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ORIGINAL PAPER

Heterologous gene silencing induced by tobacco rattle virus (TRV) is efficient for pursuing functional genomics studies in woody plants

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Abstract Virus-induced gene silencing (VIGS) is an effective tool for studying the functions of plant genes, but only a few VIGS vectors available for woody plants were reported so far. Here we present an effective heterologous VIGS system in woody plants based on tobacco rattle virus (TRV) vectors. We first tested whether the TRV-vector can be directly applied to infect woody plant species, such as *Vernicia fordii*, *Populus tomentosa* Carr. and *Camellia oleifera.* The results revealed that TRV-mediated VIGS could be effectively elicited in *V. fordii*, weakly in *P. tomentosa* Carr., but not in *C. oleifera.* TRV-based VIGS vectors with heterologous phytoene desaturase (*PDS*) sequences from various woody plant species silenced successfully the endogenous *PDS* gene in *Nicotina benthamiana* and *V. fordii*. The photobleached leaf phenotype of silenced plants significantly correlated with the down-regulation of endogenous *PDS* as compared with controls. To further confirm the reliability of VIGS in *V. fordii*, we also isolated the cloroplastos alterados 1 gene from *P. tomentosa* Carr., and the silencing pheotypes of albino leaves were observed in *V. fordii* 2 weeks after inoculation using a heterologous TRV-based VIGS system. Taken together, we have successfully developed an *Agrobacterium*-mediated VIGS assay in *V. fordii* and demonstrated that *V. fordii* as a heterologous VIGS system provides a valuable tool for functional genomic analysis in woody plant species.

Keywords Virus-induced gene silencing · Functional genomics · *Vernicia fordii* · *Populus* · *Nicotiana benthamiana* · *Camellia oleifera*

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Introduction

Since the whole-genome sequence of *Populus trichocarpa* was released in 2006 (Tuskan et al. 2006), whole genome sequencing has been performed widely in woody plants, including grapevine (Scalabrin et al. 2010), papaya (Yu et al. 2009), pear (Wu et al. 2013) and apple (Pagliarani et al. 2012). With the availability of the whole-genome sequences of increasing woody plant species, it is essential to develop novel molecular tools for large-scale analysis of gene functions at the genome-wide level. Ultimately, understanding of the molecular mechanisms of gene function and regulation will enable biomass improvement in woody plants by genetic engineering and molecular breeding.

There are various large-scale approaches, including chemical mutagenesis, irradiation mutagenesis, post-transcriptional gene silencing (PTGS), T-DNA- and transposon-based insertional mutant populations, to introduce mutations or silence of gene expression (Martienssen 1998; Waterhouse et al. 1998; Ostergaard and Yanofsky 2004; Robinson and Parkin 2009). Although these approaches have been applied successfully to study of gene functions at the genome-wide level for other model plants such as *Arabidopsis thaliana*, large collections of mutant populations is impractical to apply in woody plants due to the technical challenge of transformation, long growth cycle and large genome size. In addition, these functional genomic approaches when used in trees have also other substantial obstacles, such as lack of obvious phenotypes due to the presence of large gene families, the large populations required to disrupt a gene of interest, gene targeting complications from multiple mutations or insertions and gene duplication by polyploidy and lethality with complete loss of gene function (Borevitz and Ecker 2004; Candela et al. 2011).

Virus-induced gene silencing (VIGS) is an extremely powerful tool for large-scale functional analysis of individual gene by silencing the expression of endogenous genes (Baulcombe 1999; Dinesh-Kumar et al. 2003; Lu et al. 2003; Burch-Smith et al. 2004). This technique was developed based on the RNA-mediated PTGS that functioned as a natural defense system against various viruses in plants and other organisms (Hamilton and Baulcombe 1999; Dinesh-Kumar et al. 2003; Burch-Smith et al. 2004; Robertson 2004). Double stranded RNA (dsRNA) is of importance in the VIGS process. The dsRNAs is sheared into siRNAs whose length is from 21 to 23 nt. The siRNAs can integrate into be RNA-induced silencing complexes (RISC) and then target their homologous mRNA for cleavage, which spells the degradation of the targeted mRNA (Robertson 2004). By this mean, it is theoretically possible to knockdown almost any gene of interest if a suitable virus vector is used for the plant species under investigation. This approach also avoids the need for plant transformation and overcomes the problems of functional redundancy (Burch-Smith et al. 2004; Becker and Lange 2010).

