



Optimization of virus-induced gene silencing in pepper (*Capsicum annuum* L.)

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ABSTRACT. Virus-induced gene silencing is currently a powerful tool for the study of gene function in plants. Here, we optimized the protocol for virus-induced gene silencing, and investigated factors that affect the efficiency of tobacco rattle virus-induced gene silencing in pepper plants. Consequently, an optimal protocol was obtained by the syringe-infiltration method in the leaves of pepper plants. The protocol involves 2-leaf stage plants, preparing the *Agrobacterium* inoculum at a final OD₆₀₀ of 1.0 and then growing the inoculated plants at 22°C. Using this protocol, we achieved high efficiency in silencing *CaPDS* in different cultivars of pepper plants. We further used the *CaPOD* gene to illustrate the general reliability of this optimized protocol. Viral symptoms were observed on the leaves of inoculated plants of the Early Calwonder cultivar 25 days post-inoculation, indicating that this protocol can also be used to silence other genes in pepper plants. Real-time polymerase chain reaction analyses revealed that the expression levels of *CaPDS* and *CaPOD* were dramatically reduced in inoculated leaves compared to

control plants. These results demonstrate that the optimized protocol can be applied to functional genomic studies in pepper to investigate genes involved in a wide range of biological processes.

Key words: Pepper; Optimized protocol; Virus-induced gene silencing; Tobacco rattle virus; Phytoene desaturase

INTRODUCTION

Pepper (*Capsicum annuum* L.) is a nutritionally and economically important crop that is cultivated throughout the world as a vegetable, condiment, and food additive. Genetic transformation is extremely difficult in pepper plants, and the efficiency of transformation is very low. Although a number of transformation protocols have been published (Manoharan et al., 1998; Mihalka et al., 2000; Kim et al., 2001), investigations into the functions of pepper genes remain problematic due to the lack of stable and effective genetic transformation procedures. Moreover, transformation itself is a complex, labor-intensive procedure, with low efficiency (Baulcombe, 2004; Benedito et al., 2004; Burch-Smith et al., 2004).

These disadvantages can be avoided by adopting large-scale functional genomic approaches to analyze gene function. Virus-induced gene silencing (VIGS) provides an attractive alternative tool to investigate gene function in plants by knocking down target genes of interest without the need for transformation (Baulcombe, 1999; Dinesh-Kumar et al., 2003). This approach is considerably less time-consuming than classical stable transformation. VIGS developed from RNA-mediated post-transcriptional gene silencing (Baulcombe, 1999), which is a defense system against pathogens and is naturally present in plants and other organisms (Dinesh-Kumar et al., 2003; Burch-Smith et al., 2004; Robertson, 2004). When a virus infects a plant tissue and spreads systemically, endogenous gene transcripts with sequence identities matching the engineered sequence in the viral vector (VIGS vector) are degraded by post-transcriptional gene silencing (Baulcombe, 1999). Consequently, the plant defends itself against the virus by degrading the accumulating viral genome and, in the process, silences its own gene.

Recently, great efforts have gone into exploiting VIGS for gene function analyses in a variety of plant species. So far, VIGS has been applied to more than 30 plant species (Becker and Lange, 2010), indicating that it has become a powerful tool for functional gene analysis in plants. More than 30 VIGS vectors have been developed so far, including tobacco mosaic virus (Kumagai et al., 1995), potato virus X (Ruiz et al., 1998), tobacco rattle virus (TRV) (Ratcliff et al., 2001), barley stripe mosaic virus (Holzberg et al., 2002), and brome mosaic virus (Ding et al., 2006). These vectors have been widely used to uncover the functions of genes involved in basic cellular functions, metabolic pathways, development biology, plant-microbe interactions, and abiotic stress (Huang et al., 2012). In particular, the TRV-based vector, TRV, has been widely used in gene silencing studies involving *Nicotiana benthamiana*, tomato, pepper, eggplant, and other Solanaceous species (Kumagai et al., 1995; Brigneti et al., 2004; Fu et al., 2005; Chung et al., 2004; Liu et al., 2002a, 2004, 2012). In the TRV vector, RNA1 provides the replication and movement functions, and TRV-RNA2 from the isolate PPK20 encodes the viral coat protein (Ratcliff et al., 2001; Liu et al., 2002b). One distinct advantage of using TRV for VIGS is the ability of the virus to spread rapidly throughout the entire plant, including the meristem tissue. VIGS has therefore been used to study gene functions in leaves,

flowers (Liu et al., 2004; Chen et al., 2004), fruits (Fu et al., 2005; Orzaez et al., 2006), and roots (Valentine et al., 2004).

Despite its successful application, the silencing efficiency of VIGS varies among different plant species and even among cultivars (Burch-Smith et al., 2004; Ghazala and Varrelmann, 2007; MacFarlane, 2010). In addition, many environmental factors influence the efficiency of VIGS, such as the *Agrobacterium* inoculation method, growth stages of the plants, and the growth temperature. The VIGS system, therefore, requires optimization in order to increase the efficiency of gene silencing studies across a range of plants. The phytoene desaturase (*PDS*) reporter gene has been widely and successfully used in VIGS studies based on its observed silencing phenotype (Ratcliff et al., 2001; Liu et al., 2002a). Chung et al. (2004) were the first to achieve VIGS of pepper using a TRV-based VIGS system, but a comprehensive optimized system for VIGS is still lacking.

