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Virus-induced Gene Silencing as Tool for Functional Genomics in a *Glycine max*

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Virus-induced gene silencing (VIGS) is a recently developed gene transcript suppression technique for characterizing the function of plant genes. However, efficient VIGS has only been studied in a few plant species. In order to extend the application of VIGS, we examined whether a VIGS vector based on TRV would produce recognizable phenotypes in soybean. Here, we report that VIGS using the *Tobacco rattle virus* (TRV) viral vector can be used in several soybean cultivars employing various agro-inoculation methods including leaf infiltration, spray inoculation, and agrodrench. cDNA fragments of the soybean phytoene desaturase (*PDS*) was inserted into TRV RNA-2 vector. By agrodrench, we successfully silenced the expression of *PDS* encoding gene in soybean. The silenced phenotype of *PDS* was invariably obvious 3 weeks after inoculation with the TRV-based vector. Real-time RT-PCR analyses showed that the endogenous level of *GmPDS* transcripts was dramatically reduced in the silenced leaf tissues. These observations confirm that the silenced phenotype is closely correlated with the pattern of tissue expression. The TRV-based VIGS using agrodrench can be applied to functional genomics in a soybean plants to study genes involved in a wide range of biological processes. To our knowledge, this is the first high frequency VIGS method in soybean plants.

Keywords : agro-inoculation, agrodrench, soybean, *Tobacco rattle virus*, VIGS

Plant viruses can be used as vectors for over-expression of proteins as well as induction of sequence-specific virus-induced gene silencing (VIGS) (Baulcombe, 1999; Lindbo et al., 2001; Lu et al., 2003). In addition to allowing a better understanding of how plants defend themselves against plant viruses, VIGS has emerged as a functional genomics tool for knocking out gene expression of target plant genes in some plants (Ekengren et al., 2003; Holzberg et al., 2002; Liu et al., 2002a; Peart et al., 2002; Sharma et al., 2003). To obtain VIGS, a fragment of the gene of interesting is inserted in the virus vector either by substituting genes that are dispensable for virus replication and movement or by duplicating promoter region required for virus gene expression. In contrast to conventional mutagenesis, VIGS dose not alter the gene itself, but rather transiently suppresses the expression of the gene through degradation of mRNA transcripts. An additional advantage of VIGS over anti-sense transformation and mutagenesis is that it allows the study of genes whose functions are essential for plant viability. Thus VIGS allow, unlike stable transformation procedures, the study of genes that would normally lead to a lethal phenotype when disrupted (Lu et al., 2003).

VIGS has been successfully applied to *Arabidopsis* (Dalmay et al., 2000; Lu et al., 2003; Turnage et al., 2002) and *Solanaceae*, including *Nicotiana* species (Baulcombe, 1999; Jin et al., 2002; Liu et al., 2002b), tomato (Liu et al., 2002a), legume species (Gabriela et al., 2004), *Solanum* species (Brigneti et al., 2004), and chill pepper (Chung et al., 2004). *Nicotiana benthamiana* is by far the best-studied host for VIGS and the VIGS response is generally stronger and more persistent in *N. benthamiana* than in other plants (Lu et al., 2003). As a result, some researchers have transferred the gene controlling the pathway under study in other plants to *N. benthamiana* for experiments applying VIGS analysis. Transfer of a genetic pathway to *N. benthamiana* is only possible if the pathway is under the control of a single or a few well-characterized genes and if the heterologous pathway will be functional in *N. benthamiana*.

As a consequence of various variation, efforts have been made to develop efficient VIGS systems for other hosts including *Barley stripe mosaic virus* for VIGS in barley (*Hordeum vulgare*) (Holzberg et al., 2002), *Cabbage leaf curl virus* for VIGS in *Arabidopsis thaliana* (Turnage et al., 2002), and *Tobacco rattle virus* (TRV) for VIGS in tomato (Liu et al., 2002a). Among the several viral vector systems used to trigger VIGS, TRV-derived vectors are widely used because they produce mild symptoms on the host and TRV

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has a wide host range (Dinesh-Kumar et al., 2003; Liu et al., 2002a; Ratcliff et al., 2001).

