduct in-depth studies, assigning functions to the genes identified remains problematic.

Traditionally, forward and reverse genetics are employed to study gene function. Although forward genetics has been extremely powerful, it is time consuming in crop plants, as it requires the generation of mutant populations, screening for mutants with desired phenotypes, and mapping and cloning the genes responsible for the phenotypes (Alonso and Ecker, 2006). The relatively more rapid reverse genetics approaches are becoming increasingly important. In the past decade, reverse genetics strategies, such as T-DNA insertion mutagenesis (Azpiroz-Leehan and Feldmann, 1997), TILLING (targeting induced local lesions in genomes) (Henikoff *et al*., 2004) and RNA interference (McGinnis *et al*., 2005), have been developed. Unfortunately, reverse genetics tools are not uniformly available for economically important crop species, such as soybean. The recalcitrant nature of soybean for genetic transformation hampers the application of conventional reverse genetics approaches. Thus, to perform large-scale, high-throughput genomic studies in soybean, an economically feasible, rapid system that bypasses transformation is required.

Several plant virus-induced gene silencing (VIGS) systems have been designed to address reverse genetics needs in various plants including soybean (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; Nagamatsu *et al*., 2007; Pogue *et al*., 2002; Zhang and Ghabrial, 2006; Zhang *et al*., 2009, 2010). VIGS relies on the sequence-specific degradation of endogenous mRNA. Generally, a partial sequence of a target gene is inserted into the viral genome and the resultant virus is used to infect a host plant. Virus replication triggers plant defence responses that identify and cleave double-stranded RNA (dsRNA), an intermediate product of viral replication. dsRNA cleavage produces short interfering RNA (siRNA) which, in association with the RNA-induced silencing complex (RISC), targets homologous RNA for degradation and silences the targeted endogenous gene (Burch-Smith *et al*., 2004; Purkayastha and Dasgupta, 2009).

VIGS is used to silence genes in various plant tissues, including leaves (Liu *et al*, 2002), roots (Ryu *et al*., 2004), tubers (Faivre-Rampant *et al*., 2004), flowers (Chen *et al*., 2004; Liu *et al*., 2004) and seedlings, and can sometimes persist to progeny (Kanazawa *et al*., 2011a, b; Senthil-Kumar and Mysore, 2011; Yamagishi and Yoshikawa, 2009). To use VIGS to its full potential, an in-depth understanding of temporal and spatial silencing patterns is

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necessary. Here, we report a systematic analysis of VIGS in soybean using a *Bean pod mottle virus* (BPMV) vector (Zhang *et al*., 2010) over time in leaves, flowers, stems and roots.

Hernandez-Garcia *et al*. (2009) have developed a transgenic soybean Jack cultivar that expresses the green fluorescent protein gene (*GFP*) under the control of the *G. max* ubiquitin promoter. Silencing of this ubiquitously expressed transgene can easily be visualized and measured in various organs and tissues, and it has provided an ideal experimental system to generate a silencing atlas over time at the whole-plant level. In addition to being a visible marker, silencing of *GFP* has minimal effects on cellular processes. Here, we report the use of this transgenic line to temporally follow BPMV VIGS in soybean tissues over time. We demonstrate the feasibility of using a BPMV-based VIGS approach for reverse genetics screens in various soybean tissues, and we establish the time parameters for this VIGS system to silence genes in various shoot tissues, as well as in roots. The use of a *GFP* reporter gene allowed the assessment of temporal and spatial silencing patterns, as well as the approximate degree of silencing achieved by BPMV. The silencing of GFP in transgenic soybean plants that express GFP under the control of the *Glycine max* ubiquitin promoter facilitated the localization and quantification of the silencing pattern in various organs and tissues over time.

First, we identified the most effective GFP silencing construct to use in these experiments, because insert size, location of the target region and cloning orientation can affect the silencing potential of VIGS vectors (Bennypaul *et al*., 2011; Liu and Page, 2008; Yuan *et al*., 2011; Zhang *et al*., 2010). We made four constructs that targeted the 5′ or 3′ halves of the *GFP* coding sequence in the sense and antisense orientations using the pBPMV-IA-V1 vector (Zhang *et al*., 2010) (Fig. S1A, see Supporting Information). At 21 days post-inoculation (dpi), GFP fluorescence was reduced in plants inoculated with all four GFP silencing constructs. However, plants inoculated with the 3′-end antisense construct (1102-A) showed the most consistent and dramatic reduction of GFP fluorescence (Fig. S1B) and *GFP* mRNA expression (Fig. S1C).