In this study, we used a TRV-based VIGS vector to optimize the protocol of gene silencing in the Early Calwonder (EC) pepper cultivar, using the syringe-infiltration method. The results indicated that the growth stage, the concentration of *Agrobacterium* inoculum, and the growth temperature for inoculated plants were important factors in determining the efficiency of gene silencing. Following this optimized protocol, we achieved efficient gene silencing of the reporter gene, *CaPDS*, in different cultivars of pepper plants. This study should provide a convenient, fast, and effective method for large-scale gene silencing in pepper functional genomics.

MATERIAL AND METHODS

Plant material and growth conditions

Pepper plants were used for all experiments. Seeds were grown in a plastic tray containing a steam-sterilized soil mixture. The growth chamber conditions were maintained at 25°/18°C under a 16-h light/8-h dark photoperiod cycle with 60% relative humidity.

Plasmid construction

The pTRV1 and pTRV2 VIGS vectors (Liu et al., 2002a) were kindly provided by Dr. Dinesh-Kumar of Yale University. Two constructs were used in this study, including pTRV-*CaPDS* (*CaPDS*, phytoene desaturase from *C. annuum*, GenBank accession No. X68058.1) and pTRV-*CaPOD* (*CaPOD*, peroxidase from *C. annuum*, GenBank accession No. FJ596178).

pTRV-*CaPDS* was constructed from a 324-bp fragment of *CaPDS*, which was polymerase chain reaction (PCR)-amplified from a pepper cDNA template using 2 primers (forward: 5'-GGGGAATTCTGTTGTCAAACCTCCAAGGTCTGTA-3' with an *EcoRI* restriction site and reverse: 5'-GGGGATCCTTTCTCCCACTTGGTTCACCTTGT-3' with a *BamHI* restriction site). The resulting product was cloned into the *EcoRI*-*BamHI*-cut pTRV2 to form pTRV-*CaPDS*.

pTRV-*CaPOD* was constructed from a 485-bp fragment of *CaPOD*, which was PCR-amplified from a pepper cDNA template using 2 primers (forward: 5'-GGGTCTAGAGTGCTCAACACACACTTTATTCTTCTC-3' with an *XbaI* restriction site and reverse: 5'-GGGGGATCCCAAGAATGACAACAGAGTCCCTA-3' with a *BamHI* restriction site). The resulting product was cloned into the *XbaI*-*BamHI*-cut pTRV2 to form pTRV-*CaPOD*.

Sequences of all pTRV2 derivatives were confirmed by sequencing. For the VIGS assay, either pTRV1 or pTRV2 and its derivatives were introduced into *A. tumefaciens* strain GV3101 by the freeze-thaw method.

***Agrobacterium* inoculation**

Agrobacterium strain GV3101 containing pTRV1 or pTRV2 and its derivatives were used for the VIGS experiments. Cultures (5 mL) were grown in Luria-Bertani medium containing the appropriate antibiotic (50 mg/L kanamycin, 50 mg/L rifampicin, and 50 mg/L gentamycin), and shaken at 28°C overnight. The next day, each *Agrobacterium* culture was inoculated into 50 mL Luria-Bertani medium containing the same antibiotics, 10 mM 2-(4-morpholino)ethane sulfonic acid (MES), and 200 µM acetosyringone, and grown in a shaker at 200 rpm. After 24 h, *Agrobacterium* cells were harvested and resuspended in the *Agrobacterium* infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6, 200 µM acetosyringone). The *Agrobacterium* cells were collected by centrifugation (3000 rpm, 10 min), suspended in infiltration medium (20 mM citric acid, 2% sucrose, pH 5.6) and adjusted to OD₆₀₀ values between 0.5 and 4.0. The suspensions of *Agrobacterium* inocula containing pTRV1 and pTRV2 or its derivatives were mixed at a 1:1 ratio and incubated for 3 h at room temperature. The *Agrobacterium* was infiltrated into the cotyledons of different growth stages (2, 4, and 6 true leaves) using a 1-mL sterilized syringe without a needle (Liu et al., 2002a). The *Agrobacterium*-infiltrated pepper plants were transferred to 18°C for 2 days and grown in a growth chamber at different temperatures (22°, 25°, 28°C) under a 16-h light/8-h dark photoperiod cycle with 60% relative humidity.

RNA isolation and real-time quantitative PCR (qRT-PCR) analysis

qRT-PCR was used to quantify the abundances of *CaPDS* and *CaPOD* transcripts in the leaves of inoculated pepper plants. Total RNA was extracted from leaves of silenced and non-silenced (infiltrated with empty vectors of pTRV1 and pTRV2) 15 days after infiltration using Trizol reagents (Invitrogen), following manufacturer protocols. The first-strand cDNA was synthesized from 500 ng total RNA using a SYBR[®] Premix Ex *Taq*[™] II kit (TaKaRa). Primers that annealed outside the region targeted for silencing were used to ensure that only the endogenous gene was being tested. The ubiquitin gene, *ubi3*, served as an internal control gene. The primers specific to a 120-bp fragment of *ubi3* were 5'-TGTCCATCTGCTCTCTGTTG-3' and 5'-CACCCCAAGCACAATAAGAC-3'. Primers used in RT-PCR for *CaPDS* were 5'-AGGTCTTCTTTGGGAAGTATGAT-3' and 5'-GTTATGGAATGGGGATTACGA-3'. The primers used for qRT-PCR for *CaPOD* were 5'-CAAGGTTCAATGTGTGTTACC-3' and 5'-ATGATGATACAAATACAGTGCC-3'. qRT-PCR was performed following manufacturer protocols in the iQ5 Real-Time PCR Detection System and analyzed using the iQ5 Standard Edition Optical System Software Version 2.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Three technical replicates were run for each biological replicate.