With advantages of convenience, rapidity and efficiency, the VIGS technology achieves success for a wide range of herbaceous plants tobacco (*Nicotiana benthamiana*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), *Petunia hybrida*, pea (*Pisum sativum*), *Arabidopsis thaliana*, rice (*Oryza sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), soybean (*Glycine max*), cassava (*Manihot esculenta*), pepper (*Capsicum annuum*), and wheat (*Triticum aestivum*) (Benedito et al. 2004; Burch-Smith et al. 2004; Chung et al. 2004; Constantin et al. 2004; Faivre-Rampant et al. 2004; Fofana et al. 2004; Robertson 2004; Ding et al. 2006; Wang et al. 2006; Zhang and Ghabrial 2006; Doja and Koonin 2013). Unfortunately, VIGS is not effective in many other plant species, especially in trees, due to the lack of a compatible VIGS vector (Robertson 2004). To date, there are only a few studies on effective VIGS-inducing virus vectors that are able to be used for woody plant species. These viruses include poplar mosaic virus (PopMV) (Naylor et al. 2005), *Plum pox* virus (Lansac et al. 2005), apple latent spherical virus (ALSV) (Sasaki et al. 2011) and *Grapevine leafroll-associated virus-2* (GLRaV-2) (Kurth et al. 2012). However, it is still unknown whether these vectors are effective VIGS inducers and can be used for analysis of gene functions in other woody plants. Therefore, a high-throughput VIGS-mediated method for assessing gene function is not yet available for most of woody plant species.

Previously, it has been demonstrated that heterologous VIGS is a promising approach for functional genomic analysis in model plants as long as there is minimal nucleotide sequence homology between the gene sequences (Ekengren et al. 2003; Fofana et al. 2004; Ryu et al. 2004; Senthil-Kumar and Udayakumar 2006). For instance, a fragment of phytoene desaturase (*PDS*) gene, encoding an enzyme required for the biosynthesis of carotenoid pigments that protect chlorophyll from photobleaching, from a monocot (*Lilium longiflorum*) caused the silencing of endogenous *PDS* in *N. benthamiana* by VIGS in spite of the remote evolutionary relationship between these two species (Benedito et al. 2004). The endogenous *PDS* genes of tomato, *N. tabacum* and *Petunia hybrida* were successfully silenced using the *N. benthamiana PDS* gene sequence (Ryu et al. 2004). A late embryogenic abundant-4 (*lea4*) gene from peanut (*Arachis hypogaea*) was used to efficiently silence its ortholog in tomato (Senthil-Kumar and Udayakumar 2006). Similarly, a DEAD box helicase gene from *Dunaliella salina* was used to silence its ortholog in a distantly related species, *N. benthamiana* (Howes and Kumagai 2005). A recent study has demonstrated that tobacco rattle virus (TRV)-mediated VIGS can be performed in a wide range of *Solanaceous* plant species and that heterologous gene sequences from far-related plant species can be used to silence their respective orthologs in the VIGS-efficient plant *N. benthamiana* (Senthil-Kumar et al. 2007). However, it is still unknown whether TRV-mediated VIGS is an effective genetic tool for rapid assessment of plant gene functions in woody species, especially when heterologous gene sequences are used.

TRV-mediated VIGS has been commonly used in many plants, including dicots and monocots (Burch-Smith et al. 2004; Brigneti et al. 2004; Becker and Lange 2010). The system has also been successfully applied in some woody

plant species. For example, Ye et al. (2009) reported that TRV-based vector could trigger virus-induced gene silencing in *Jatropha curcas* L., which is a small tropical, woody tree belonging to the Euphorbiaceae family. Recently, an *Agrobacterium*-mediated VIGS assay was successfully developed in several cotton cultivars with various genetic backgrounds (Gao et al. 2011). Therefore, TRV is an alternative tool for functional genomic analyses of many tree species by VIGS. To extend the use of TRV-VIGS vector to diverse woody plant species, in this study, we first tested the possibility of employing TRV vector to silence genes in Chinese white poplar (*P. tomentosa* Carr.) and wood-oil trees including tung tree (*Vernicia fordii*) and tea-oil tree (*Camellia oleifera*) with the marker gene *PDS*. Using TRV-based VIGS vectors, we also reported silencing of endogenous *PDS* and cloroplastos alterados 1 gene (*CLA1*) in tung tree with heterologous gene sequences from poplar, respectively. The loss-of-function assay based on heterologous VIGS developed in this study provides an alternative tool for functional genomics studies of woody plant species.