Soybean (*Glycine max*) is one of the most economically important crops following wheat, maize and rice. Accordingly, development of functional genomics tools for this plant is in demand. Attempts to silence genes in soybean using VIGS had been unsuccessful in the past, and there are no reports in the literature of VIGS employed in soybean for gene function studies. Recently, 570 soybean genes that expressed differentially during SMV infection processes were identified from susceptible (Geumjeong 1) and resistant (Geumjeong 2) cultivars against SMV strain G7H (Jeong et al., 2005). Functional characterization of these genes will likely facilitate the elucidation of defense signal transduction and biological function in SMV-infected soybean plants. Therefore, VIGS could potentially be a fast and efficient way to assess candidate gene phenotypes in this important crop species. While the model plants *Lotus japonicus* and *Medicago truncatula* are readily transformed (Stiller et al., 1997; Trieu et al., 2000), methods are still sought for analysis of essential genes (Andersen et al., 2003) and for high throughput analysis by transient RNA silencing (Kumagai and Kouchi, 2003). There is a need to transfer results from this model plants to the agriculturally important soybeans. Unfortunately, none of the recognized VIGS vectors are known to infect soybeans efficiently.

In order to develop a VIGS system in a soybean, we decided to test the TRV as a silencing vector in several soybean cultivars. TRV contains bipartite positive-sense RNA genome (Matthews, 1991). RNA-1 encodes the two viral replication proteins, a movement protein and a seed transmission factor while RNA-2 encodes the coat protein and a nematode transmission factor (Visser and Bol, 1999). TRV is a soil-borne virus transmitted by nematodes and accumulates to high titer in the root system (MacFarlane and Popovich, 2000). In the VIGS procedure, agroinoculation is extensively used to deliver VIGS vectors into plants for RNA silencing (Liu et al., 2002a; Peart et al., 2002). Leaf infiltration is the most common methods of agroinoculation used for VIGS (Dinesh-Kumar et al., 2003; Lu et al., 2003). However, the leaf infiltration method has limitations. Leaf infiltration is laborious for large-scale screening and certain plants, such as soybean and maize, are difficult to infiltrate. Leaf infiltration also normally utilizes fully expanded leaves (Liu et al., 2002b; Ratcliff et al., 2001). In this study, various agro-inoculation approaches including agrodrench (Ryu et al., 2004), agro-infiltration, and spray inoculation were employed to test efficiency of VIGS in soybean and present a highly efficient TRV-based VIGS as a good alternative to genetic transformation and efficient agro-inoculations on soybean. This technique should provide a powerful tool for identifying gene functions that has so far been unraveled by the low efficiency of genetic transformation and agro-inoculation.

Materials and Methods

Plasmid construction. All the constructs used for VIGS were built into pTRV2 vector (Liu et al., 2002a). pTRV1 and pTRV2 VIGS vectors were obtained from Dr. Dinesh-Kumar, Yale University. A 330-bp soybean *PDS* fragment was amplified by RT-PCR with primers, prPDS-F: 5'-GCTCTAGATTCAGGAGAACATGGTTC-3' and prPDS-R: 5'-GCCGAGCTCGTTTTC-3' from *G. max* cv. Somyung. The PCR-amplified soybean *PDS* (GenBank accession No. M64704) fragment was cloned into the *Xba*I and *Sac*I site of pTRV2 in a anti-sense orientation to produce pTRV2:GmPDS. Sequence of the pTRV2:GmPDS was confirmed by sequencing at the NICEM, SNU. Plasmids were introduced into *Agrobacterium tumefaciens* strain GV2260 by electroporation.

Plant material and growth conditions. *G. max* cv. Somyung, was used for all experiments, although silencing of Gm*PDS* was also observed in other soybean cultivars (data not shown). Soybean cultivar Somyung and *N. benthamiana* seeds were germinated and grown in a pot of soil in a growth chamber at 25°C with a 16 h light and 8 h dark photoperiod cycle. One to 3-week-old plants were used for silencing experiments. To obtain sterile soybean plants, seeds were surface-sterilized with 70% ethanol for 1-2 min followed by treatment with 1% sodium hypochlorite for 20 min.

***Agrobacterium* inoculation methods.** For *Agro*-inoculation, pTRV1, pTRV2 and the recombinant plasmids pTRV2:GmPDS were transformed into *A. tumefaciens* GV2260 by electroporation. A 5 ml culture of each strain was grown overnight at 30°C in YEP (50 μg/ml of kanamycin and 50 μg/ml of rifampicin). The overnight culture was inoculated into 50 ml of YEP medium and grown at 30°C shaker overnight. The cells were sedimented by centrifugation (3000 rpm, 15 min, 20°C), resuspended in inoculation medium (20 mM citric acid, 2% sucrose, pH 5.2), and adjusted O.D. to 1.0. They were then exposed to 200 μm acetosyringone at 22-25°C with shaking for 3-5 h.

