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ORIGINAL PAPER

Sprout vacuum-infiltration: a simple and efficient agroinoculation method for virus-induced gene silencing in diverse solanaceous species

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Abstract Virus-induced gene silencing (VIGS) is a robust technique for identifying the functions of plant genes. Tobacco rattle virus (TRV)-mediated VIGS has been commonly used in many plants. In order to overcome the limitations of existing agroinoculation methods, we report an easy and effective method of agroinoculation for virus-induced gene silencing—sprout vacuum-infiltration (SVI). Using sprout vacuum-infiltration, we have successfully silenced the expression of phytoene desaturase and Mg-protoporphyrin chelatase genes in four important solanaceous crops, including tomato, eggplant, pepper, and *Nicotiana benthamiana*. The gene-silenced phenotypes are conspicuous in 1-week-old plants. The method is simple, low cost and rapid compared to other techniques such as leaf infiltration or agrodrench. It may be more practical for studying gene function in the early stages of plant growth. An important aspect of SVI is that it will be used for high-throughput VIGS screens in the future. SVI will be an effective tool to overcome the limitations of current inoculation methods and to facilitate large-scale VIGS analysis of cDNA libraries.

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Key message SVI is a simple, low cost agroinoculation method for VIGS. It is practical for studying the function of genes expressed in early stages of plant growth and high-throughput VIGS screens.

Keywords Sprout vacuum-infiltration · Virus-induced gene silencing · Agroinoculation · Solanaceous species

Introduction

Virus-induced gene silencing (VIGS) is a transcript suppression technique used for loss-of-function analysis of plant genes. The technique offers an attractive alternative for knocking down target genes of interest and avoids the need for transformation (Baulcombe 1999; Dinesh-Kumar et al. 2003). It is considerably less time-consuming than classical stable transformation approaches. When the virus infects a plant tissue and spreads systemically, the endogenous gene transcripts with sequence identity to the engineered sequence in the viral vector (VIGS-vector) are degraded by post-transcriptional gene silencing (PTGS) (Baulcombe 1999).

Many plant viruses have been developed as VIGS vectors to study gene function in plants, such as tobacco mosaic virus (TMV) (Kumagai et al. 1995), tobacco rattle virus (TRV) (Ratcliff et al. 2001; Liu et al. 2002a), potato virus X (PVX) (Ruiz et al. 1998; Lu et al. 2003; Faivre-Rampant et al. 2004), bean pod mottle virus (BPMV) (Zhang and Ghabrial 2006), barley stripe mosaic virus (BSMV) (Holzberg et al. 2002), tomato golden mosaic virus (TGMV) (Peele et al. 2001) and cabbage leaf curl virus (CaLCuV) (Turnage et al. 2002). Because of wide host range and relatively mild symptoms, TRV-based VIGS vectors had been widely used to silence gene in

Solanaceous plant species (Ratcliff et al. 2001; Liu et al. 2002a, b; Brigneti et al. 2004; Chung et al. 2004), *Arabidopsis* (Burch-Smith et al. 2006; Wang et al. 2006), opium poppy (Hileman et al. 2005), thalictrum (Di Stilio et al. 2010), cotton (Gao et al. 2011), etc. In the TRV vector, RNA1 provides the replication and movement functions, TRV-RNA2 from isolate PPK20 encodes the viral coat protein (cp) (Ratcliff et al. 2001; Liu et al. 2002b). One distinct advantage of using TRV for VIGS is the ability of virus to spread rapidly throughout the entire plant, including meristem tissue, and it has been used to study gene function 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). *Phytoene desaturase* (*PDS*) gene encodes an enzyme that catalyzes an important step in the carotenoid biosynthesis pathway (Cunningham and Gantt 1998). The *PDS* as a reporter gene had been widely and very successfully used because of the silencing phenotype in the past studies (Ratcliff et al. 2001; Liu et al. 2002a; Turnage et al. 2002). In addition, environmental conditions, such as temperature and humidity, play an important role in the VIGS efficiency (Fu et al. 2006).

Plant transformation mediated by *Agrobacterium tumefaciens* has become the most useful method for the introduction of foreign genes into plant cells. Agroinoculation is also a widely used method to deliver VIGS vectors into plants for RNA silencing (Liu et al. 2002b; Gossele et al. 2002; Peart et al. 2002). However, the current agro-infiltration methods have their limitations. Among the existed methods, leaf infiltration method is widely used to introduce the infiltration solution into seedling leaves using a needle-less syringe, vacuum machine, or high-pressure spray gun. However, they are laborious for large-scale VIGS screens. Also, the silencing efficiency of target gene is not similar in different kinds of plants, especially in plants or tissues that are difficult to infiltrate, such as soybean, eggplant and pepper, infiltration operation is very hard and the silencing efficiency is very low. On the other hand, the agrodrench method has been described in Ryu et al. (2004), in which the *Agrobacterium* suspension is used to drench the soil surrounding the plant roots directly. Compared with the leaf infiltration, it is labor-saving, but the target plant requires much more time to get silencing, approximately 1–2 weeks. And TRV2-NbPDS did not silence the *PDS* orthologs of pepper and eggplant. In addition, agrodrench method is more costly and requires substantial bacterial cultures to be applied to the soil adjacent to the crown part of plants. VIGS technology is an excellent tool to identify the function of genes in plants, however, these limitations of these infiltration methods above need to be overcome for the widespread use of VIGS in the future, especially for large-scale screening of candidate genes.