Chlorophyll content measurement

Chlorophyll was measured immediately after preparing the extracts, as described by Lichtenthaler (1987) and Hajirezaei et al. (2002), with slight modifications. Leaves (approximately

0.1 g) were extracted in 80% (v/v) aqueous acetone, followed by an additional step with 100% acetone. Samples were then incubated at room temperature until they were white in appearance. Absorbance was recorded at 663 and 645 nm using a UV-visible spectrophotometer. Total chlorophyll was expressed as the percentage reduction relative to the corresponding control.

Statistical analysis

The data were subjected to analysis of variance using the JMP software, version 4.0.4 (SAS Institute Inc., Cary, NC, USA). When a significant F-test was obtained ($P \leq 0.05$), separation of treatment means was accomplished by Fisher's protected least significant difference. Values are reported as means \pm SD of 3 independent experiments.

VIGS efficiency analysis was conducted in triplicate with 30 plants per experiment. The efficiency of gene silencing was calculated with the following formula: gene silencing efficiency (%) = (number of plants showing the photobleaching phenotype / total number of tested plants) \times 100%.

RESULTS

To estimate the efficiency of VIGS in pepper, we used the *PDS* gene from pepper in a pTRV2 vector. *PDS* encodes an enzyme involved in the carotenoid biosynthesis pathway (Cunningham and Gantt, 1998). The silencing of *PDS* has previously been used as a visual marker for the effectiveness of VIGS in several plants, such as tomato (Liu et al., 2002a), *Arabidopsis thaliana* (Turnage et al., 2002), *N. benthamiana* (Ratcliff et al., 2001), and *C. annuum* L. (Chung et al., 2004). The silencing of *PDS* produces a photobleaching phenotype, which occurs in the absence of the *PDS* gene product (Turnage et al., 2002). In this study, a 324-bp fragment of the *CaPDS* gene was obtained through RT-PCR, which was used to form a pTRV2-*CaPDS* construct in order to silence the *PDS* gene in pepper. The effects of different factors on VIGS efficiency in pepper plants were then estimated.

Effect of different cultivars of pepper on VIGS efficiency in agroinoculated plants

Cultivars of the same species often vary in their susceptibility to TRV infection (Ghazala and Varrelmann, 2007; MacFarlane, 2010) and in TRV-VIGS efficiency (Burch-Smith et al., 2004; Deng et al., 2012; Bennypaul et al., 2012). To estimate the efficiency of VIGS in different cultivars of pepper, we examined gene silencing in 3 agroinoculated cultivars of pepper: CM334 (A5), PBC137 (A3), and EC. The *Agrobacterium* inoculum was infected into 4-leaf stage plants with a final OD₆₀₀ of 1.0. Next, *Agrobacterium*-inoculated plants were transferred to 18°C for 2 days and grown in a growth chamber at 22°C under a 16-h light/8-h dark photoperiod cycle with 60% relative humidity. The photobleaching symptom was observed on the leaves of infected pepper plants 15 days postinoculation. The efficiency of VIGS in the different pepper cultivars is shown in Figure 1. EC exhibited the most intensive *PDS*-silencing phenotype and had the highest observed gene-silencing efficiency of 94%, while A5 and A3 showed gene silencing efficiencies of 64 and 77%, respectively. Based on these results EC was used as the target plant for agroinoculation in all subsequent gene silencing experiments.