To test silencing on soybean, we approached three major agro-inoculation methods including agro-infiltration, spray inoculation, and agrodrench. The induced *Agrobacterium* mixtures of pTRV1 and pTRV2 or pTRV2:GmPDS (1:1 ratio) were infiltrated with a needle-less 1 ml syringe into cotyledons of germinating plants and the youngest pair of

leaves of 2-week-old plants and sprayed using an artist's airbrush connected to a portable air compressor set at 75 psi. For agrodrench, mixture of *Agrobacterium* strains containing TRV1 and TRV2 was drenched, 3-5 ml each, with a 10-ml pipette into the crown part of each plant. Accumulation of virus in the upper uninoculated leaves of the plant was detected, 3 weeks after inoculation, by PCR with TRV coat protein-specific primers, prTRV CP-F: 5'-CTGAATCACTTGCGCTAAT-3' and prTRV CP-R: 5'-CAGAGTTCACGTCCTTAAA-3' and TRV RNA-dependent RNA polymerase-specific primers, prTRV RdRp-F: 5'-TCTTCACAGCCTGTATGACT-3' and prTRV RdRp-R: 5'-TAGAACACATTCCCTTGGTA-3'. The experiment was repeated at least three times with 5-6 plants for each experiment.

RNA extraction and RT-PCR analysis. Total RNAs from leaves were isolated from silenced and non-silenced (infiltrated with empty vector pTRV1 and pTRV2) plants 3 weeks post-inoculation using TRIzol™ reagent (Giboco BRL, USA) and treated with DNase I (TaKaRa, Japan) to remove the genomic DNA contamination. An RT-reaction was performed on 1-5 μg of total RNA with 0.5 units of M-MuLV Reverse Transcriptase (New English BioLabs, USA), 250 ng of gene-specific primers and 2.5 mM dNTPs in a final volume of 20 μl. The primers used for amplifying the *PDS* gene from soybean were prPDS-F: 5'-GCTCT-AGATTCAGGAGAACATGGTTC-3' and prPDS-R: 5'-GCCGAGCTCGTTTTC TGTCAAACCATA-3'. The primers used to amplify the β-tubulin gene from soybean were prTubulin-F: 5'-GACAGCATCAGCCATGTTCA-3' and prTubulin-R: 5'-AACCTCCTCCTCATCGTACT-3'. As a control for silenced and non-silenced plant, parallel reactions were conducted to normalize *PDS* and β-tubulin transcripts from various soybean cultivars. Each sample was run in triplicate and repeated four times from pooled samples of three independently silenced and non-silenced plants.

Results

Construction of a TRV-based VIGS system. It has been reported that agro-inoculation is the most efficient method for introducing cDNA-derived viral RNA into plants (Lu et al., 2003). In order to develop an efficient VIGS system, we tested a modified TRV vector (Liu et al., 2002a). TRV is a bipartite positive sense RNA virus consisting of RNA-1 and RNA-2 (Matthews, 1991). For VIGS, both a pTRV1 vector containing the RNA-1 gene driven by a double 35S *Cauliflower mosaic virus* (CaMV) promoter in a T-DNA construct, and a construction vector, pTRV2, are required. Using pTRV2, we constructed pTRV2:GmPDS (antisense orientation) (Fig. 1) to assess the ability of the TRV vector to suppress the expression of the endogenous *PDS* gene in soybean and *N. benthamiana* plants.

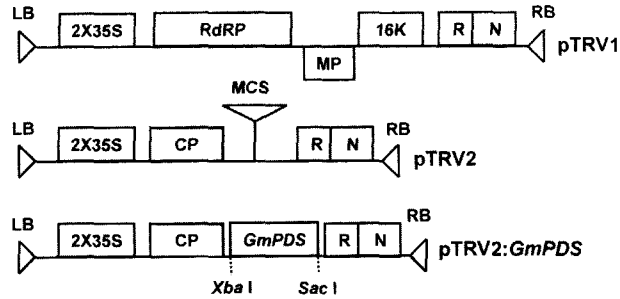

Fig. 1. TRV-based VIGS vectors used in soybean plants. The TRV-based vectors are described in Liu et al. (2002). TRV cDNA clones were placed between duplicated CaMV 35S promoters (2 x 35S) and the nopaline synthase terminator (N) in a T-DNA vector. pTRV2:GmPDS with a 333 bp cDNA fragment of *Glycine max* phytoene desaturase (*PDS*) inserted in the pTRV2 vector.