In recent study, we reported a novel highly efficient agroinoculation method, sprout vacuum-infiltration (SVI) for VIGS in plants. Sprouts about 0.5–1 cm in length are infiltrated by vacuum with bacteria containing the TRV-based VIGS vector. SVI can elicit VIGS in 1-week-old germinated seedlings including tomato, pepper, and *Nicotiana benthamiana*, the loss-of-function phenotypes are observed on the first true leaves. The results indicated that we could rapidly identify evidence of in-plant gene function which will only need one-fifth of the time required for other methods. SVI is a cost-efficient and robust agroinoculation method for VIGS to study gene function in plants. It will make VIGS library screening simple and feasible.

Materials and methods

Plant materials and growth condition

Tomato (*Solanum lycopersicum* cv. Ailsa Craig and *S. lycopersicum* cv. Micro-Tom), *N. benthamiana*, pepper (*Capsicum frutescens* cv. Mantianhong), and eggplant (*S. melongena* cv. Changqieyihao) were used for the experiments. Seeds were germinated in flask with sterile water. The flask was placed in a 25 °C shaker for 24 h at the speed of 100 rpm/min. The seeds were transferred to a flat with filter paper and sterile water. After approximately 24 h, the germinating seeds reached 0.5–1 cm in length, and were subjected to SVI. Treated sprouts were sown in pots in a growth chamber (23 ± 2 °C, 20–30 % RH) under a 16/8 h light–dark photoperiod at 600 μE/m^2/s. Fertilizer including soluble trace element mix was applied with water.

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from leaves of wild-type plants *S. lycopersicum* cv. Ailsa Craig and *S.* cv. Changqieyihao) using TRIzol reagent (Invitrogen Co.) according to the manufacturer's manuals, and then was treated with RNase-free DNase (RQ1; Promega Co.). First-strand cDNA was performed using 2 μg of total RNA with M-MLV reverse transcriptase (Promega Co.), oligo d (T) primer and dNTPs in a final volume of 25 μL, and stored at −20 °C.

Plasmid construction

All the constructs used for VIGS were assembled into the TRV-RNA2 vector (Liu et al. 2002a).

pTRV2-LePDS construction

To generate TRV2-*LePDS*, a 421-bp *PDS* fragment was amplified from *S. lycopersicum* cv. Ailsa Craig cDNA by

RT-PCR with primers, p*PDSEco*RIF with an engineered *Eco*RI restriction site and p*PDSBam*HIR with an engineered *Bam*HI restriction site. The resulting PCR product was cleaved with restriction endonucleases *Eco*RI and *Bam*HI and inserted into the *Eco*RI and *Bam*HI sites of the cleaved template plasmid pTRV2.

pTRV2-SmChlH construction

To generate TRV2-*SmChlH*, a 547-bp *Chl H* fragment was amplified from *S. melongena* cv. Changqieyihao cDNA by RT-PCR with primers, p*ChlHEco*RI with an engineered *Eco*RI restriction site and p*ChlHBam*HI with an added *Bam*HI restriction site. The resulting PCR product is cleaved with restriction endonucleases *Eco*RI and *Bam*HI and inserted into the *Eco*RI and *Bam*HI-cleaved template plasmid pTRV2. All primers were listed in Supplemental Table SII.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from leaves of wild-type, silenced and non-silenced (infiltrated with empty vector pTRV1 and pTRV2) plants using TRIzol reagent (Invitrogen Co.) according to the manufacturer's protocols. For semi-quantitative RT-PCR, primers were designed to anneal outside the region targeted for silencing to ensure that only the endogenous gene would be detected. *S. lycopersicum Actin* gene (Genbank no. AB199316-1) severed as an internal control for RNA quantity in RT-PCR with primers, *Slactin*F and *Slactin*R. Semi-quantitative RT-PCR was performed using primers listed in Supplemental Table SI. The primer concentration used for RT-PCR in this assay was 10 μM.

Sprout vacuum-infiltration, leaf infiltration and agrodrench methods

For VIGS TRV1, TRV2 and its derivatives were introduced into *A. tumefaciens* strain GV3101 by freeze–thaw method. Bacterial cells were grown at 28 °C on Luria–Bertani (LB) agar medium or LB broth with appropriate antibiotics.

For sprout vacuum-infiltration, a single colony was selected and inoculated in 2 mL liquid culture of LB with appropriate antibiotics. Bacterial cells were incubated in 14-mL Falcon round bottom polyethylene tubes with shaking at 200 rpm at 28 °C for 10 h. And then, 500 μL of culture was inoculated into a 2–3 mL LB medium containing antibiotics, 10 mM MES and 20 μM acetosyringone, placed in clearly labeled Falcon tubes. The culture was incubated with shaking at 200 rpm at 28 °C for 10 h. Each *Agrobacterium* strain containing TRV1 and TRV2 vectors or its derivatives (TRV-*PDS* or TRV-*chl H*) were mixed in 1:1 ratio. The bacteria cells were harvested by centrifugation and resuspended individually into infiltration buffer with 10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone, pH 5.6, adjusted to an OD of 1.0 and left at room temperature for 3–4 h before infiltration. Silwet L77 was added to a concentration of 0.05 % (v/v) (1 μL per 2 mL of solution) and mixed well immediately. The infiltration mixture and germinating seeds (about 10 seeds) were placed in each 1.5 or 2.0 mL centrifuge tubes. And then, centrifuge tubes were placed into vacuum dryer. *Agrobacterium* was infiltrated into sprouts using sprout vacuum-infiltration system [a vacuum dryer connected to a portable air compressor (GAST. INC)] set at relative vacuum degree of −25 kPa for tomato, eggplant, and pepper, and −18 kPa for *N. benthamiana*. Vacuum pressure was maintained for about 10 s and then released rapidly to atmospheric pressure. This operation was repeated once or twice. Treated sprouts were sown in nutritional soil in pots.