Materials and methods

Plant material and growth conditions

Seeds of tobacco (*Nicotiana benthamiana* L.) were germinated at 25 °C and seedlings were grown in plastic pots (10 cm diameter) containing potting mixture. Plants were grown in growth rooms at 22–25 °C with 60 % relative humidity and a 12 h light: 12 h dark photoperiod cycle with light intensity ranging from 300 to 400 μmol m^{-2} s^{-1}.

Seeds of tung tree (*Vernicia fordii*) and tea-oil tree (*Camellia oleifera*) were stored at 4 °C and germinated seeds were sown in soil and grown in a growth chamber (25 °C, 16 h: 8 h light:dark photoperiod).

Adventitious shoots of Chinese white poplar (*Populus tomentosa* Carr.) clone 741 were regenerated in woody plant medium (WPM) (Lloyd and McCown 1980). Rooted plantlets were transferred to potting mix and grown in the greenhouse. Two-month-old seedlings were used for further experiments.

Cloning of *PDS* and *CLA1* genes

Total RNA was isolated from leaves of woody plants using RN09-EASYspin RNA Plant Mini Kit (Aidlab Biotechnologies Co., Ltd, Beijing, China), following the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg DNase-treated RNA with RT-AMV transcriptase (TaKaRa, Dalian, China) in a total volume of 20 μl using oligo d(T) at 42 °C for 30 min. In order to get the accurate *PDS* fragments from *V. fordii* and *C. oleifera*, gene-specific primers were designed based on ESTs of *PDS* from *Jatropha curcas* (Ye et al. 2009) and *Camellia sinensis*, respectively. The genome of poplar has been completed (Tuskan et al. 2006), therefore, sequences of *PtoPDS* and *PtoCLA1* were used as the templates to design the primers. The fragments of the *PDS* genes from different plants were amplified by *Taq* DNA polymerase (TakaRa, Dalian, China), respectively. Positive clones of every fragment were confirmed by DNA sequencing (BGI, Shenzhen, China). All *PDS* and *PtoCLA1* gene sequences were deposited in GenBank with the accession numbers and primer sequence information and fragment sizes of the PCR products used in this study are listed in Supplementary Material Table S1.

Sequence alignment and identity calculation

The alignments of the nucleotide sequences of *PDS* and *CLA1* were performed using Clustal X 1.81 (http://www.clustal.org/). The parameters of alignment are used as follows: gap opening penalty, 10.00 (both in pairwise alignment and multiple alignment); gap extension penalty, 0.20 (both in pairwise alignment and multiple alignment); protein weight matrix, gonnet; residue-specific penalties, ON; hydrophilic penalties, ON; gap separation distance, 0; end gap separation, ON; use negative matrix, OFF; and delay divergent cutoff (%), 30. Identity calculation between sequences was performed using DNAman software (http://www.lynnon.com/).

Construction of TRV plasmids

The DNA fragments of *VfPDS*, *CoPDS* and *PtoPDS* were amplified using a pair of primers containing *Xho*I and *Sac*I. *NbPDS* and *PtoCLA1* fragments were generated by RT-PCR with primers containing *Xho*I and *Eco*RI restriction sites. The pTRV1 and pTRV2 vectors described by Liu et al. (2002) were used in this study. The DNA products were double-digested and ligated into pTRV2 with the same enzymes, respectively. The resulting vectors were designated as pTRV2-*VfPDS* (*PDS* from *V. fordii*), pTRV2-*CoPDS* (*PDS* from *C. oleifera*), pTRV2-*PtoPDS* (*PDS* from *P. tomentosa* Carr.), pTRV2-*NbPDS* (*PDS* from *N. benthamiana*) and pTRV2-*PtoCLA1* (*CLA1* from *P. tomentosa* Carr.). Plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 by freezing-thawing method (Höfgen and Willmitzer 1988).

