Biological Sciences

Effectively organ-specific virus induced gene silencing in tomato plant

Tian Wang, Li Wei Wen, Hong Liang Zhu*

The College of Food Science and Nutritional Engineering, China Agricultural University, No. 17 Tsinghua East Road, Beijing 100083, China

Virus-induced gene silencing (VIGS) is a valuable tool for identification and characterization of genes function. To improve the efficiency of VIGS in different organ, we developed an organ-specific VIGS that could be applied to tomato (*Solanum lycopersicum* cv Micro Tom) leaves, flowers and fruits respectively. With *phytoene desaturase* (*PDS*) as a reporter gene, almost up to 100% of efficiency of VIGS was achieved in tomato leaves, flowers and fruits. The suppression of *PDS* gene in these organs was also up to 90% compared with control tomato plants. In addition, a few sucrose in infiltration buffer is important for VIGS in flowers and fruits, which could help the *Agrobacterium* culture attached to organ longer and largely increase the time of infection. In short, organ-specific VIGS would shed light on rapid characterization of key genes function in whole tomato growth and reproductive development. *Journal of Nature and Science, 1(1):e34, 2015.*

Solanum lycopersicum | virus-induced gene silencing | phytoene desaturase gene | tobacco rattle virus

Virus-induced gene silencing (VIGS) is a powerfully handy tool for identification of genes function (Senthil-Kumar and Mysore 2011), which is an easy, rapid, reliable and transformation-free method (Lange *et al*. 2013). Normally, partial sequence information of one gene is sufficient to silence itself. Recombinant virus vectors carried part of a gene is agro-infiltrated into a plant and induce the obvious phenotype only in a few weeks (2-4 weeks) (Lange *et al*. 2013). VIGS has been widely used for many plant species(Huang *et al*. 2012) such as *Arabidopsis*, tobacco, tomato, rice, maize, grape, apple and pear, and for validation of functional genes involved in plant development (Liu *et al*. 2010), virus infection(Caplan *et al*. 2008), disease resistance(Rowland *et al*. 2005), insect resistance(Mantelin *et al*. 2011), abiotic stress tolerance(George *et al*. 2010) and nutrient stress(Pacak *et al*. 2010). It is so difficult to characterize key metabolic and regulatory genes at late developmental stages by classical plant transformation method, whose loss-of-function mutants show lethality and severe growth arrest at early developmental stages(Burch-Smith *et al*. 2004). Many researches suggest VIGS is one of the most powerful tools for the analysis of key genes which mutations cause embryonic and seedling-lethality(Robertson 2004). However, Short duration of VIGS in plant is not good for validation the function of genes from seedlings to the terminal growth stage and reproductive development(Senthil-Kumar and Mysore 2011). Long-duration VIGS is likely to be potentially substitute for the need for mutants or stable RNA interference (RNAi) lines(Senthil-Kumar and Mysore 2011), but it depends on appropriate conditions. Moreover, low silencing efficiency in long-duration VIGS is another problem for study the gene function in whole plant development. Organ-specific VIGS may be possible to address above problems, which can induce the silencing of one gene in leaves, flowers and fruits respectively.