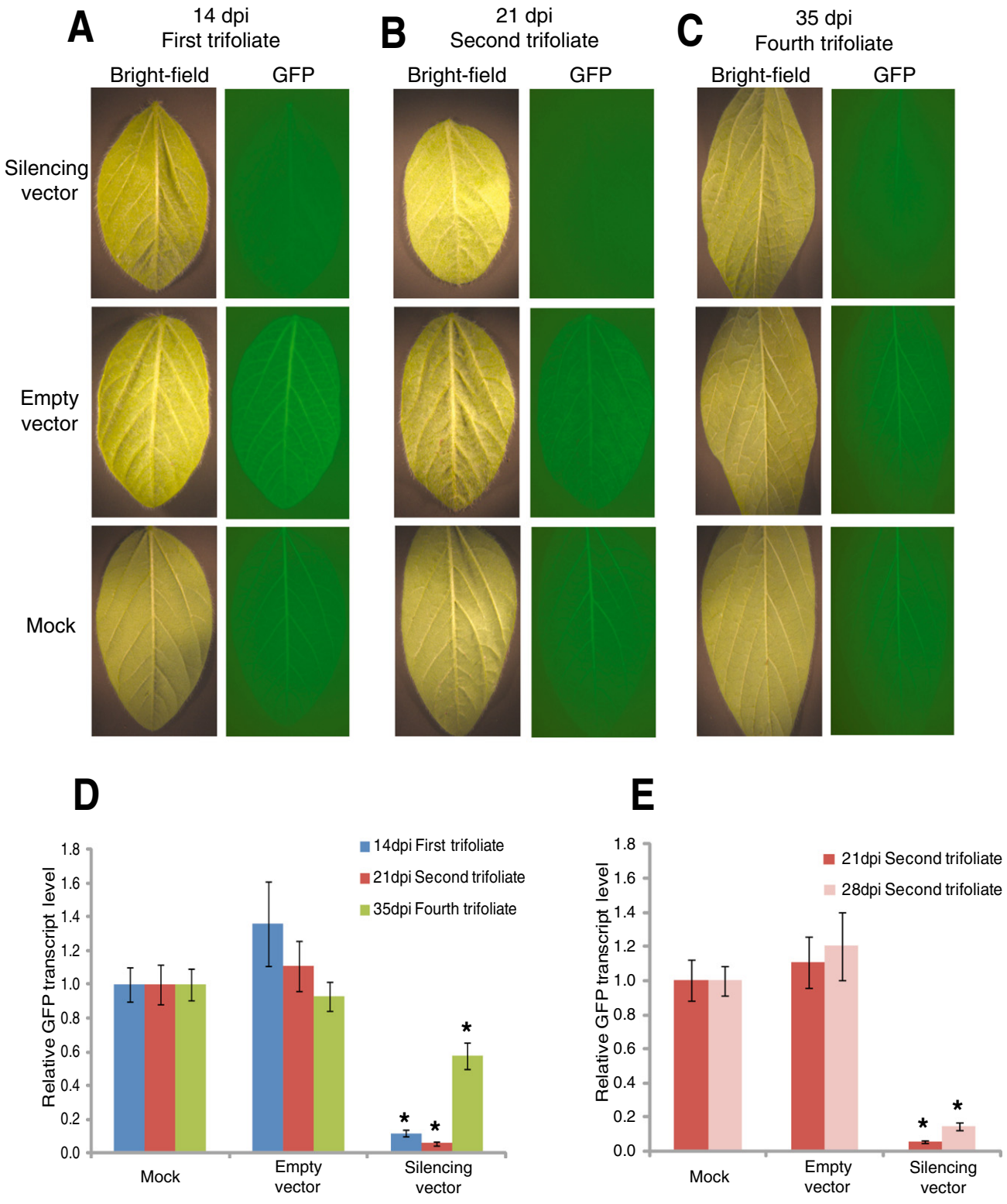
To examine the temporal and spatial GFP silencing patterns induced by BPMV in different soybean tissues, we used construct 1102-A, because it was the most effective. A significant reduction in GFP fluorescence was observed in the leaflets of the first trifoliate as early as 14 dpi (Fig. 1A). Examination of the second and fourth trifoliates at 21 and 35 dpi, respectively, showed significant silencing up to 35 dpi when compared with controls (Fig. 1B,C), documenting a long-lasting VIGS effect. However, the relative levels of GFP mRNA demonstrated that the magnitude of GFP silencing at 35 dpi was not as strong as that at 14 and 21 dpi (Fig. 1D) (Appendix S1, see Supporting Information).

In order to examine the duration of silencing at a particular location, we monitored the GFP fluorescence in one leaflet from the first trifoliate at 14 dpi and in a second leaflet from the same trifoliate at 21 dpi. Similarly, GFP fluorescence was monitored in two leaflets of the second trifoliate at 21 and 28 dpi and in the third trifoliate at 28 and 35 dpi. Although significant silencing was observed in the inoculated plants up to 35 dpi when compared with the controls, no significant differences occurred within the leaflets of the same trifoliate (Fig. S2, see Supporting Information), showing a sustained level of VIGS during the observation period. The GFP observations were supported by quantitative polymerase chain reaction (qPCR) analysis of GFP transcript levels in leaflets of the second trifoliate collected at 21 and 28 dpi (Fig. 1E).