Agrobacterium infiltration

For the VIGS assay, a 5-mL culture of *Agrobacterium* strain GV3101 containing pTRV1 and pTRV2 was grown

overnight at 28 °C in Luria-Broth (LB) medium containing three antibiotics (40 mg L^{-1} rifampicin, 50 mg L^{-1} gentamycin and 50 mg L^{-1} kanamycin). The next day, 500 µL of each *Agrobacterium* culture was inoculated into a 50 mL LB medium containing antibiotics, 10 mM MES (2-(4-Morpholino)-Ethane Sulfonic Acid) and 20 µM acetosyringone. The culture was grown overnight in a 28 °C shaker at the speed of 200 r min^{-1}. *Agrobacterium* cells were harvested and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 µM acetosyringone, pH 5.6) to a final $O.D._{600}$ of 1.0. *Agrobacterium* cultures containing pTRV1 and pTRV2 or its derivatives were mixed at 1:1 ratio and incubated for 3 h at room temperature. The syringe method operated the same as described previously (Burton et al. 2000; Liu et al. 2002).

Effectiveness of gene silencing

The effectiveness of gene silencing, in *N. benthamiana* that showed photobleaching symptoms, was calculated by comparing the number of leaves that showed symptoms with the total number of leaves on the plant. The formulas and method are described previously (Senthil-Kumar et al. 2007).

RT-PCR analysis

To detect the presence of TRV virus, RNA1 and RNA2 of TRV were amplified by two pairs of specific primers as follows: TRV1-forward primer: 5′-GTAGGAGGAAGA GACCGAAG-3′, TRV1-reverse primer: 5′-TAGTCGAAT CAGTAGCAACC-3′, TRV2-forward primer: 5′-GTATGT CAGTGATCGCAGTAG-3′, TRV2-reverse primer: 5′-CGT CCGTTTAGACGCTTGCGTAGG-3′. To quantify *PDS* and *CLA1* transcript abundances in TRV2::*PDS* (*PDS* from different plant species) inoculated plants, semi-quantitative RT-PCR was performed as described before (Burton et al. 2000; Liu et al. 2002). Primers that annealed outside the region targeted for silencing were used to ensure that only the endogenous gene would be tested and excluded the interference of the sequences carried on the TRV2 derivatives. The *18S* gene was used as an internal control for RNA quantity in semi-quantitative RT-PCR. PCR products were separated on a 1 % TAE gel and visualized by EtBr staining. At least three silencing plants for each construct were chosen for RT-PCR. Three technical replicates were performed for each sample. All the primers used for RT-PCR are shown in Supplementary Material Table S2.

Extraction and measurement of chlorophyll

Total chlorophyll was extracted from 200 mg of leaf tissue in an acetone:dimethyl sulphoxide (DMSO) (1 : 1 v/v) mix. The supernatant was made up to 1 mL using this mix. The absorbance was recorded at 663 and 645 nm using UV–visible spectrophotometer Model DU800 (Shimadzu Corporation, Kyoto, Japan). Total chlorophyll was measured as described previously (Hiscox and Israelstam 1979) and expressed as the percentage reduction relative to the corresponding control (wild-type or mock-infiltrated). Three silencing plants were examined and all tests were conducted by performing three technical replicates for each sample in the assay.

Statistical analysis

The Student's *t* test program (http://www.graphpad.com/quickcalcs/ttest1.cfm) was used for statistical analysis of the data in the experiments of quantitative RT-PCR and measurement of chlorophyll. In all these experiments, it was found that the quantitative differences between the two groups of data for comparison were statistically significant ($P < 0.05$).

Results

TRV-mediated VIGS in *V. fordii*

In order to investigate whether the TRV vector can directly infect tung tree, a mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2 constructs in a 1:1 ratio was infiltrated into two or three leaves of three-week-old tung tree. The seedlings infected were grown at 25 °C. Four weeks after agroinfiltration, more than twenty plants inoculated with TRV1 and TRV2 vectors showed no obvious differences in overall shoot and leaf morphology compared with the control (Fig. 1a).

To assess the infection and spread of TRV virus, total RNA was isolated from the newly developed true leaves of these infiltrated plants. RT-PCR with TRV1-RNA1-specific and TRV2-RNA2-specific primers showed that TRV virus was detected in all tested seedlings, while no specific product was detected in control plants (Fig. 1b), indicating that tung tree could be infected by TRV virus. These results suggested that TRV-based VIGS vector could be used for silencing genes in tung tree.