Due to its consumption, today tomato absolutely occupies a economically important position in world vegetable production (FAOSTAT 2012; http://faostat.fao.org/). The tomato genome sequencing being recently finished, it becomes more convenient to find the sequence information of genes and more urgent to determine functions of many yet to be characterized genes.. VIGS is increasingly being used in tomato to elucidate gene function(Sahu *et al*. 2012). Various key regulating factors involved in ethylene synthesis and leaf abscission have been functional exploration as well as fruit color development(Sahu *et al*. 2012; Fantini *et al*. 2013), such as *LeHB-1*, *SlEBF1/2*, *LeEIN2*, *LeRIN*, *SlTAPG* and *SlMYB12*. However, VIGS could not completely repress the expression of target gene, and phenotype may not at all be observed in the silenced plant. In addition, the level of silencing by VIGS may also vary between plants and experiments. Compared to *S. lycopersicum*, *S. pennellii* showed more efficient gene silencing and the phenotype of silencing was clearly showed on stems, flowers and fruits(Senthil-Kumar and Mysore 2011). It will be difficult for the analysis of results when the VIGS does not produce a visible phenotype. Normally, a marker gene could be use for visualization of the silenced regions, such as *phytoene desaturase* (*PDS*)(Liu *et al*. 2002), *green fluorescence protein* (*GFP*)(Quadrana *et al*. 2011) and *Antirrhinum majus Delila* and *Rosea1* expressing transcription factors(*Del*/*Ros1*)(Orzaez *et al*. 2009). PDS catalyzes a key step in the carotenoid biosynthesis pathway, and is important for chlorophyll. Inhibition of *PDS* expression results in the decrease of carotenoids and the destruction of chlorophyll(Kumagai *et al*. 1995), which induces that leaves turn to white(Liu *et al*. 2002). Here we show organ-specific VIGS in tomato plants using *PDS* as marker gene, almost completely repressing *PDS* in leaves, flowers and fruits. It would shed light on rapid characterization of key genes function in whole tomato growth and reproductive development, especially for the genes whose mutation is lethal for plant development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The seeds of tomato cultivar MicroTom were planted in commercial tomato-cultivated soil. All plants were grown in the greenhouse at 22°C with 75% relative humidity under a 16 h light/8 h dark cycle.

Constructs and VIGS Treatments

For the VIGS of tomato Micro-Tom, the pTRV1 and pTRV2 vectors were adopted. The construction of pTRV2-*PDS* was described before(Fu *et al*. 2005). Agrobacterium strain GV3101 containing pTRV1, pTRV2 and pTRV2-*PDS* vectors were grown at 28 °C in LB medium (pH 5.6) containing 10 mM Morpholineethanesulfonic acid and 20 μM acetosyringone with kanamycin, gentamycin and rifampicin antibiotics. After shaking for 16 h, cultures were harvested and resuspended in infiltration buffer (10 mM $MgCl_2$, 200 μM acetosyringone) to a final O.D.600 of 1.8. For injection of flower and fruit, the infiltration buffer contained 5% sucrose. Resuspensions of pTRV1 and pTRV2 or pTRV2-*PDS* were mixed at a ratio of 1:1 and left at room temperature for 3 h. *Agrobacterium* was infiltrated into the cotyledon of seedling, the shoot of seedling and the carpopodium of fruit respectively with a 1 mL syringe. Tomato infiltrated with pTRV1 and pTRV2 was used as control. Each inoculation was carried out three times and each time six different organs were infiltrated. When VIGS phenotype was visible, tomato leaves, flowers and fruits were collected and stored in -80℃.

Conflict of interest: No conflicts declared.

*Corresponding Author: Professor Hong Liang Zhu, Ph.D., The College of Food Science and Nutritional Engineering, China Agricultural University, No. 17 Tsinghua East Road, Beijing 100083, China. Email: hlzhu@cau.edu.cn

TABLE 1 List of Primers Used in This Study

Primer Name	Sequence (5'-3')
TRV RT primer	GGGCGTAATAACGCTTACGTAGGC
CP For	CTGACTTGATGGACGATTCTT
CP Rev	TGTTCGCCTTGGTAGTAGTA
TRV2-*PDS* For	GTCGACGCTTTACCCGCTCCTT
TRV2-*PDS* Rev	ATCGATTGCACTACCGTCACTC
PDS For for qRT-PCR	AGTTAGTCGGAGTACCTGTG
PDS Rev for qRT-PCR	AGTGAGCTTCTGCTGAAGAG
Actin For for qRT-PCR	CAGCAGATGTGGATCTCAAA
Actin Rev for qRT-PCR	CTGTGGACAATGGAAGGAC

For for forward; Rev for reverse.