To test silencing in flowers, GFP fluorescence in floral organs from plants inoculated with the silencing vector was compared with that of control treatments. Under our experimental conditions, plants produced their first flowers at 7 weeks post-virus inoculation. At this time, flowers were assayed for GFP fluorescence. After this 7-week period, high levels of silencing were observed in all floral parts, including petals, sepals and reproductive whorls (Fig. 2A,B). The 95% reduction in GFP mRNA levels in the most silenced flowers confirms the silencing efficiency in floral tissues (Fig. 2C). When GFP fluorescence was compared among all flowers collected from each individual plant in the experiment, silencing was observed in all flowers, indicating that silencing in floral tissue was independent of the flower's developmental stage and/or its location on the plant (Fig. S3, see Supporting Information). The fact that BPMV-based VIGS attained high levels of silencing and sustained the silencing for significant durations makes it possible to employ this system to dissect the molecular mechanisms underlying numerous biological phenomena in leaves and flowers.

The duration of silencing in leaves and flowers is dependent in part on the stability of the target gene sequence cloned into the vector. Insertion of GFP into the BPMV RNA2 polyprotein between the movement protein and large subunit of the coat protein has been shown to be stable after four passages (Diaz-Camino *et al*., 2011). However, the stability of inserts cloned into the 3′ end of BPMV after the stop codon has not been investigated systematically. We performed reverse transcription-polymerase chain reaction (RT-PCR) on RNA samples from leaves at 14, 21, 28 and 35 dpi and flowers collected at 49 dpi using two sets of primers. The first primer set included a reverse primer in GFP and a forward primer in the viral genome, and the second primer set included forward and reverse primers in the BPMV genome that flanked the insertion site. The expected PCR products were obtained using these primers in all leaf and flower samples (Fig. S4, see Supporting Information). These data demonstrated that the GFP insert was stable in the BPMV-IA-V1 vector for the tissues and time courses used in the presented studies.

Mosaic patterns of infection are prominent features of virus infection in plants. In addition, viruses may not infect all cell types equally, which may result in sectored silencing or silencing confined to certain regions. For example, a *Tobacco rattle virus* (TRV) vector could not invade meristematic tissue and was excluded from lateral roots (Valentine *et al*., 2002). Our previous results