Silencing of the *PDS* gene from *V. fordii* by *Agrobacterium*-mediated VIGS

The phytoene desaturase (*PDS*) gene, which is highly conserved in various plant species (Liu et al. 2002; Senthil-Kumar et al. 2007), has been widely used as a VIGS marker in various plant species (Ratcliff et al. 2001; Liu et al. 2002; Turnage et al. 2002). The silencing of *PDS*

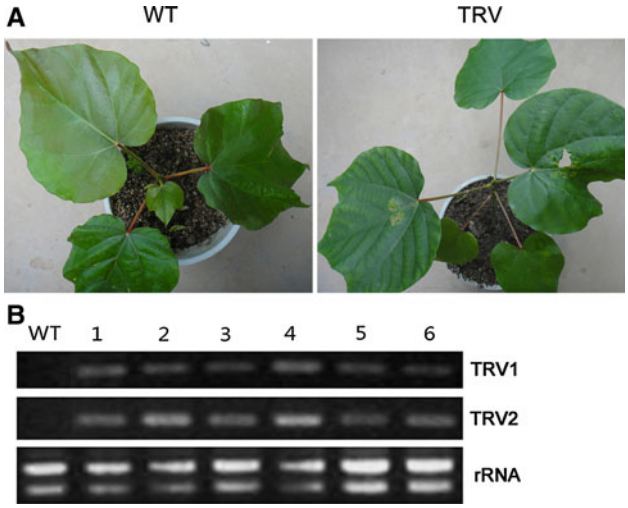

Fig. 1 Agroinfection of tung tree plant with tobacco rattle virus (TRV) vector. **a** Tung tree plant infiltrated with mixed *Agrobacterium* cultures containing pTRV1 and pTRV2 (*right*) and untreated plant (*left*). **b** Viral transcripts (TRV1 and TRV2) in leaves by Reverse Transcriptase (RT)-PCR. Six mock-treated plants were determined and all of them contained TRV transcripts. rRNAs was used as internal controls

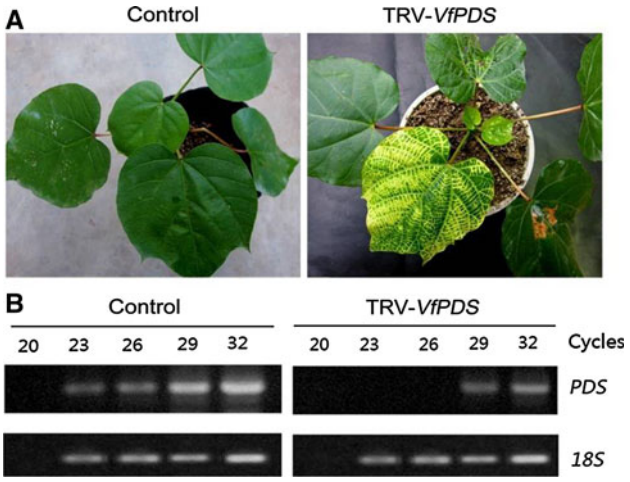

Fig. 2 Silencing of endogenous phytoene desaturase (*PDS*) gene in seedlings of tung tree. **a** Control and TRV-*VfPDS*-treated plants 25 days after infiltration. Photobleached phenotype was observed in the plant (*right*) infected with TRV-*VfPDS* while the plant treated with TRV alone (*left*) remained green. **b** RT-PCR analysis in *PDS* silenced plant. PCRs with cycles of 20, 23, 26, 29 and 32

results in white leaves caused by photobleaching, which occurs in the absence of the gene product. In a previous study, Ye et al. (2009) successfully developed high throughput screening of gene function by TRV-based VIGS in *Jatropha curcas*, a woody oil plant belonging to the Euphorbiaceae family. To determine if endogenous gene silencing can also be elicited by TRV-mediated VIGS in other Euphorbiaceae species, we amplified a 261-bp fragment of *VfPDS* from *V. fordii* by a standard homology-based cloning technique. Sequencing results revealed that the partial coding sequence of *VfPDS* cloned shares 83.14 and 83.52 % nucleotide sequence identities with those of *Arabidopsis thalianan* and *Nicotiana benthamiana*, respectively (Supplementary Material Fig. S1). The fragment of *VfPDS* was inserted into a pTRV-RNA2 VIGS vector, pYL156 (Liu et al. 2002). The TRV vectors were delivered to the leaf cells of young tung tree plants by a simple agrobacterium infiltration. All of these plants infiltrated with pTRV2-*VfPDS* exhibited photo-bleaching symptoms on the upper leaves 25 days post agroinfiltration and the symptoms continued to appear in the leaves which subsequently developed one month later (Fig. 2a). In addition, the bleaching seems to be more in the phloem or in the veins which may be involved in vascular movement of plant virus (Séron and Haenni 1996). While plants infected with the pTRV2 empty vector did not display any photobleaching phenotype and grew normally (Fig. 2a).