Gene Expression Analysis

Total RNA was isolated from TRV control and TRV-*PDS* samples using DeTRNa reagent (EarthOx, CA, USA). The RNA concentration and purity were measured using a NAS-99 spectrophotometer (ATCGene, NJ, USA). The RNA integrity was checked by agarose gel electrophoresis. 2 μg total RNA was used for the first-strand cDNA synthesis using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Trans, Beijing, China) with oligo(dT) primer or TRV RT primer. RT-PCR was performed using EasyTaq PCR SuperMix (Trans, Beijing, China) with PCR system T-100 (Bio-rad, CA, USA). RT-PCR conditions for *CP* and *PDS* were as follows: 94 °C for 10 min, followed by 24 cycles of 94 °C for 30 s, 55 °C for 30 s and 72°C for 30 s. The quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix with a BIO-RAD real-time PCR System CFX96 (Bio-rad, CA, USA). *PDS* primers that anneal outside the region targeted for silencing were used to ensure that only the endogenous gene would be used. qRT-PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Fluorescence changes of SYBR Green were monitored automatically in each cycle, and the threshold cycle (Ct) over the background was calculated for each reaction. Samples were normalized using *Actin* and the relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ analysis method. Oligonucleotide primers used in this study are listed in Table 1. All RT-PCR data presented are representative of three independent experiments.

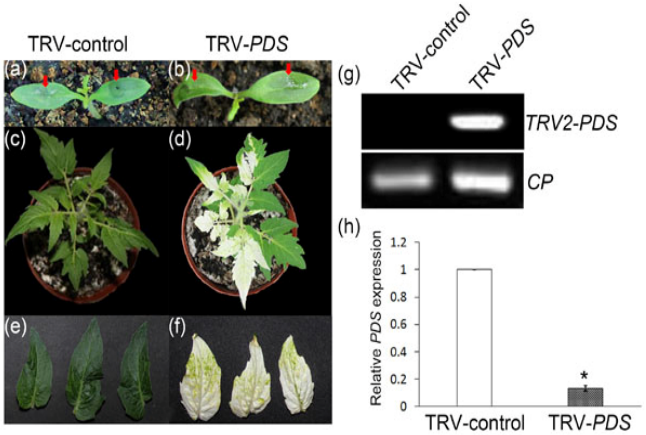

Fig 1. Efficient *PDS* silencing in tomato leaves.
(a) and (b), the sites of injection on cotyledon of tomato infiltrated with TVR control and TRV-*PDS* respectively. Red arrows indicated the sites of injection. (c), TRV control tomato plant. (d), *PDS* silenced tomato plant showing photo bleaching phenotype. (e) and (f), close up from control tomato leaves and *PDS* silenced leaves respectively. (g), RT-PCR detection of recombinant TRV RNA in upper uninjected leaves. RNA samples were extracted from TRV-*PDS* and TRV control tomato plant, and RT-PCR was performed with TRV2-*PDS* and *CP* primers. (h), qRT-PCR analysis of *PDS* transcript in TRV control and TRV-*PDS* tomato plants. *Actin* expression values were used for internal reference. The relative level of *PDS* transcripts was normalized to that in TRV control plants where the amount was arbitrarily assigned a value of 1. Error bars indicate ±SD of three biological replicates, each measured in triplicate. Asterisks indicate significant difference as determined by Student's t-test ($P \leq 0.01$).