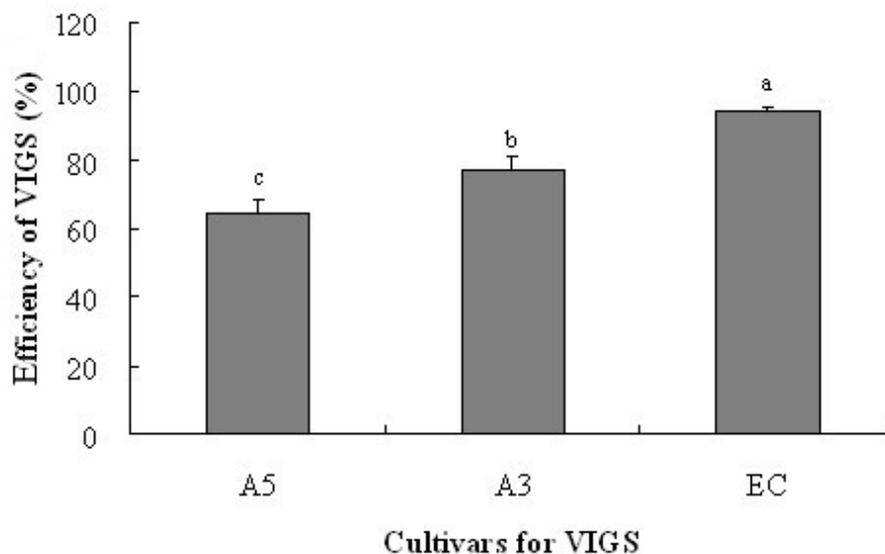


Figure 1. Effect of different cultivars for agroinoculated plants on virus-induced gene silencing (VIGS) efficiency in pepper. The *Agrobacterium* inocula with $OD_{600} = 1.0$ were injected by the syringe-infiltration method into 4-leaf stage plants of different cultivars, A5 (CM 334), A3 (PBC 137), and EC (Early Calwonder). Then, inoculated pepper plants were transferred to 18°C for 2 days and grown in a growth chamber at 22°C under a 16-h light/8-h dark photoperiod cycle with 60% relative humidity. The efficiency of silencing *CaPDS* was analyzed at 15 days postinoculation, which was calculated in accordance with the formula described in Statistical analysis. Letters indicate significant differences using the Fisher LSD test at $P \leq 0.05$.

Effect of plant growth stage on VIGS efficiency in agroinoculated pepper

The developmental stage of a plant at the time of inoculation is a known critical factor for VIGS (Chung et al., 2004; Hileman et al., 2005; Burch-Smith et al., 2006; Deng et al., 2012). To investigate the effect of the growth stage of agroinoculated pepper plants on gene silencing efficiency, we attempted to silence the gene at different growth stages (2-, 4-, 6-, and 8-leaf) in the EC cultivar. The *Agrobacterium* inoculum, with a final OD_{600} of 1.0, was injected into pepper leaves through the syringe-infiltration method. Plants were then grown at 22°C under a 16-h light/8-h dark photoperiod cycle with 60% relative humidity. The *CaPDS* gene silencing efficiency results are shown in Figure 2. The photobleaching symptom was observed on the leaves of 2-, 4-, and 6-leaf stage plants at 15 days postinoculation, and gene silencing efficiencies were 96, 90, and 58%, respectively. In contrast, leaves of 8-leaf stage plants showed no obvious photobleaching at this time, which was observed 10 days later than the phenotype first appeared in 2-leaf stage plants. These results demonstrate that younger plants are preferable for TRV-based silencing in pepper. Therefore, because the 2-leaf stage of pepper plants was the most competent for *Agrobacterium* infection, it was used in subsequent experiments for gene silencing.

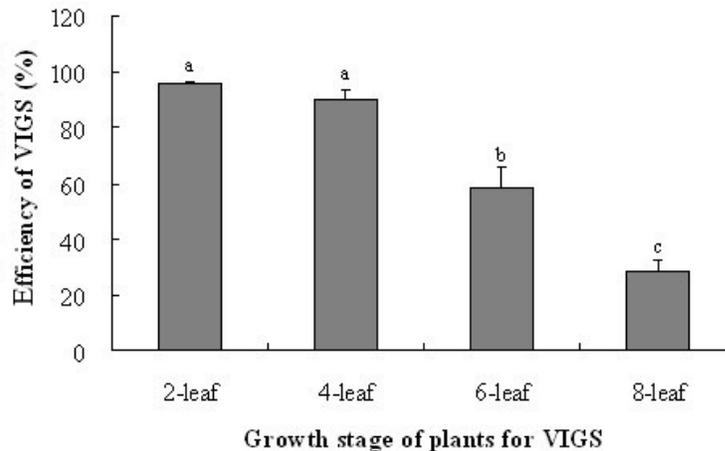


Figure 2. Effect of different growth stage of plants for agroinoculation on virus-induced gene silencing (VIGS) efficiency in pepper. The agroinocula with a final $OD_{600} = 1.0$ were injected into different growth (2-, 4-, 6-, 8-leaf) stage plants through the syringe-infiltration method, which were then grown at 22°C under a 16-h light/8-h dark photoperiod cycle with 60% humidity. The efficiency of silencing *CaPDS* was analyzed at 15 days postinoculation, which was calculated in accordance with the formula described in Statistical analysis. Letters indicate significant differences using the Fisher LSD test at $P \leq 0.05$.

Effect of different concentrations of agroinocula on VIGS efficiency in pepper

Previous studies have shown that in some plant species, the concentration of agroinoculation affects gene silencing efficiency (Chung et al., 2004; Burch-Smith et al., 2006; Zhou et al., 2006). In order to determine the optimal agroinoculation concentration, different concentrations of *Agrobacterium* inocula (OD_{600} values of 0.5, 0.8, 1.0, 2.0, and 3.0) were syringe-infiltrated into 2-leaf stage EC plants, which were then grown in growth chambers at 22°C. The resulting *CaPDS* gene silencing efficiencies from the different concentrations of agroinocula are shown in Figure 3. The highest gene silencing efficiencies were 88 and 83%, with final OD_{600} of 1.0 and 0.8, respectively. When the agroinoculation concentration was lower than an OD_{600} of 0.8, the gene silencing efficiency decreased to 47%. When the concentration of agroinoculation was $OD_{600} = 2.0$, the gene silencing efficiency was 38%. In addition, higher death rates occurred on plants infected with a final OD_{600} of 3.0. These results confirmed that a concentration with a final OD_{600} of 1.0 was suitable for gene silencing in pepper plants.