For leaf infiltration, mixture of *Agrobacterium* strain containing TRV1 and TRV2 or its derivatives was infiltrated to the leaves of 2 to 3-week-old plants with 1 mL needle-less syringe.

For agrodrench, mixture of *Agrobacterium* strains containing TRV1 and TRV2 or its derivatives was drenched into the crown part of each plant, 5 mL mixture each seedling.

The extraction and measurement of chlorophyll

0.2 g samples from *PDS* silencing plants and control plants were grinded in 1 mL of 100 % acetone with the help of calcium carbonate and silica sand. The homogenized seriflux was filtered and transferred to a 50 mL volumetric flask. The tubes and filter paper were washed twice or three times with 80 % acetone. The wavelength setting was checked regularly by means of the hydrogen line at 656.3 nm. Absorbance values of extracts in 1 cm pathlength cuvettes were read to three decimal places at the wavelength of 645 and 653 nm using DR3900UV-visible spectrophotometer (Hach Co., USA).

Results

Sprout vacuum-infiltration may be utilized for infecting tomato sprouts with TRV

TRV can infect the different plant tissues such as leaves (Liu et al. 2002a; Dinesh-Kumar et al. 2003), carpopodia (Fu et al. 2005), roots (Ryu et al. 2004) and fruits (Orzaez et al. 2006) by agroinjection (fruit), agrodrench (root), agroinfiltration with a needleless syringe, vacuum and

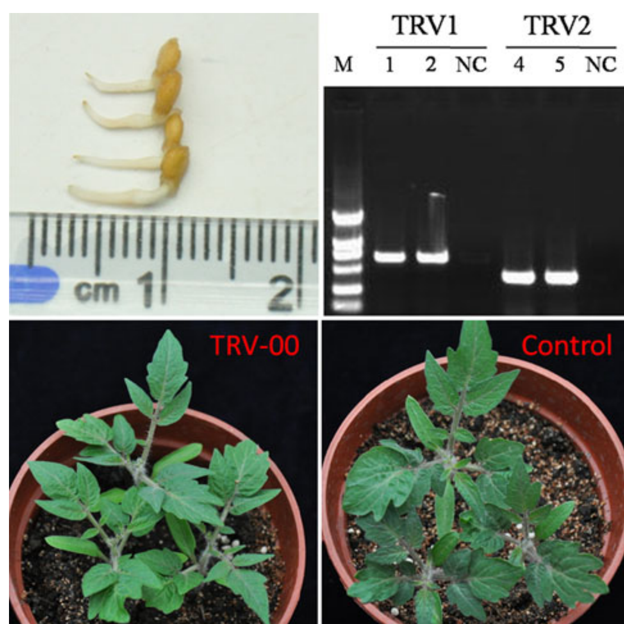

Fig. 1 Recombinant TRV infects tomato sprout. **a** The sprouts about 0.5–1 cm in length were infiltrated with *Agrobacterium* containing TRV1 and TRV2. **b** RT-PCR analysis of RNA1 and RNA2 of TRV in tomato seedling. RNA was extracted from the tomato seedlings (infected and control) and was used to generate the first-strand cDNA for use as a template in RT-PCR. *Lane 1* and *2* represents TRV–RNA1-specific product. *Lane 4* and *5* represents TRV–RNA2-specific product. Lane NC represents the negative control in which RT-PCR mix with the cDNA from no-infiltration tomato seedling. *Lane M* is the DNA size marker (DL2000). **c** Three-week-old tomato seedlings of treatment group (*left*) and control group (*right*). No different phenotypes were observed between two groups

high-pressure spray (leaves). The sprouts come from seeds and develop to seedlings. And roots, leaves and fruits of plant are all after sprouts. On the other hand, sprouts are easy to obtain in short time about 2 days, which are all consistent and young. Taking into account its advantages above, it may be an idea subject for initiating VIGS. To test whether the TRV-vector can directly infect the tomato sprouts, a mixture of *Agrobacterium* (GV3101) cultures containing pTRV1 and pTRV2 constructs in a 1:1 ratio was vacuum-infiltrated into the sprouts using a infiltration system [a vacuum dryer connected to portable air compressor (GAST. INC)] (Appendix S1). Sprouts approximately 0.5–1.0 cm in length were appropriate for VIGS experiments (Fig. 1a). Multiple agroinfiltration experiments were performed simultaneously. Total RNA was extracted from the newly developed leaves (2 weeks after sowing), and was used to generate the first-strand cDNA for use as a template in RT-PCR. Evidence of successful infiltration was observed as the TRV-RNA1 and TRV-RNA2-specific PCR products were detected only in the TRV-infiltrated seedlings, and were absent in the control (no infiltration) (Fig. 1b). The identity of the PCR products was further confirmed by sequencing, and the resulting sequence was consistent with the fragment of RNA2 included in the plasmid pYL156 (GenBank accession number AF406991, data not shown). These results clearly demonstrated that recombinant TRV could efficiently infect tomato sprouts by vacuum-infiltration. No different phenotypes were observed between treatments and control groups 3 weeks after infection (Fig. 1c). This showed that the sprouts infection would not affect plant normal development later. The results above suggested that sprout vacuum-infiltration could be used to establish the VIGS system in tomato.