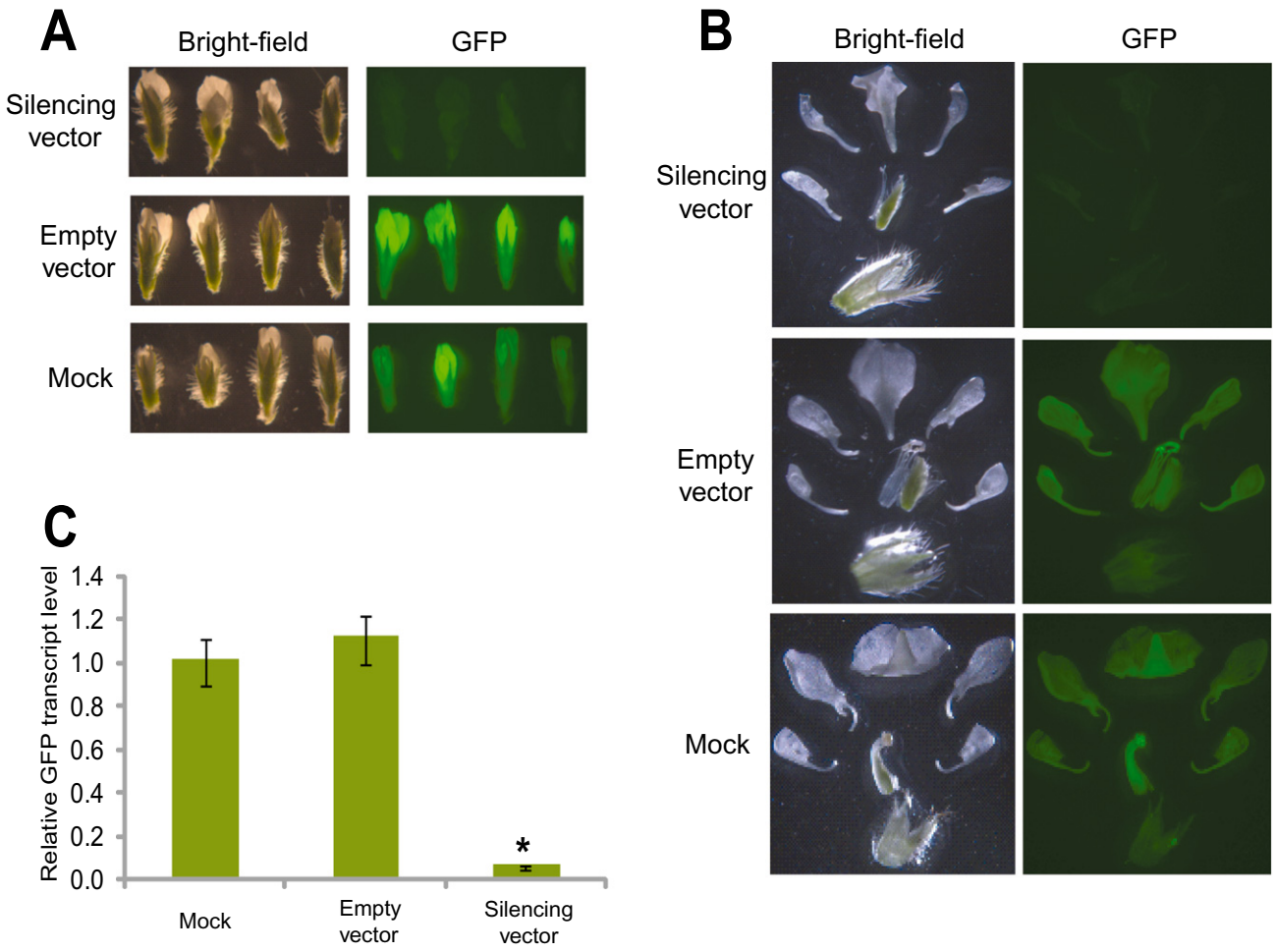


Fig. 2 *Bean pod mottle virus* (BPMV)-induced gene silencing of the green fluorescent protein transgene (*GFP*) in floral tissue. (A) GFP silencing in flowers. Flowers at different developmental stages were collected from transgenic GFP soybean plants treated with silencing vector (top panel), BPMV empty vector (middle panel) or mock (bottom panel) and visualized under white light (left panel) and UV light (right panel) and photographed (1-s exposure on a Zeiss Stemi SV11 stereoscope). Similar results were obtained from three independent experiments. (B) GFP silencing in floral whorls. Hand-dissected flowers were collected from transgenic GFP soybean plants inoculated with silencing vector (top panel), BPMV empty vector (middle panel) or mock (bottom panel) at 7 weeks post-virus inoculation and visualized under white light (left panel) and UV light (right panel) and photographed (0.5-s exposure on a Zeiss Stemi SV11 stereoscope). Note the reduced GFP fluorescence in the flowers and floral whorls collected from the transgenic plants inoculated with silencing construct compared with those obtained from the control treatments. (C) Quantification of gene silencing induced by BPMV in floral tissues using quantitative polymerase chain reaction (qPCR). The mRNA expression levels of *GFP* were measured in RNA samples isolated from floral tissues of the transgenic GFP plants at 7 weeks post-virus inoculation and compared with those treated with empty vector or mock. The expression level was calculated using the $2^{-\Delta\Delta CT}$ method. Data are the average of three biological samples, each with three technical replicates. Mean values significantly different from the mock are indicated by an asterisk as determined by paired *t*-test ($P < 0.01$).

Fig. 1 *Bean pod mottle virus* (BPMV)-induced gene silencing of the green fluorescent protein transgene (*GFP*) in leaves. (A–C) Silencing of GFP in the first trifoliate (A), second trifoliate (B) and fourth trifoliate (C) at 14, 21 and 35 days post-inoculation (dpi), respectively. Leaves inoculated with silencing vector (top panel), BPMV empty vector (middle panel) or mock (bottom panel) were visualized under white light (left panel) and UV light (right panel) and photographed (3-s exposure on a Zeiss Stemi SV11 stereoscope). Similar results were obtained from two independent experiments. (D) Quantification of *GFP* silencing in the first, second and fourth trifoliates at 14, 21 and 35 dpi, respectively. Quantitative polymerase chain reaction (qPCR) was used to quantify the silencing of *GFP* in RNA isolated from the first, second and fourth trifoliates treated with silencing vector, empty vector or mock. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and represent changes in mRNA abundance in BPMV-inoculated plants relative to the mock-treated control. Data are the average of three biological samples, each with three technical replicates. Mean values significantly different from the mock are indicated by an asterisk as determined by paired *t*-test ($P < 0.01$). (E) Quantification of *GFP* silencing in the second trifoliates at 21 and 28 dpi using qPCR. RNA samples were isolated from the second trifoliates treated with the silencing vector, empty vector or mock at the 21- and 28-dpi time points. The expression levels of *GFP* were calculated using the $2^{-\Delta\Delta CT}$ method and represent changes in mRNA abundance in BPMV-inoculated plants relative to the mock-treated control. Data are the average of three biological samples, each with three technical replicates. Mean values significantly different from the mock are indicated by an asterisk as determined by paired *t*-test ($P < 0.01$).