Effect of different growth temperatures of agroinoculated plants on VIGS efficiency in pepper

The growth temperature of agroinoculated plants is another important factor that influences several aspects in VIGS, such as *Agrobacterium* performance, viral spread, accumulation of short-interfering RNAs, and ultimately, plant growth (Burch-Smith et al., 2004; Robertson, 2004; Hartl et al., 2008). The particular effect of temperature is species-specific. For example, several *Solanum* spp favor temperatures between 16° and 20°C (Liu et al., 2002a; Brigneti et al., 2004), whereas *A. thaliana* favors higher temperatures, between 22° and 24°C (Wang et al., 2006).

To assess the influence of the growth temperature of agroinoculated pepper plants on gene silencing, 2-leaf stage agroinoculated plants, with a final OD_{600} of 1.0, were grown in plant growth chambers at different temperatures (18°, 20°, 22°, 25°, and 28°C). The results of temperature on the efficiency of *CaPDS* gene silencing are shown in Figure 4. The pTRV2-*PDS*-silenced plants grown at 20°, 22°, and 25°C demonstrated the highest gene silencing efficiencies of 86, 88, and 80%, respectively, while plants grown at the lowest (18°C) and the highest (28°C) temperatures displayed the lowest gene silencing efficiencies; 28 and 11%, respectively.

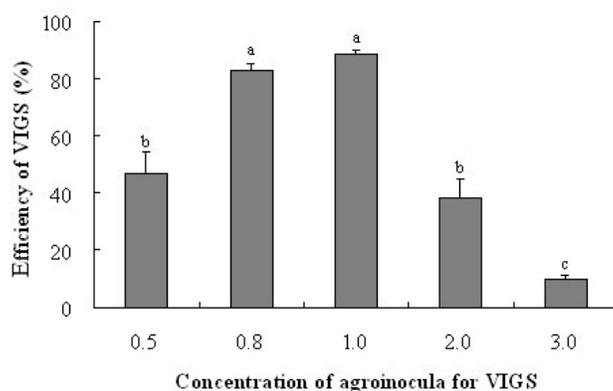


Figure 3. Effect of the different concentrations of agroinocula for agroinoculation on virus-induced gene silencing (VIGS) efficiency in pepper. The agroinocula with different concentration (OD_{600} = 0.5, 0.8, 1.0, 2.0, 3.0) were injected into 2-leaf stage plants through the syringe-infiltration method, which were then grown at 22°C under a 16-h light/8-h dark photoperiod cycle with 60% humidity. The efficiency of silencing *CaPDS* was analyzed at 15 days postinoculation, which was calculated in accordance with the formula described in Statistical analysis. Letters indicate significant differences using the Fisher LSD test at $P \leq 0.05$.

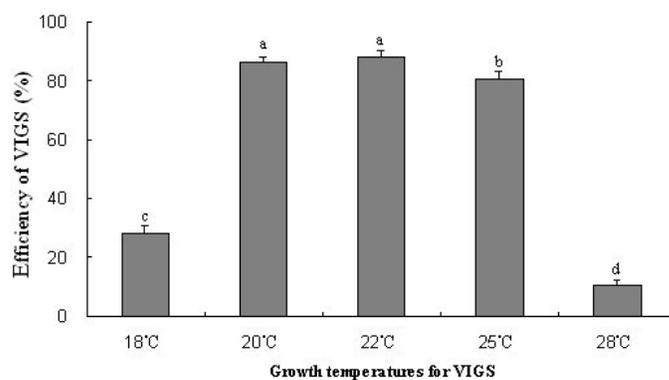


Figure 4. Effect of the different growth temperatures of agroinoculated plants on virus-induced gene silencing (VIGS) efficiency in pepper. The agroinocula with a final OD_{600} = 1.0 were injected into 2-leaf stage plants through the syringe-infiltration method, which were then grown at different temperatures (18°, 20°, 22°, 25°, and 28°C) under a 16-h light/8-h dark photoperiod cycle with 60% humidity. The efficiency of silencing *CaPDS* was analyzed at 15 days postinoculation, which was calculated in accordance with the formula described in Statistical analysis. Letters indicate significant differences using the Fisher LSD test at $P \leq 0.05$.

In addition, the earliest sign of photobleaching was observed in agroinoculated plants grown at 22°C, whereas the silencing phenotype only appeared on plants grown at 20° and 25°C 2-3 days later. The expression of the photobleaching symptom was delayed for 7-10 days in plants grown at 18° and 28°C, which had substantially reduced leaf areas relative to those grown at 22°C. These results demonstrated that 22°C was the optimal temperature for plant growth after agroinoculation, which is within the natural temperature range of pepper growth.