SVI can elicit VIGS in 1-week-old tomato seedlings and extend to the reproductive organs (flowers and fruit)

The TRV-based VIGS system had been established in tomato seedlings (Leaf infiltration) (Liu et al. 2002a) and fruit (carpopodia-injection) (Fu et al. 2005). To test whether SVI can be used to silence target genes in tomato seedlings, the endogenous *PDS* gene was used as a reporter gene. *PDS* silencing will inhibit carotenoid biosynthesis and causes silencing plants to exhibit a photo-bleached phenotype (Liu et al. 2002a). A mixture of *Agrobacterium* cultures containing pTRV2 carrying tomato *PDS* (pTRV2-*siPDS*) and pTRV1 was vacuum-infiltrated into the tomato seed sprouts with 0.5–1 cm length, then the infected sprouts were sowed into soil and kept for normal growth in standard condition. Photo-bleaching was observed on the first true leaves of tomato seedling 1 week post-infection and sowing (Fig. 2a). Compared with described leaf infiltration methods, the photo-bleaching phenotype was more obvious in the plants agroinoculated by SVI. All true leaves of some infected plants displayed the white phenotype 2 weeks post-infection (Fig. 2b), however, *PDS* silencing effect cannot be achieved by the use of leaf infiltration and agrodrench. The above results showed that SVI was particularly practical for studying gene function in the early stages of plant growth.

To further confirm *siPDS* suppression at the molecular level, semi-quantitative RT-PCR was performed. Primers were designed for regions of the *PDS* gene outside the region targeted for silencing to assess the accumulation of the endogenous transcript. As shown in Fig. 2c, *siPDS* RNA in the photo-bleached leaves was reduced by more than 90 % when compared with the control plants infiltrated with TRV alone. In contrast, the level of *Actin* gene mRNA was similar in *siPDS*-silenced and control leaves demonstrating that the affected plants were not displaying a pleiotropic loss of mRNA stability, suggesting that the effects were transcript specific. In addition, the photo-bleached phenotype is directly related to low content of the total chlorophyll. Compared with the control leaves, total