To monitor the silencing level of *VfPDS*, we performed semi-quantitative RT-PCR with specific primers of pTRV1 and pTRV2. The result revealed that the *PDS* transcript levels in photobleaching leaves were reduced by more than 80 % compared to the controls infected with TRV alone (Fig. 2b).

TRV-mediated VIGS in *Populus* and *C. oleifera*

In poplar, a candidate for development of a VIGS vector is poplar mosaic virus (*PopMV*), which is a RNA carlavirus that naturally infects species and hybrids in the genus *Populus*. Recently, Naylor et al. (2005) demonstrated that a VIGS vector based on the genome sequence of *PopMV* (Smith and Campbell 2004) was successfully applied in *N. benthamiana* for suppression of a GFP reporter gene. However, it is not clear whether this virus vector can suppress effectively the expression of endogenous genes in poplar so far. To test the infection ability of TRV in poplar, *PtoPDS* was isolated from Chinese white poplar (*P. tomentosa* Carr.) based on homology (Supplementary Material Fig. S1). Three weeks after agroinfiltration, approximately 30 % of poplar plants treated with pTRV2-*PtoPDS* developed photobleaching symptoms on the upper newly-grown leaves (Fig. 3a), but silencing symptoms rapidly disappeared on the newly formed leaves one month post inoculation (Fig. 3b). Plants infiltrated with the pTRV2 empty vector did not exhibit any photobleaching phenotype and grew normally (Fig. 3a). Endogenous transcript levels of *PtoPDS* were assessed by RT-PCR. The results showed that a slight decrease in *PDS* transcript levels was detected by 20 days post infiltration in these plants infiltrated with pTRV2-*PtoPDS*. But transcript suppression was recovered partially after 40 days (data not shown). These data

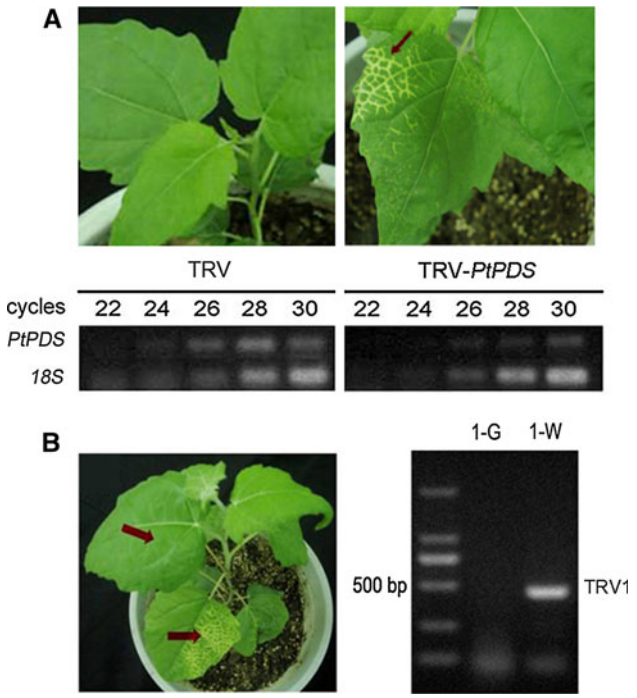

Fig. 3 Silencing of *PDS* in seedlings of *Populus*. **a** The control plant infected with mixed *Agrobacterium* cultures containing pTRV1 and pTRV2 remained green 3 weeks after infiltration while photobleached phenotype (shown with *red arrow*) was observed in the plant infiltrated with TRV-*PtoPDS* whose phenomenon was not so strong. *PtoPDS* expression was analyzed by RT-PCR in silenced plant. PCRs with cycles of 22, 24, 26, 28 and 30 were performed by *PtoPDS*-specific primers. **b** Photobleached phenotype was observed in the old leaves but not the newly ones (shown with *red arrows*). TRV1 transcripts were visible in leaves by RT-PCR. 1-G: green newly leaves, 1-W: old leaves with photobleached phenotype. (Color figure online)

indicated that TRV-based VIGS is not effective to silence endogenous genes in *Populus*.