VIGS of *CaPDS* in pepper

To assess the general effectiveness of the TRV-based VIGS protocol for gene silencing that was optimized in the pepper cultivar EC, we examined *CaPDS* gene silencing in 3 other pepper cultivars: A5, A3, and B12. The optimized protocol for gene silencing in pepper involves agroinoculating, via syringe-infiltration, *Agrobacterium* with an OD₆₀₀ of 1.0 into 2-leaf growth stage plants at 22°C. Following this protocol, the photobleaching symptom was exhibited in all inoculated pepper plants just as was observed in EC (Figure 5A). These results therefore demonstrate that the optimized protocol for gene silencing in the EC cultivar is suitable for other cultivars of pepper.

Furthermore, we used chlorophyll content as another measure of silencing efficiency. More chlorophyll degradation indicates higher silencing efficiency and vice versa. Chlorophyll content was assessed in the third or fourth leaf from the upper infected leaves showing the photobleaching phenotype, and was compared with the chlorophyll content of leaves from the mock-inoculated plants (pTRV-00; empty vector) that did not show photobleaching. As shown in Figure 5B, the contents of chlorophyll a, chlorophyll b, and total chlorophyll in the leaves of *CaPDS*-silenced plants were substantially reduced relative to those of control plants (pTRV-00; empty vector). The highest reductions in total chlorophyll were observed in the *CaPDS*-silenced leaves of EC (86%) and B12 (54%), whereas the silenced leaves of A5 and A3 showed only 38 and 44% reductions, respectively.

To further confirm gene silencing of *CaPDS* at the molecular level, qRT-PCR was performed with gene specific primers (see Materials and Methods for details). Total RNA was extracted from the third or fourth photobleached leaf from the upper leaves of inoculated plants and from the non-photobleached leaves of the control plants (pTRV-00; empty vector). The results of gene expression patterns are shown in Figure 5C. The expression of the *CaPDS* genes in silenced plants A5, A3, EC, and B12 were dramatically reduced 51, 75, 91, and 85%, respectively, compared to control plants. These results further demonstrate that the optimized protocol efficiently silences *CaPDS* in pepper plants.

Virus-induced gene silencing of *CaPOD* in pepper

To validate that the gene silencing protocol optimized for *CaPDS* is also applicable to other genes, we attempted to silence the *CaPOD* gene in the EC cultivar. The optimized protocol for gene silencing in pepper involves agroinoculating, via syringe-infiltration, *Agrobacterium* with an OD₆₀₀ of 1.0 into 2-leaf growth stage plants at 22°C. Following this protocol, the *CaPOD*-inoculated EC plants exhibited viral symptoms (Figure 6A). This result demonstrated that the optimized protocol in EC is also applicable to other genes for virus-induced gene silencing in pepper plants.