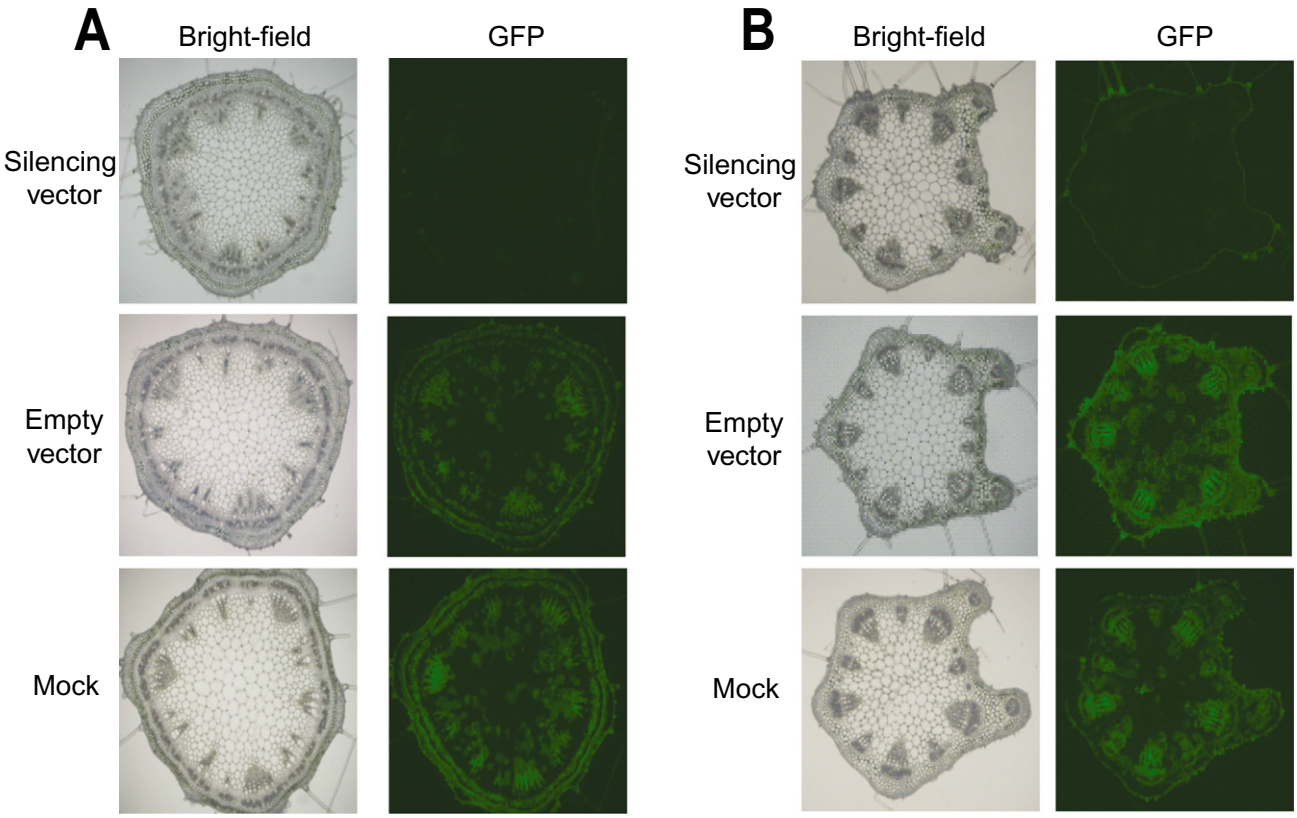


Fig. 3 *Bean pod mottle virus* (BPMV)-induced gene silencing of the green fluorescent protein transgene (*GFP*) in internal tissues. (A, B) GFP silencing in the stem (A) and leaf petiole (B). Cross-sections (thickness, 80 μm) prepared from transgenic GFP soybean plants inoculated with the silencing vector (top panel), BPMV empty vector (middle panel) or mock (bottom panel) were visualized under white light (left panel) and UV light (right panel) and photographed (500-ms exposure on a Zeiss Axiovert 100 microscope). Similar results were obtained from two independent experiments.

using a BPMV construct tagged with GFP that also silenced the phytoene desaturase gene (PDS) indicated that the most extensive silencing occurred in tissues that contained GFP fluorescence and, presumably, BPMV (Zhang *et al*., 2010). This observation was consistent with a mosaic pattern of silencing. However, in the present study, we observed that the silencing of GFP by BPMV VIGS was surprisingly uniform. To verify the uniformity of silencing triggered by the BPMV system, we examined fluorescence in cross-sections of stem regions between the second and third trifoliates and in petioles of the second trifoliates at 21 dpi. Although cross-sections of the mock and control plants showed bright GFP fluorescence in various cell types and tissues, such as vascular tissue, parenchyma, chlorenchyma, pith as well as cortical cells, the cross-sections from plants inoculated with silencing constructs showed almost complete, and, most importantly, uniform and section-wide silencing of GFP (Fig. 3A,B). One potential explanation for the uniformity of GFP silencing is that in tissues that are not directly infected by BPMV, the transgene may be more effectively silenced than endogenous genes.