Tea oil tree (*C. oleifera*) is mainly cultivated in tropical and subtropical climates and its seeds can be pressed to yield edible oil. To determine whether the TRV-vector efficiently mediates gene silencing in tea oil tree, we cloned a 366-bp fragment of *CoPDS* by PCR amplification based on the homologous sequence from *Camellia sinensis.* Sequence comparison of *CoPDS* and its homologs in *J. curcas*, *Populus*, tobacco and tomato revealed a nucleotide acid identity level of 85–93 % (Supplementary Material Fig. S1). We used the *Agrobacterium*-infiltration method mentioned above to induce VIGS. The photobleaching phenotype of *PDS* was not visible on upper leaves of treated plants even 2 months after infection (Supplementary Material Fig. S2A). Furthermore, we did not detect TRV1 or TRV2 transcripts in all treated plants (Supplementary Material Fig. S2B). This result suggested that tea-oil tree could not be infected by TRV-based vectors.

Silencing of the *PDS* gene in *N. benthamiana* using heterologous *PDS* gene sequences from woody plant species

It has been well established that TRV-mediated VIGS is highly efficient in *N. benthamiana* (Lu et al. 2003; Burch-Smith et al. 2004). Senthil-Kumar et al. (2007) demonstrated that heterologous *PDS* gene sequences from a wide range of plant species could be applied to suppress the expression of their orthologs in *N. benthamiana*. To test whether heterologous gene sequences from woody plant species can be used to silence their respective orthologs in *N. benthamiana*, *PDS* fragments from *P. tomentosa* Carr., *C. oleifera* and *V. fordii* were inserted into the TRV2 vector and used to perform VIGS in *N. benthamiana*, respectively. As controls, *PDS* sequences from *N. benthamiana* and tomato (*S. lycopersicum*) were also been cloned into the TRV2 vector. We infiltrated tobacco leaves with the TRV-vectors containing these *PDS* genes from various plant species, respectively. A visible bleached phenotype was observed in all treated plants but the extent of photobleaching varied from mild to almost complete bleaching among the different *PDS* sequences used for gene silencing (Fig. 4a).

We investigated the frequency of gene silencing in *N. benthamiana* when infected with different *PDS* sequences. The gene silencing effectiveness was calculated by comparing the number of photobleaching leaves with the total number of leaves inoculated with TRV2::*PDS*. A wide range of variation in silencing effectiveness was found among the plant species (Fig. 4b). *N. benthamiana* showed the highest effectiveness (95 %) of silencing and *SlPDS* sequences caused photobleaching in approximately 90 % of the plants tested. *C. oleifera* and *V. fordii* showed silencing frequencies of only 51 and 63 %, respectively. The effectiveness of heterologous *PDS* silencing was the lowest (46 %) in *Populus* (Fig. 4b). These results suggested that the effectiveness of gene silencing was relatively low in *N. benthamiana* when heterologous gene sequences from woody plant species, especially for *Populus*, were used for VIGS.

Furthermore, we determined the efficiency of gene silencing triggered by heterologous *PDS* gene sequences in *N. benthamaina*. qRT-PCR analysis showed that a marked reduction in gene expression occurred in these plants infected with TRV2-*NbPDS* and TRV2-*SlPDS*, but only a reduction of twofold in plants infected with TRV2-*PtoPDS* compared with the control (Fig. 4c). Chlorophyll was extracted from both green control leaves and the whole photobleaching leaves. Plants infected with TRV2-*NbPDS* showed almost 100 % reductions of total chlorophyll compared with wild-type plants. The content of chlorophyll was reduced by 87 % in the TRV2-*SlPDS*