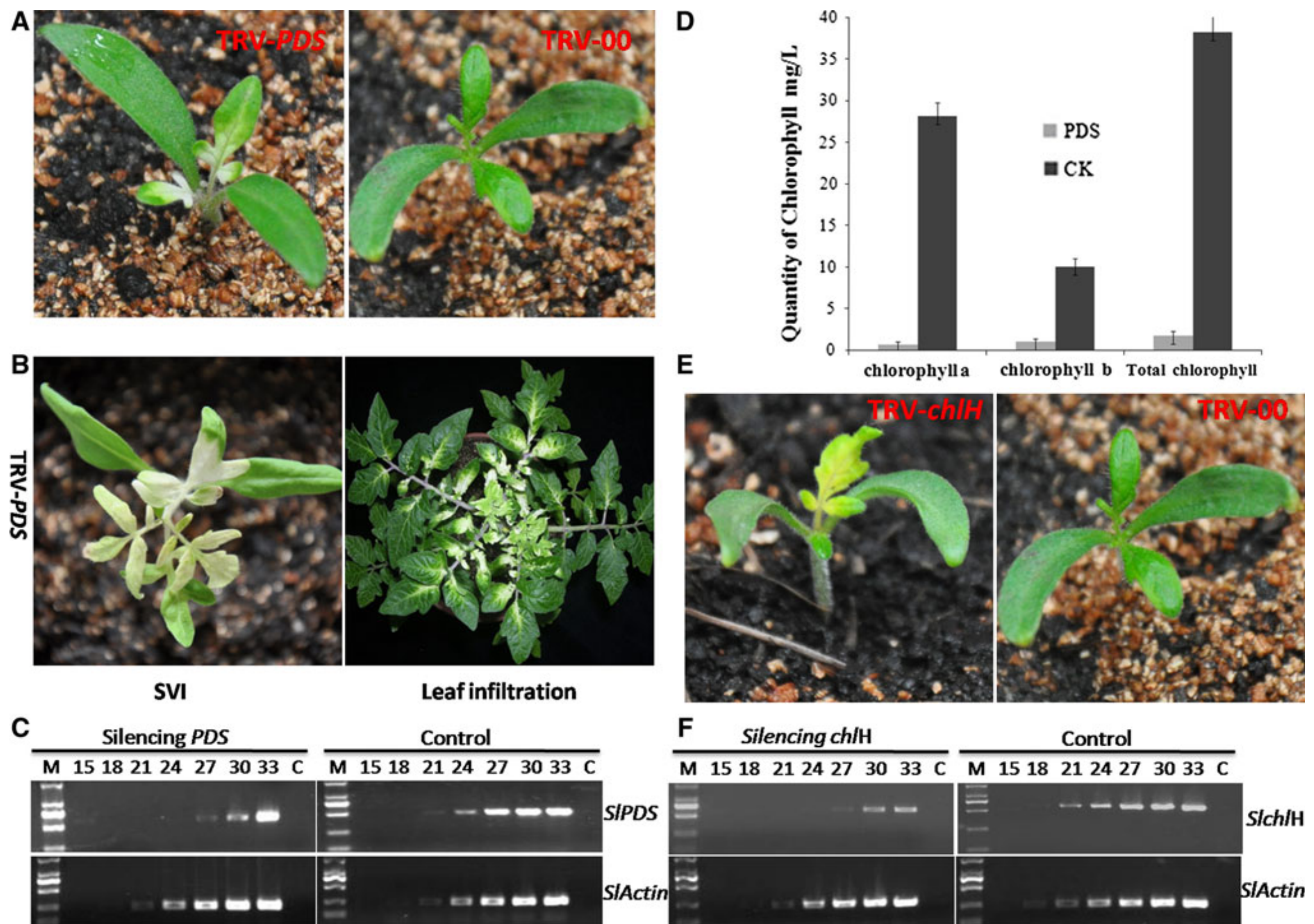

Fig. 2 Silencing of tomato *PDS* and *Chl H* causes visible phenotypic symptoms. **a** Sprout vacuum infiltration allows efficient silencing of *SlPDS* as shown by the photo-bleaching on the first true leaves of silenced plant (*left*). Control plant infiltrated with empty TRV2 vector (*right*). **b** Photo-bleaching phenotype of tomato seedlings treated with leaf infiltration (*right*) and sprout vacuum-infiltration (*left*). **c** The semi-quantitative RT-PCR analysis mRNA levels in control (*right*) and *LePDS*-silenced (*left*) tomato leaves effective silencing of gene indicated. Number of PCR cycles is indicated *above* lanes. *Lane M* is the DNA size marker (DL2000) and *C* is no RT control. Actin is a loading control. **d** Content of chlorophyll *a*, chlorophyll *b* and total chlorophyll in *PDS* silenced and control plants. Compared with control plant, the total chlorophyll content was reduced nearly 87 % in the *PDS* silenced plant. **e**, **f** Suppression of *Chl H* gene causes *yellow* color leaves in tomato, and the semi-quantitative RT-PCR indicate effective silencing of genes indicated. Photograph was taken 7 days after inoculation (color figure online)

chlorophyll content was reduced 87 % in photo-bleached leaves (Fig. 2d). The method was further validated by silencing a separate transcript. Identical protocols were employed to silence the transcript from the Mg-protoporphyrin chelatase (*Chl H*), which was a key gene required for chlorophyll biosynthesis (Hudson et al. 1993). The first true leaves of treated tomato seedlings exhibited yellow within 1 week of treatment (Fig. 2e), consistent with a reduction in transcription level of *Chl H* gene (Fig. 2f). The results showed that sprout vacuum infiltration is an effective agroinoculation method for VIGS in very young seedlings; the target endogenous genes can be silenced efficiently in 1-week-old plants.

In addition, VIGS based on TRV had been used to study gene function in reproductive organs, such as flowers (Liu et al. 2004; Chen et al. 2004), and fruits (Fu et al. 2005; Orzaez et al. 2006). In our results, about 2 months after agroinfiltration, systemic silencing of *PDS* gene can be achieved in carpopodium, petal, sepal and fruit in *S. lycopersicum* cv. Micro-Tom (Fig. 3). These results showed that SVI was a simple and effective agroinoculation method to elicit VIGS in the entire tomato plant, including the vegetative tissues (leaves) and the reproductive organs (flowers and fruits).

SVI is more cost effective than agrodrench and leaf infiltration of agroinoculation for VIGS in plant

It was important to test the gene silencing efficiency of the SVI method compared to other agrointroduction methods such as leaf infiltration or agrodrench. One-hundred seeds or seedlings were tested using these methods over five