Comparison of inoculation methods for silencing soybean *PDS* gene. We chose phytoene desaturase (*PDS*) for the test of TRV-based silencing of an endogenous gene in soybean, because this gene has successfully been targeted in other VIGS systems (Holzberg et al., 2002; Liu et al., 2002a; Ratcliff et al., 2001). PDS is essential for the production of carotenoids that protect plants from photo-bleaching (Demmig-Adams et al., 1996). Leaves of plants in which PDS is silenced turn white as a result of the lack of carotenoids and destruction of chlorophyll by photo-oxidation (Kumagai and Kouchi, 1995). A 330 bp fragment of *GmPDS* was sequenced (GenBank accession no. M64704; Fig. 1) and cloned into pTRV2 vector generating pTRV2:GmPDS. In order to choose the most efficient way of silencing in soybean, we tested the following inoculation methods.

Three methods of inoculation including spray inoculation of the *Agrobacterium tumefaciens* cell suspension (Liu et al., 2002a), direct agro-infiltration (English and Sweatt, 1997) and agrodrench (Ryu et al., 2004) were used. The three methods of inoculation were compared on Somyung cultivar that is susceptible soybean cultivar against SMV-G7H. Two-week-old soybean or *N. benthamiana* plants were agro-inoculated with pTRV2:GmPDS using three inoculation methods along with pTRV2 as a negative control. Two-to-three weeks post-inoculation, plants inoculated with pTRV2:GmPDS showed chlorosis on upper tissues that inoculated by agrodrench (Fig. 2, panel B). Representative upper leaves harvested 30 dpi by agrodrench are shown in Fig. 2C. In contrast, all leaves of plants inoculated with other methods remained green. At the 45 dpi, approximately 95% of upper leaves displayed chlorosis.

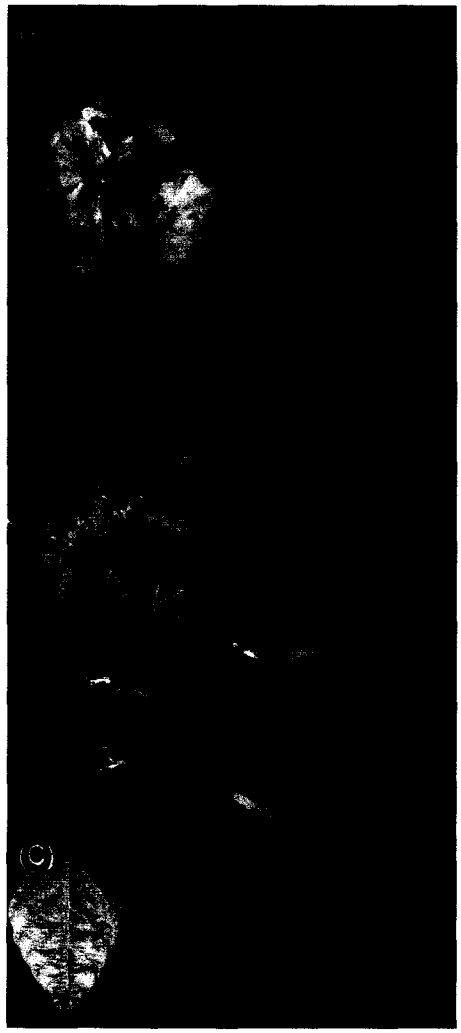

Fig. 2. Infection of *Nicotiana benthamiana* (A) and *G. max* (B and C) plants with the TRV-based vectors. (**A**) Infection with pTRV2:GmPDS silencing endogenous *PDS* in *N. benthamiana* plants (left panel). Photo-bleaching phenotype caused by the inhibition of carotenoid biosynthesis was observed. (**B**) PDS silencing using different agro-inoculation in soybean. pTRV2:GmPDS silencing with agrodrench (top left), infiltration (bottom left), and spray inoculation (bottom right). Plants inoculated with pTRV2 are shown in right and top right position of panels A and B, respectively. (**C**) Enlarged images of one soybean leaf inoculated with pTRV2:GmPDS by agrodrench, infiltration, and spray inoculation (left to right, respectively). Soybean leaf inoculated with pTRV2 is shown on far right position.

Chlorosis continued to develop on emerging leaves beyond 45 dpi. The frequency of VIGS was determined by the number of plants that show silencing phenotype after inoculation with pTRV2:GmPDS. By agrodrench method, 100% of the infected soybean plants showed the silencing phenotype. Our results indicate that the agrodrench inoculation method was effective in VIGS than those of other inoculation methods.