In general, most applications of VIGS systems, including BPMV, have been directed towards traits associated with the shoots, such as leaves, flowers and fruits. Some vectors, such as *Pea early browning virus* (PEBV), have been shown to be effective at silencing genes in the roots, as well as aerial plant tissues. PEBV VIGS can be used to dissect genetic requirements for symbiotic relationships with *Rhizobium* species and vesicular–arbuscular mycorrhizae (Constantin *et al*., 2008; Grønlund *et al*., 2010). In contrast, studies with other viruses have suggested that VIGS might not be as effective in roots (Dalmay *et al*., 2000; Kaloshian, 2007; Palauqui *et al*., 1997; Saedler and Baldwin, 2004; Sonoda and Nishiguchi, 2000; Valentine *et al*., 2002). Because we have an interest in developing BPMV as a tool to investigate interactions between soybean and soybean cyst nematode, we chose to investigate silencing in the roots. Previous silencing experiments with essential genes, such as actin and ribosomal proteins, have suggested that root growth is inhibited and the accumulation of transcripts is reduced (Zhang *et al*., 2009). However, the dramatic effect of the silencing of these genes on the shoot could have affected the expression of these genes in the root. The use of the *GFP* transgene in our present study overcame these complications, because there was no apparent effect on the shoot from the silencing of GFP. Thus, the silencing responses in the