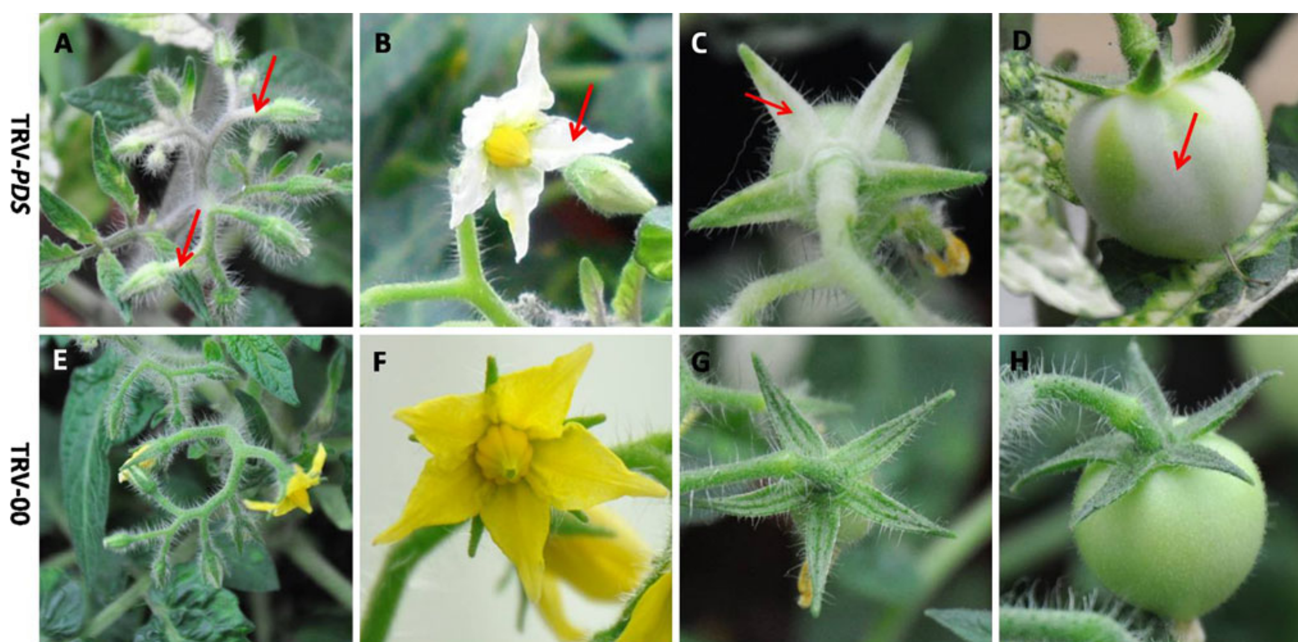

Fig. 3 Phenotype of *PDS* silencing was observed in carpopodium, petal, sepal and fruit in *Solanum lycopersicum* cv. Micro-Tom. Photograph was taken from eight to 12 weeks after agroinfiltration. Photo-bleaching in **a**–**d** indicated efficient silencing *PDS* in carpopodium, petal, sepal and fruit, respectively. **e**–**h** were control, agrobacterium cultures transformed with TRV alone were mixed in a 1:1 ratio. *Red arrows* indicate the photo-bleaching site (color figure online)

independent replicates. The resulting plants were visually screened for evidence of *PDS* silencing. Nearly 100 % infected plants showed the silencing phenotype. In addition, the detailed data for each method were recorded, including the amount of medium (LB) and infiltration buffer, the infiltration time and total cost. The results are shown in Table 1, and show that compared with leaf infiltration and agrodrench, SVI required less infiltration solution and could be performed at a lower cost. On average, SVI required only 6.25 % of the bacterial culture medium and infiltration buffer compared to leaf infiltration and 1 % the amount required for agrodrench. Therefore, the cost was much lower than other methods, and the space to grow such substantial cultures was not required. An important advantage of SVI for VIGS was that the handling time was greatly reduced compared with other methods. It required only half a minute for agroinoculation for a large number of sprouts using SVI. The savings of materials and time were substantial advantages to the SVI method.

Table 1 Statistical results on the amount of LB medium, infiltration buffer, operating time and total cost of three infiltration methods (including leaf infiltration, agrodrench and sprout vacuum) for 100 seedlings or sprouts

Method	Prepared bacterial (mL)	Infiltration solution (mL)	Operating time (min)	Cost ($)
Leaf infiltration	80	100	200	25
Agrodrench	500	500	30	80
Sprout vacuum	5	5	0.5	1.5

The time from seed-sowing to initial presentation of the photo-bleaching phenotype was also recorded in detail for all experiments. The VIGS *PDS* phenotype could be observed in 1-week-old tomato seedlings. However, for leaf infiltration or agrodrench, we first needed to prepare 2–3 weeks old seedings for infection. And these infected plants could appear *PDS* silencing phenotype around 2 week post-infection. It means that the VIGS *PDS* phenotype performed by these two methods could be observed in 4- to 5-week-old tomato seedlings. The SVI method reduced the time required to observe gene silencing phenotypes by approximately 3–4 weeks (Fig. 4).

Our statistical results suggested that SVI was a simple, fast and low cost agroinoculation method for VIGS experiments in tomato, with minimizing manpower and laboratory resources. Compared with leaf infiltration and agrodrench methods, the new method had substantial advantages of objectivity.

SVI is effective in various Solanaceae species

The TRV-based VIGS system had been successfully used in many *Solanum* plants using several different agroinfiltration methods (Brigneti et al. 2004; Ryu et al. 2004), but

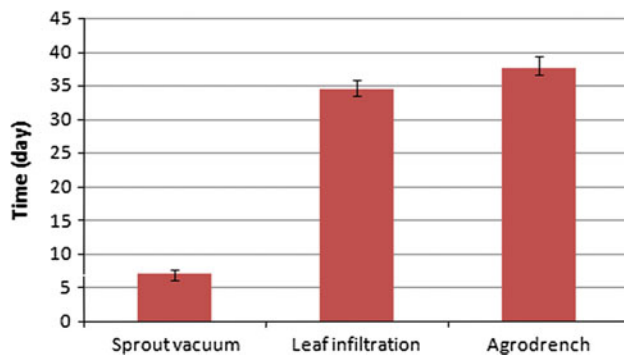

Fig. 4 Statistical results on days required for the appearance of silencing phenotype using sprout vacuum infiltration, leaf infiltration and agrodrench of agroinoculation methods. Experiment was conducted five times with 100 young seedlings or germinated seeds in each experiment. The phenotype of silencing *PDS* was used as a visual marker

the efficiency of gene silencing varies greatly in different species using these methods. For example, tobacco leaves were more easily infiltrated than other Solanaceae species using leaf infiltration. Perhaps variation in leaf anatomical structures made some plant leaves were difficult to infiltrate. However, for newly sprouted seeds, the structures were similar even between distantly related species, so the SVI method may present a widely applicable solution for VIGS vector introduction.