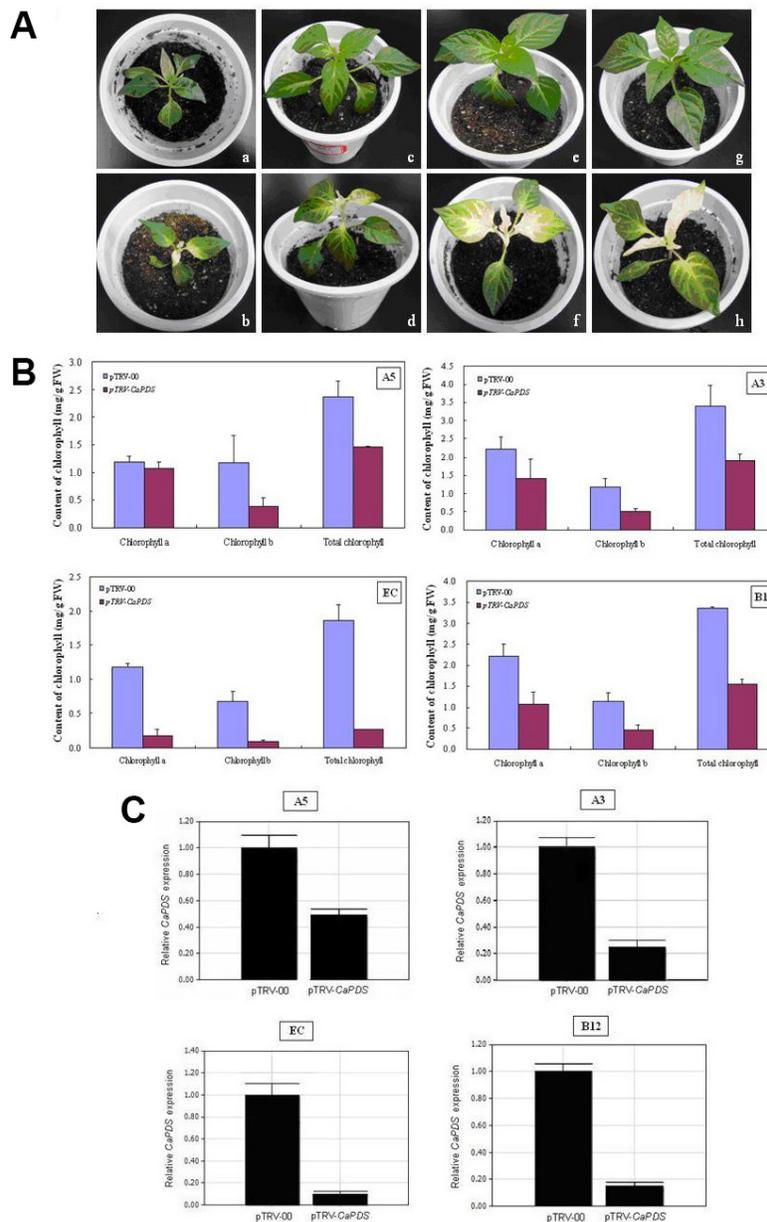


Figure 5. TRV-induced gene silencing of the *CaPDS* gene in 4 cultivars of pepper. The photographs were taken 20 days postinoculation. **A.** Symptoms of inoculated plants: **a.** control plant (pTRV-00, empty vector) of A5; **b.** pTRV-*CaPDS*-infected plant of A5; **c.** control plant (pTRV-00, empty vector) of A3; **d.** pTRV-*CaPDS*-infected plant of A3; **e.** control plant (pTRV-00, empty vector) of EC; **f.** pTRV-*CaPDS*-infected plant of EC; **g.** control plant (pTRV-00, empty vector) of B12; **h.** pTRV-*CaPDS*-infected plant of B12. **B.** Comparison of the content of chlorophyll: chlorophyll a; chlorophyll b, and total chlorophyll between the control (pTRV-00, empty vector) and pTRV-*CaPDS*-silenced plants. **C.** Real-time RT-PCR analysis of *CaPDS* gene expression in the control (pTRV-00, empty vector) and pTRV-*CaPDS*-silenced plants.

To confirm the silencing of the *CaPOD* gene at the molecular level, expression of the *CaPOD* gene was determined by RT-PCR. Total RNA was extracted from the third or fourth leaves of injected and control (pTRV-00; empty vector) plants. Expression of *CaPOD* genes in infected plants decreased by more than 26% compared to that of control plants (Figure 6B). These results further demonstrate that the optimized protocol is efficient for silencing other genes in pepper plants.

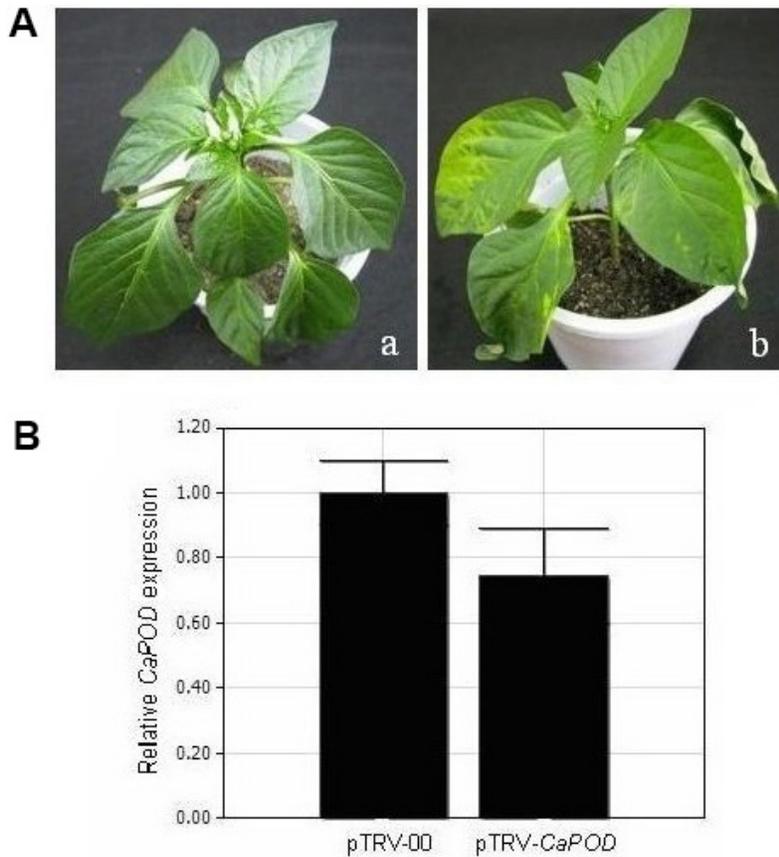


Figure 6 TRV-induced gene silencing of *CaPOD* in pepper cultivar Early Calwonder. **A.** Symptoms of inoculated plants. The photographs were taken 30 days postinoculation. **a.** control plant (pTRV-00, empty vector); **b.** pTRV-*CaPOD*-infected plant. **B.** Real-time RT-PCR analysis of *CaPOD* gene expression in the control (pTRV-00, empty vector) and pTRV-*CaPOD*-silenced plants.

DISCUSSION

VIGS technology has shown its potential as a reverse genetic tool, especially for plants lacking a high throughput transformation system. This is mainly because VIGS is a very simple and robust method that does not require stable transformation and extensive gene

sequence information (Sasaki et al., 2011). Successful gene silencing depends upon a dynamic interplay between virus spread and plant growth, both of which can be influenced by environmental conditions. Factors related to the rapid accumulation of the TRV can greatly affect the efficiency of TRV-induced gene silencing. In particular, most authors have emphasized the choice of vector, the inoculation method, plant age, and plant growth temperature as key parameters (Brigneti et al., 2004; Saedler and Baldwin, 2004; Burch-Smith et al., 2006; Wang et al., 2006). It is, therefore, important to invest a substantial amount of effort to determine optimal inoculation levels and plant growth conditions prior to conducting VIGS studies (Burch-Smith et al., 2004). However, once these factors are optimized, it is fairly simple to obtain reproducible levels of silencing plants.