RESULTS AND DISCUSSION

Efficient VIGS of *PDS* Gene Induced Bleach Phenotype in Tomato Leaves

PDS silencing by VIGS inhibits carotenoid biosynthesis, causing the plants to exhibit a photo-bleached phenotype. Compared to 100% efficiency in tobacco, the *Agrobacterium* infiltration method of infecting TRV-*PDS* resulted in the *PDS* silencing phenotype in only five out of 10 tomato plants (50% efficiency) (Liu *et al*. 2002). In addition, another spray method improved the efficiency of silencing in tomato to 90%(Liu *et al*. 2002). However, the spray method needs more instrument such as airbrush and air compressor, as well as good hand to get representative results. Considering compact architecture of the young tomato leaves, we wondered whether tomato cotyledon could be a good target for *Agrobacterium* infiltration. In order to do so, we infiltrated *Agrobacterium* cultures containing pTRV2-*PDS* and pTRV1 into the cotyledon of one-week tomato seedling (Fig. 1a and 1b). After two weeks, compared with TRV control plants (Fig. 1c), all the 20 seedling infiltrated with TRV-*PDS* exhibited the photo-bleached phenotype (Fig. 1d), the efficiency of silencing is almost 100%. Young leaves of TRV control plants kept green (Fig. 1e), whereas the young true leaves of tomato totally turned to white (Fig. 1f), which phenotypes were exactly the same as those observed in tomato by VIGS(Liu *et al*. 2002). To further determine whether photo-bleached phenotype was induced by the silencing of *PDS*, we firstly detect the TRV-*PDS* virus of young leaves in RT-PCR assay, a result clearly indicating recombinant TRV can efficiently replicate and spread systemically in tomato plants (Fig. 1g). Secondly, qRT-PCR analysis suggested *PDS* was silenced by 87% in white leaves compared with leaves infiltrated with TRV alone (Fig. 1h). Together these results indicated infiltration through cotyledon is more efficient in the induction of silencing in tomato leaves, which suggested that other important genes in leaves could be targeted for silencing in a similar manner.

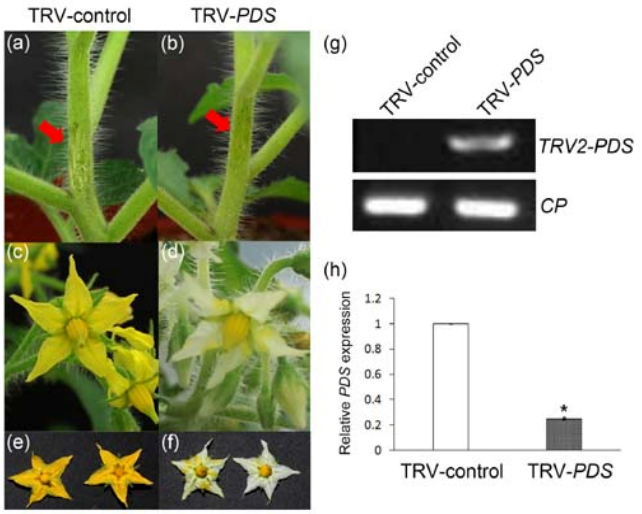

Fig 2. Induced silencing of *PDS* gene by VIGS in tomato flowers.
(a) and (b), the sites of injection on shoot of tomato infiltrated with TVR control and TRV-*PDS* respectively. Red arrows indicated the sites of injection. (c), TRV control tomato flowers. (d), *PDS* silenced tomato flower petals showing photo bleaching phenotype. (e) and (f), close up from control tomato flowers and *PDS* silenced flowers respectively. (g), RT-PCR detection of recombinant TRV RNA in upper uninjected flowers, which were conducted as in Fig 1g. (h), qRT-PCR analysis of *PDS* transcript in TRV control and TRV-*PDS* tomato flowers, which were conducted as in Fig 1h. Asterisks indicate significant difference as determined by Student's t-test ($P \leq 0.01$).