In order to test the usefulness of the SVI method in other species, we tested the effect in several Solanaceae species including *N. benthamiana*, pepper and eggplant. Previous studies have shown that it is not possible to silence *PDS* in eggplant using agrodrench (Ryu et al. 2004). Here *Chl H* and *PDS* were again used as visual markers for endogenous gene silencing in our experiments. Considering the gene coding sequences are sufficiently conserved among Solanaceae species, we used TRV-*SlPDS* and TRV-*SmChlH* (partial sequence of *Chl H* clone from eggplant) to silence the endogenous *PDS* and *Chl H* orthologs in this range of species. An identical protocol for SVI was implemented. Germination approached 100 % for all species in 2 days. Photo-bleaching was observed on the first true leaves of *N. benthamiana* and pepper after 7 days, and eggplant after 2 weeks. Similarly, yellow leaves were observed on newly developed leaves infiltrated by TRV1 and TRV-*SmChlH* system (Fig. 5a). Approximately 100 % of the infected plants showed gene silencing phenotypes in these species. The appearance of silencing phenotype developed later in eggplant seedlings, which may be due to the time required for vegetative growth during early plant development. We further tested *PDS* and *Chl H* suppression at the molecular level by semi-quantitative RT-PCR. RNA was isolated at 10 days post-inoculation in *N. benthamiana*, pepper, and 15 days in eggplant. RNA was also isolated from leaves of TRV2-*siPDS*, TRV2-*siChlH* and TRV2-empty vector-inoculated plants as controls. The RT-PCR results showed that expression of target genes *PDS* (Fig. 5b) and *Chl H* (Fig. 5c) was significantly inhibited. The above results indicate that SVI is easily applied to other Solanaceae

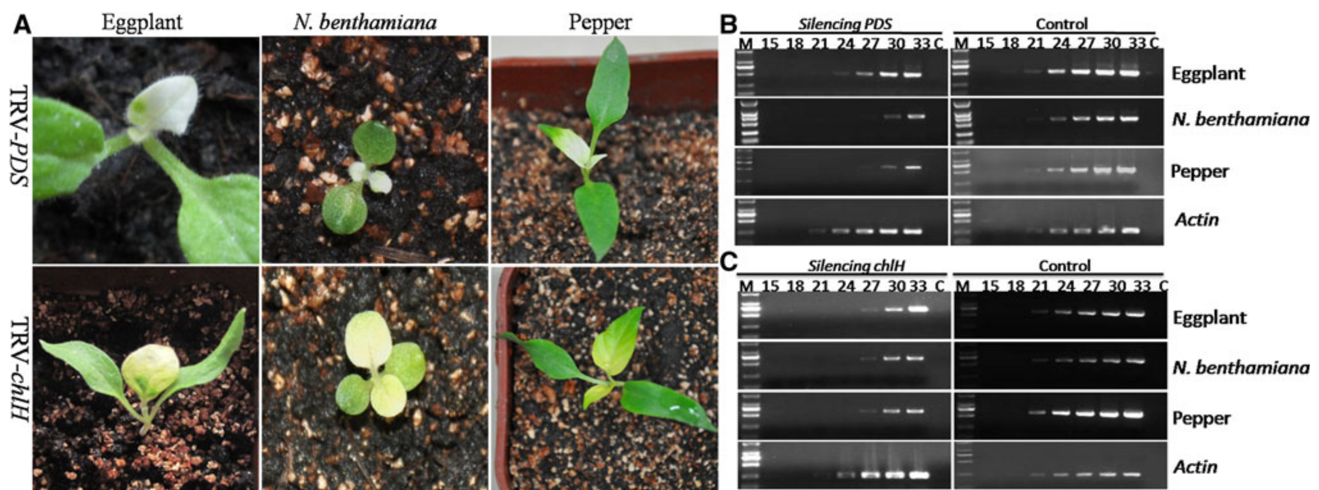

Fig. 5 Selected phenotypes observed with silencing of *PDS* and *Chl H* in eggplant, *N. benthamiana*, pepper. **a** Silencing *PDS* and *Chl H* resulting in photo-bleaching and yellow color leaves in eggplants, *N. benthamiana*, pepper. *Agrobacterium* containing either TRV-*LePDS* or TRV-*SmChl H*, along with *Agrobacterium* containing pTRV1, was applied for agroinoculation by the sprout vacuum-infiltration method. Pictures of *N. benthamiana* and pepper were taken at 1 week post-inoculation, and the picture of eggplant was taken at 2 weeks post-inoculation. **b** mRNA levels of *PDS* in control (*right*) versus silenced (*left*) tissue indicate effective silencing of genes indicated. Number of PCR cycles is indicated *above* lanes. *Lane M* is the DNA size marker and *C* is no RT control. Actin is a loading control. **c** mRNA levels of *Chl H* in control (*right*) versus silenced (*left*) tissue indicate effective silencing of genes indicated. Number of PCR cycles is indicated *above* lanes. *Lane M* is the DNA size marker and *C* is RT control. Actin is a loading control

species, and produces evidence of gene silencing at high frequency.