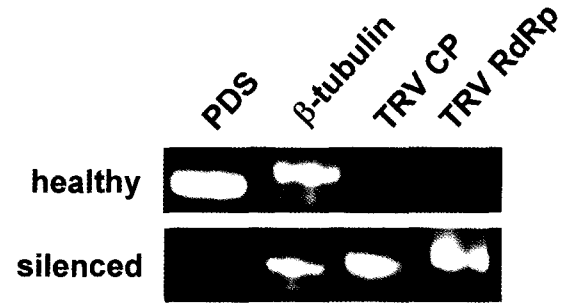

Fig. 3. Detection of PDS, β-tubulin, TRV CP, and TRV RdRp. Total RNAs extracted from healthy and pTRV2:GmPDS silenced soybean leaves were used for RT-PCR using specific oligonucleotides.

Silencing of the soybean *PDS* gene by agrodrench using TRV-VIGS vector. Phytoene desaturase mRNA levels were quantified by real-time RT-PCR using β-tubulin as an internal control. Four samples from three independent experiments were analysed. Total RNAs extracted from infected plants was first transcribed in an RT reaction. RT-PCR was then used in order to assess the mRNA levels in plants inoculated with pTRV2:GmPDS. The results from these experiments, showed that PDS mRNA levels in tissue samples showing chlorosis from soybean plants inoculated with pTRV2:GmPDS by agrodrench method were significantly reduced compared to those inoculated with pTRV2 control (Fig. 3).

Discussion

Virus-induced gene silencing is an excellent functional genomics tool for gene function analyses. The most widely used vectors are based on PVX (Lu et al., 2003; Sharma et al., 2003; Slaymaker et al., 2002) or TRV (Liu et al., 2002a; Peart et al., 2002). However, the applications of these silencing vectors have so far mainly restricted to *N. benthamiana*. This reflects not only the efficiency of VIGS in *N. benthamiana* but also the difficulty of developing efficient VIGS vectors for other hosts (Lu et al., 2003).

Our results indicate that we have overcome these problems by developing efficient inoculation methods using TRV as a VIGS vector for soybean plants. Recombinant TRV induced an efficient and reliable gene silencing in soybean. The work described here is intended to provide guidelines for the use of VIGS in soybean and its wild relatives. For the VIGS in soybean, we used various agro-inoculation methods including agrodrench, agro-infiltration, and spray inoculation. We revealed that agrodrench is a novel and simple agro-inoculation method to elicit VIGS in soybean. Due to the easiness and high efficiency of VIGS caused by agrodrench inoculation, it would be a good solution for high-throughput gene silencing and for plants

which are difficult to infiltrate.

Agrodrench method was developed for efficient VIGS in various *Solanaceae* species (Ryu et al., 2004). We used the *PDS* gene as indicator of gene silencing in foliar tissue. This gene, with known function and a clear mutant phenotype, was targeted in soybean by inoculation with recombinant TRV. *GmPDS* has a single gene homologue in the sequenced soybean and, therefore, is likely to exist as a single copy gene or as low copy gene in soybean plant. Although the complete photo-bleaching routinely observed in *N. benthamina* was not observed, the silencing phenotype, systemic and consistent chlorosis, of *PDS* gene was clearly displayed. When the effect on mRNA levels was measured in soybean agrodrenched with TRV carrying *GmPDS*, we observed that agrodrenching significantly reduced mRNA levels. As expected, the *PDS* transcripts level in agrodrench was dramatically reduced than those of the other samples. This may allow us to do gene functional analyses during root-microbe interactions such as plant-*Rhizobium* symbiosis, plant-mycorrizal symbiosis, plant-rhizobacteria, and plant-pathogen interaction. As noted in Fig. 3, silencing by agrodrench did not completely shut down transcript levels. Although we have not yet been able to silence target gene, future experiments will involve characterization of effects caused by other factors including soybean cultivars and environmental conditions. We are currently examining the efficiency of VIGS using the other soybean genes by agrodrenching pTRV2 derivatives.

In summary, we present a rapid and efficient TRV-based VIGS system in soybean plants. Silencing of PDS expression in soybean plants was close to 100% efficient about 3 weeks post-inoculation by agrodrench method and lasted through their life cycle. At the mRNA level, a significant reduction in PDS expression was observed in the silenced plants. This agrodrench method will be useful for functional analysis of many genes of interest and for advances in the functional genomics of soybean.

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