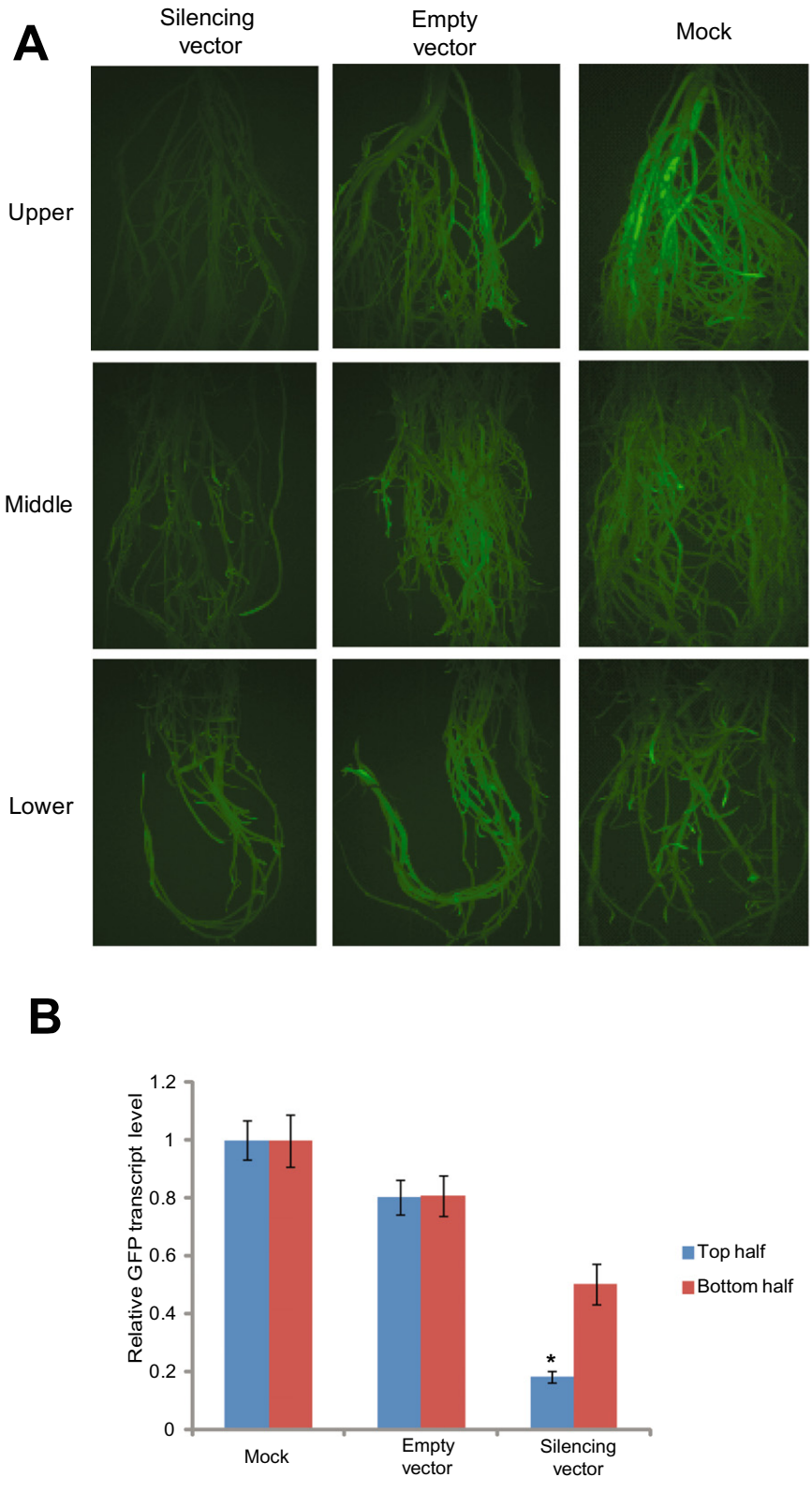


Fig. 4 *Bean pod mottle virus* (BPMV)-induced gene silencing of the green fluorescent protein transgene (*GFP*) in roots. (A) BPMV triggered higher silencing in the upper part of the roots than in the lower root tissues. Root tissues were collected from transgenic GFP soybean plants treated with silencing vector (left panel), BPMV empty vector (middle panel) or mock (right panel) and GFP fluorescence was visualized in the upper, middle and lower parts of the roots under UV light. Note the gradual decrease in GFP fluorescence from the upper to lower region of the root system of the silenced plants (left panel) compared with control treatments (middle and right panels).Similar results were obtained from two independent experiments. (B) Comparison of silencing efficiency induced by BPMV in the upper and lower halves of the roots. Quantitative polymerase chain reaction (qPCR) was used to measure the abundance of *GFP* transcript in RNA samples isolated from the upper and lower halves of the root system. The root tissues were collected from transgenic GFP soybean plants inoculated with BPMV vector, BPMV empty vector or mock at 21 days post-inoculation (dpi). The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and represent changes in mRNA abundance in BPMV-inoculated plants relative to the mock-treated control. Data are the average of three biological samples, each with three technical replicates. Mean values significantly different from the mock are indicated by an asterisk as determined by paired *t*-test ($P < 0.01$).

root were not expected to be coupled necessarily to pleiotropic effects in the shoot. As maximum silencing in foliar tissues was observed between 14 and 28 dpi, root assays were conducted at 21 dpi. When compared with the control, distinct GFP silencing was observed in roots from plants inoculated with the silencing construct. The qPCR analysis confirmed that GFP mRNA was reduced by over 65% (Fig. S5, see Supporting Information).

Closer observation of the GFP fluorescence revealed greater silencing in the upper, more mature, root tissue than in the lower, newly growing, tissues (Fig. 4A). To provide additional evidence for this observation, we cut the root system of treatment and control plants approximately at the midpoint and processed the upper and lower halves separately for RNA extraction and qPCR analysis. Data obtained from three biological replicates showed more extensive gene silencing in the upper root tissues relative to the lower region. More specifically, although GFP silencing in the upper root regions was about 80% of the control treatments, in the lower root region, GFP silencing was calculated to be 50% (Fig. 4B). Significant GFP silencing induced by BPMV in soybean roots demonstrates the potential of this system to accelerate functional and genomics studies of root biology. However, our results from experiments involving BPMV VIGS in soybean roots also show that silencing is not uniform throughout the root system.

Another key question that we were interested in was the timing and duration of BPMV VIGS. Silencing of target genes using VIGS systems can be detected in some cases as early as 3 dpi (Hein *et al*., 2005; Scofield *et al*., 2005). However, between 1 and 2 weeks is generally the time span required to observe efficient silencing using the majority of VIGS systems. In a few cases, 4 weeks post-virus inoculation is required to see efficient silencing (Bennypaul et al., 2011; Turnage *et al*., 2002). Similar to the majority of VIGS systems, we were able to detect significant silencing caused by BPMV VIGS within 2 weeks post-inoculation. Over time the silencing was reduced, but was still significant. An interesting question that remains is whether BPMV VIGS can extend to the seed or, possibly, to the next generation, as has been observed for TRV in *Nicotiana benthamiana* and tomato and *Cucumber mosaic virus* (CMV) in petunia, *N. benthamiana* and tomato (Kanazawa *et al*., 2011b; Senthil-Kumar and Mysore, 2011). Nagamatsu *et al*. (2007) have shown that CMV can induce VIGS of the chalcone synthase gene (*CHS*) in the soybean seed coat, demonstrating that VIGS in soybean seeds is a possibility.