Discussion

We report here the new agroinfiltration method, sprout vacuum-infiltration, for virus-induced gene silencing. This method allows rapid, efficient to achieve silencing of target genes and has the added advantage of doing so at markedly reduced costs. By comparing the time and space consuming, the bacterial culture medium and infiltration buffer required for different infiltration methods show that SVI method described in this report would be an improvement for in plants loss-of-function tests of gene function, which is particularly practical for studying the gene function in the early stages of plant growth, especially suitable for plants that are difficult to be infiltrated. Previously described VIGS methods have presented evidence of transient suppression of gene expression in different periods during plant growth and development, such as in foliar tissues (Ratcliff et al. 2001), flowers (Liu et al. 2004; Chen et al. 2004), fruits (Fu et al. 2005), and roots (Valentine et al. 2004). However, 2- to 3-week-old seedlings were commonly used for VIGS in these agroinoculation methods, and it requires more than 10 days for gene silencing phenotypes to develop and by that time the age of the plant is at least 5–6 weeks. Therefore, current agroinoculation method limits the application of the technology to studies of in-plant gene function during early stage of vegetative growth, especially for 1- to 3-week-old seedlings. In our results, SVI could elicit VIGS in 1-week-old tomato, pepper, and *N. benthamiana* seedlings; photo-bleaching was shown on the first true leaves of diverse solanaceous species. Therefore, we concluded SVI would be a robust agroinfiltration method for virus-induced gene silencing. These findings make the approach especially applicable for studies of important seedling behaviors during establishment.

Most importantly, the silencing effect of the SVI method was higher than the leaf infiltration method, and the photo-bleaching phenotype resulting from silencing of *PDS* gene was observed on carpopodium, petal, sepal and fruit. These results suggested that sprout infection operation would not affect plant normal development. The virus could spread throughout all organ of the infected plants and elicit gene silencing. Furthermore, the two current methods would require a significant investment in labor and material resources, as well as space and time. However, all of these potential barriers may be overcome using the SVI method, seed germination are sowed directly into the soil and do not need a separate nursery space.

In addition, VIGS in roots had been demonstrated using the TRV Vector system (Ryu et al. 2004) and modifications of the TRV Vector system (Valentine et al. 2004). As agroinoculation had been performed before root morphogenesis, we speculated that SVI would be a useful agroinoculation method for VIGS in roots. More studies are required to determine the accuracy of the speculation. Taken together, these results indicate that the sprout vacuum-infiltration is an excellent method for introduction of *Agrobacterium* into plants for VIGS, providing a way to systemically silence endogenous genes rapidly, with symptoms clearly evidence on 1-week-old seedlings. Therefore, SVI is an improved method to elicit VIGS in young plants, which can elicit VIGS after 1 week of tomato seed germination. The great advantage is the ability to rapidly supplant, or perhaps complement, studies in stable transgenic plants.

Further, high-throughput screening (HTS) will be a method for scientific experimentation especially used in studying the function of genes. The large number of expressed sequence tags (ESTs) deposited in public databases is a valuable resource to develop special genes expressed in specific stages of plant development. And a large number of candidate genes of interest could be examined at the levels of transcriptome, proteome, and metabolome in present. Furthermore, suppression subtractive hybridization (SSH) cDNA library is an advance in screening and cloning novel genes expressed differentially between two tissues, experimental conditions or treatment (Diatchenko et al. 1996). Using existing methods, it is possible to construct VIGS cDNA libraries (Liu and Page 2008; Todd et al. 2010), only that the T-vector in the classic SSH cDNA library techniques is replaced by the TRV-RNA2 plasmid (pYL156). TRV2-LIC vector was readily accessible for generating large-scale gene silencing vectors for VIGS (Dong et al. 2007). Therefore, we can generate hundreds of silencing vectors for VIGS based on TRV-LIC background (Dong et al. 2007) and VIGS cDNA libraries (Liu and Page 2008; Todd et al. 2010).

Agroinoculation methods for VIGS had been reported in the literatures (Liu et al. 2002b; Gossele et al. 2002; Ryu et al. 2004). However, agroinfiltration currently used including leaf infiltration and agrodrench were laborious and time consuming for large-scale VIGS experiments. In our results, *Agrobacterium* containing TRV1 and TRV2-*PDS* or TRV2-*Chl H* could be infiltrated into sprouted seeds of four solanaceous species, respectively, at a time using the new agroinfiltration method. It only took half a minute to complete the whole agroinfiltration process. Agroinfiltration of newly germinated seeds with TRV1 and multiple TRV2 constructs carrying genes of interest or candidate clones from a VIGS cDNA library could be performed simultaneously by the vacuum agroinfiltration system. SVI method presents significant practical advantages for large-scale screening of candidate genes

compared to parallel methods, such as simple, timesaving, cost-effective, etc. Therefore, a high-throughput phenotypic screen based on TRV2-LIC-ESTs and VIGS cDNA libraries candidates would be simpler using SVI than other existing infiltration methods. TRV can spread in seedlings, petal, sepal and fruit resulting from the infiltrated sprouts. Therefore, SVI will be a better agroinoculation method to implement large-scale VIGS experiments with minimal manpower and materials, in a relatively short time. TRV2-LIC vector and VIGS-cDNA libraries from subtractive hybridization will be powerful tool for both forward and reverse genetics experiments in plants using the SVI method.

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