In this study, we optimized a TRV-based virus-induced gene silencing protocol via syringe-infiltration for pepper plants. The factors affecting the efficiency of VIGS in pepper plants included the specific cultivar used, growth stage, the concentration of *Agrobacterium* inoculum, and the growth temperature. Several studies have shown that TRV infection causes relatively mild disease symptoms and often induces intensive and uniform silencing phenotypes (Ratcliff et al., 2001; Brigneti et al., 2004; Burch-Smith et al., 2006). Therefore, the TRV was used for silencing pepper plant genes in our experiment.

Genetic differences among cultivars of the same species may result in differences in their susceptibility to TRV infection (Ghazala and Varrelmann, 2007; MacFarlane, 2010) or TRV-based virus-induced gene silencing (Chen et al., 2004). We confirmed this observation as the different pepper cultivars sampled showed large variations in their TRV-based gene silencing responses. EC showed obvious photobleaching symptoms 13 days postinoculation, whereas A5 exhibited this silencing phenotype 7 days later.

The developmental stage of the plant at the time of inoculation was also found to strongly affect silencing efficiency (Burch-Smith et al., 2006; Deng et al., 2012). VIGS has mostly been used to investigate gene function in young seedlings or during seedling development (Liu et al., 2002; Chung et al., 2004). This implies that the host plant may have a particularly competent stage for *Agrobacterium* infiltration. Through comparisons of inoculation via syringe-infiltration across life stages, we concluded that young seedlings of pepper plants show the highest efficiency in gene silencing. The reason may be that the *Agrobacterium* inoculum is more easily injected in younger leaves.

It has previously been shown that the concentration of agroinoculation affects gene silencing efficiency in some plant species (Chung et al., 2004; Burch-Smith et al., 2006; Zhou et al., 2006). From our results, the *Agrobacterium* inoculum with a final OD₆₀₀ of 1.0 was the most efficient for gene silencing in pepper plants. When the *Agrobacterium* concentration was lower than 1.0, the efficiency of plant infection was low and the photobleaching symptom was not evident. In contrast, when the *Agrobacterium* concentration was higher than 2.0, the host plants expressed more dead blots on the leaves. This is probably because, under these optical conditions, the infectious TRV is more rapidly available in plant cells, causing increased infection.

Temperature is one of the most important factors for good viral spread and effective silencing (Burch-Smith et al., 2004). Several *Solanum* species favor lower temperatures, ranging from 16° to 20°C (Liu et al., 2002a; Brigneti et al., 2004), but the best results in *A. thaliana* are achieved at temperatures of 23°C or higher. Therefore, temperature is an important consideration when optimizing VIGS in new plants species. In our experiment, we observed that the development of good silencing phenotypes with TRV on pepper occurs at 22°C, and it

must be grown at 18°C for 2 days after injection. The low temperature treatment after injection may actually be one of the contributing reasons for the high efficiency observed (Chung et al., 2004). In fact, plants generally grew more vigorously under cooler conditions. Additionally, Fu et al. (2005) showed that conditions of low temperature (15°-18°C) and low humidity (30-40%) enhanced the silencing of *PDS* throughout inoculated tomato plants, flowers, and fruits.

The silencing phenotype of *PDS* has been used as a visual marker for the effectiveness of VIGS in several plants, such as tomato (Liu et al., 2002a), *A. thaliana* (Turnage et al., 2002), *N. benthamiana* (Ratcliff et al., 2001), and pepper (Chung et al., 2004). The silencing of *PDS* produces a typical white color, which occurs in the absence of the *PDS* gene product. Using this optimal protocol, we achieved highly efficient VIGS for *CaPDS* in 4 pepper cultivars (Figure 5A). Moreover, to further verify the efficiency of VIGS, we also examined the chlorophyll content of inoculated plants (Figure 5B). More chlorophyll degradation indicates higher silencing efficiency, and vice versa. Finally, RT-PCR was performed (Figure 5C). From these results, we concluded that the optimized protocol is applicable to other cultivars of pepper. Furthermore, silencing of another gene, *CaPOD*, which is involved in the defense response to pathogens, was also investigated (Figure 6). At 30 days postinoculation, the pTRV-*CaPOD*-inoculated plants showed viral symptoms, but the control plants (pTRV-00; empty vector) had not changed. Therefore, VIGS can likely be used to exploit several significant genes in pepper, such as genes related to disease-resistance.

CONCLUSIONS

VIGS is a widely used, powerful technique for reverse genetics. Vectors derived from the TRV are among the most popular for VIGS. In this study, we optimized the protocol for TRV-induced gene silencing in pepper plants. The optimized TRV-based protocol can be used to effectively silence the reporter gene, *CaPDS*, in different pepper cultivars. Importantly, TRV-based VIGS is also efficient in silencing *CaPOD*, which is a gene involved in the defense response to pathogens. Therefore, this TRV-based gene silencing system provides a rapid and high-throughput technique platform for gene function analysis in pepper.

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