In conclusion, our report details the characteristics of BPMV VIGS in soybean and establishes an atlas of VIGS over a 5-week time course. We have demonstrated that BPMV VIGS is effective in achieving long-lasting, uniform and consistent gene silencing in various tissues, including leaves, flowers and roots. These results can be used to guide the design of experiments that utilize BPMV VIGS in a variety of soybean tissues. This information is critical for extending and maximizing the utility of BPMV VIGS for large-scale genomic studies in soybean and other legumes, such as common bean (Diaz-Camino *et al*., 2011; Zhang *et al*., 2010).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Experimental procedures

Fig. S1 Sequence location and cloning orientation influence silencing potential triggered by *Bean pod mottle virus* (BPMV). (A) Schematic representation of partial green fluorescent protein (GFP) sequences used for transgene silencing. Two GFP fragments targeting the 3′ or 5′ half were cloned in sense or antisense orientation in the BPMV vector and the silencing responses were quantified using quantitative polymerase chain reaction (qPCR). The silencing efficiencies are indicated. Arrows indicate the orientation of the insert. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and represent changes in mRNA abundance in BPMV-inoculated plants relative to the mock-treated control. Data are the average of three biological samples, each with three technical replicates. Mean values significantly different from the mock are indicated by an asterisk as determined by paired *t*-test ($P < 0.01$). (B) Quantification of gene silencing induced by BPMV in leaves using qPCR. The expression levels of *GFP* mRNA were measured in RNA samples isolated from the second trifoliate of the transgenic GFP plants at 21 days post-inoculation (dpi) and compared with those receiving empty vector or mock. The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and represent changes in mRNA abundance in BPMV-inoculated plants relative to the mock-treated control. Data are the average of three biological samples, each with three technical replicates. Mean values significantly different from the mock are indicated by an asterisk as determined by paired *t*-test ($P < 0.01$). (C) Silencing of GFP in the second trifoliate at 21 dpi by the indicated silencing constructs. Mock leaves, leaves inoculated with empty vector and silencing vectors 1101-A, 1101-C, 1102-A and 1102-D are shown. The leaves were visualized under UV light and photographed (3-s exposure on a Zeiss Stemi SV11 stereoscope; Carl Zeiss, Thornwood, NY, USA). Note the strongest reduction in GFP fluorescence in the trifoliates from the plants inoculated with silencing construct 1102-A compared with the other treatments.

Fig. S2 Sustained *Bean pod mottle virus* (BPMV)-induced silencing in foliar tissue. (A, B) Silencing of green fluorescent protein (GFP) in the first trifoliate at 14 days post-inoculation (dpi) (A) and 21 dpi (B). (C, D) Silencing of GFP in the second trifoliate at 21 dpi

(C) and 28 dpi (D). (E, F) Silencing of GFP in the third trifoliate at 28 dpi (E) and 35 dpi (F). In each group, the top panel shows a single leaflet from an individual mock plant and the bottom panel shows a single leaflet from an individual plant inoculated with the silencing construct. Each leaflet was visualized with UV light and photographed (3-s exposure on a Zeiss Stemi SV11 stereoscope). Note the persistent silencing in the leaflets from all three spatial locations for the duration of 7 days.

Fig. S3 *Bean pod mottle virus* (BPMV)-induced silencing of the transgene in flowers. Flowers at different developmental stages from individual transgenic green fluorescent protein (GFP) soybean plants treated with silencing vector (top three panels), BPMV empty vector (middle three panels) or mock (bottom three panels) visualized under white light (left panels) and UV light (right panels) and photographed (1-s exposure on a Zeiss Stemi SV11 stereoscope) at 7 weeks post-inoculation. Each panel shows flowers from a single plant. Similar results were obtained from three independent experiments.

Fig. S4 Long-lasting insert stability of *Bean pod mottle virus* (BPMV) virus-induced gene silencing (VIGS) in soybean. Panels show amplified BPMV RNA2 products. Each bracket denotes three individual polymerase chain reactions (PCRs) conducted on cDNA from a transgenic soybean plant inoculated with a virus harbouring either a silencing construct or an empty vector, or a mock-inoculated plant, respectively. The vector harbouring the 1102-A silencing construct was used as a positive control. Top panel: PCR products were amplified using a vector-specific forward primer (BP-R2-3195F) and an insert-specific reverse primer sGFP-349F (Table S1). Bottom panel: PCR products were amplified using vector-specific primers (BP-R2-3195F and BP-R2-3603R) that flank the insert cloning site.

Fig. S5 *Bean pod mottle virus* (BPMV)-induced silencing of the transgene in roots. Silencing efficiency induced by BPMV in roots. Quantitative polymerase chain reaction (qPCR) was used to measure the abundance of green fluorescent protein (*GFP*) transcript in RNA samples isolated from root systems. The root tissues were collected from transgenic GFP soybean plants inoculated with BPMV vector with silencing construct and BPMV empty vector at 21 days post-inoculation (dpi). The expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and represent changes in mRNA abundance in silenced plants relative to the empty vector inoculated control. Data are the average of three biological samples, each with three technical replicates. Mean values significantly different from the mock are indicated by an asterisk as determined by paired *t*-test ($P < 0.01$).

Table S1 Primer sequences used in this study.

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