Directly Induced Silencing of *PDS* Gene by VIGS in Tomato Flowers

Normally，plant development can be divided into vegetative and reproductive growth phases. Flowering is critical for the reproduction of angiosperms(Luo *et al*. 2013). VIGS has been used to identify the function of genes involved in flower development

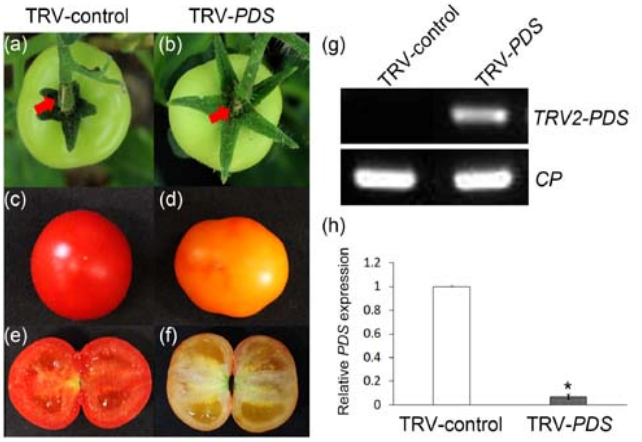

Figure 3. *PDS* gene silencing in whole tomato fruit.
(a) and (b), the sites of injection on carpopodium of tomato fruit infiltrated with TVR control and TRV-*PDS* respectively. Red arrows indicated the sites of injection. (c), TRV control tomato fruit. (d), *PDS* silenced tomato fruit showing yellow color. (e) and (f), close up from control tomato fruit and *PDS* silenced fruit respectively. (g), RT-PCR detection of recombinant TRV RNA in upper uninjected fruits, which were conducted as in Fig 1g. (h), qRT-PCR analysis of *PDS* transcript in TRV control and TRV-*PDS* tomato fruits, which were conducted as in Fig 1h. Asterisks indicate significant difference as determined by Student's t-test ($P \leq 0.01$).

and senescence(Spitzer-Rimon *et al*. 2013; Chang *et al*. 2014; Jiang *et al*. 2014). However, the efficiency of VIGS in flowers is not good as in leaves. In order to improve the efficiency of VIGS in flower, we infiltrated into shoot (Fig. 2a and 2b) with *Agrobacterium* culture containing sucrose, which could help the culture attached the shoot longer and not evaporated easily. After three weeks post infiltration, all the petals of flowers turn to white (Fig. 2d), whereas TRV control flowers kept yellow (Fig. 2c), which indicated the efficiency of our VIGS in flower is almost 100%. RT-PCR and qRT-PCR analysis suggested recombinant virus could rapid spread from shoot to flower and silence *PDS* gene by 75% in tomato flowers (Fig. 2g and 2h). These results clearly showed adding sucrose in infiltration buffer is good for VIGS, especially for the infiltration that it is not easy to inject *Agrobacterium* culture into the inside of plants.

PDS gene was Silenced in Whole Tomato Fruit

Tomato fruit is a model for fleshy fruit development. VIGS is one of widely used tools to identify gene function in tomato fruit development and ripening(Fu *et al*. 2005; Fernandez-Moreno *et al*. 2013). However, tomato plants inoculated at the leaves show silenced symptoms in about half of their fruit(Fu *et al*. 2006). When injecting *Agrobacterium* culture into the carpopodium of fruit, most silenced sectors of tomato fruit represented only 30% to 70% of the fruit surface(Fu *et al*. 2005). Although the higher percentage of silenced fruit reached up to 90% when tomatoes are agroinjected directly in the fruit(Orzaez *et al*. 2006), it is not good that *Agrobacterium* culture which is inside of fruit would induce artificial phenotype. To improve the distribution of silenced sectors of tomato fruit by VIGS, we also added sucrose into *Agrobacterium* culture and injected through the carpopodium of mature green stage fruit (Fig. 3a and 3b). Fortunately, after two or three weeks whole tomato fruit injected with TRV-*PDS* turned from green to yellow, not red (Fig. 3c and 3d). The silenced sectors of tomato fruit represented 90% to 100% of the fruit surface. Furthermore, RT-PCR and qRT-PCR analysis suggested recombinant virus could rapidly spread from carpopodium to fruit and silence *PDS* gene by 95% (Fig. 3g and 3h). All these results convincingly indicated our VIGS in tomato fruit above is another potential and alternative tool to characterize the function of genes involved fruit development and ripening, which is more convenient, efficient and precise.

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