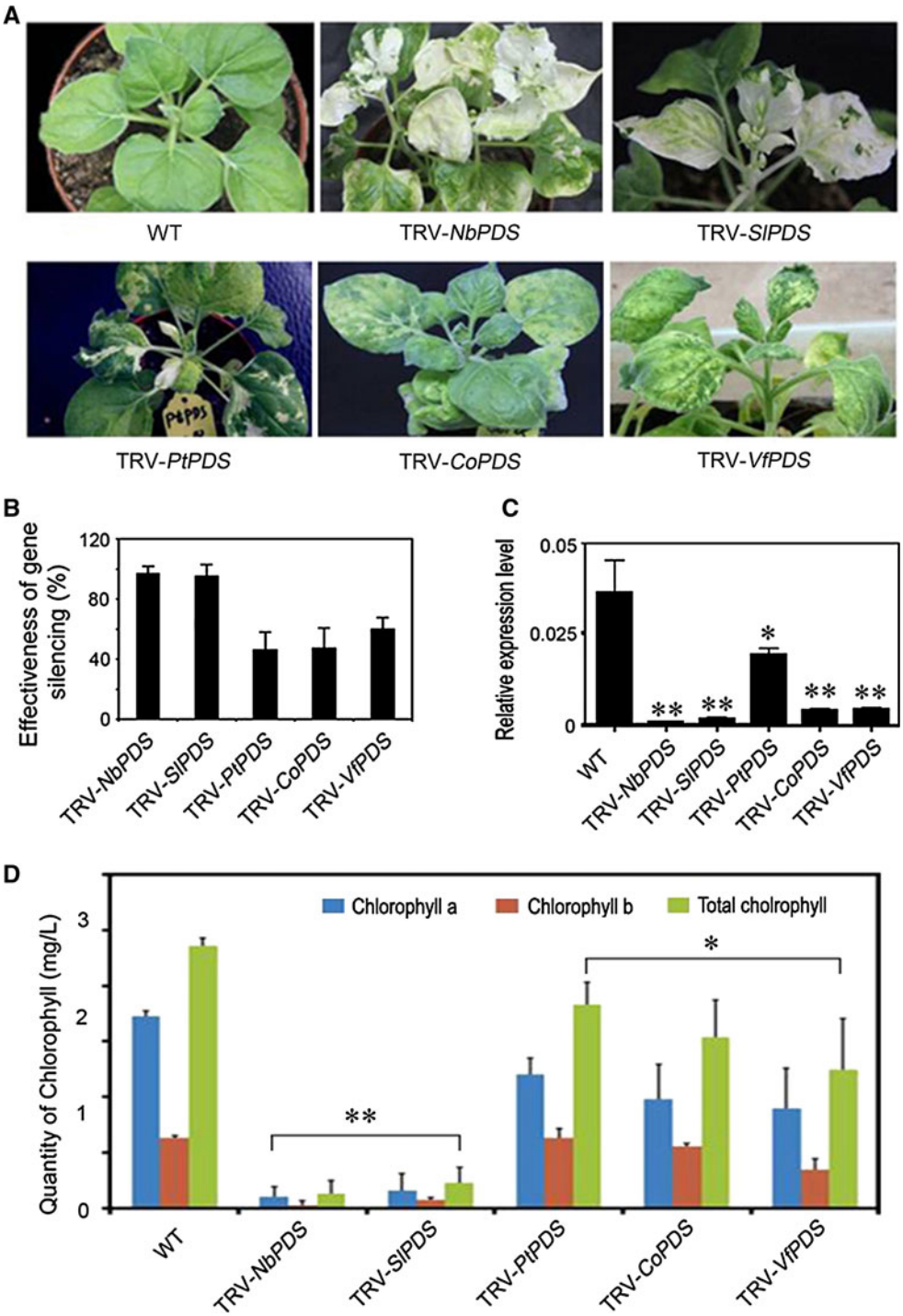

Fig. 4 Silencing of the *PDS* gene in *N. benthamiana* using heterologous *PDS* gene sequences from different plant species by TRV-mediated VIGS. Plants were infiltrated with TRV1 + TRV2-*PDS* (*PDS* from different species). **a** The photobleaching phenotype was photographed at 20 days post infiltration. **b** The effectiveness of gene silencing was calculated by counting the number of leaves that showed photobleaching on a gene-silenced plant at 20 days post infiltration. **c** qRT-PCR analysis in *PDS*-silenced plant. **d** The quantification of chlorophyll content in gene-silenced plants at 20 days post infiltration. The values are means of three independent experiments and the *bars* represent standard error. Statistical differences were determined using the Student's *t* test; * $P < 0.05$

silenced plants. While plants inoculated with the *PtoPDS*, *CoPDS* and *VfPDS* clones showed reductions of less than 20, 45 and 60 % in chlorophyll content, respectively (Fig. 4d). These results clearly demonstrated that heterologous gene sequences from woody plants could not be used to effectively silence their respective orthologs in *N. benthamiana*.

Silencing of heterologous *PDS* gene in tung tree by *CoPDS* gene sequences

We have previously demonstrated that TRV virus could infect and spread rapidly in tung tree (Fig. 1). To investigate whether heterologous virus-induced gene silencing is an effective tool in functional genomic analysis of woody

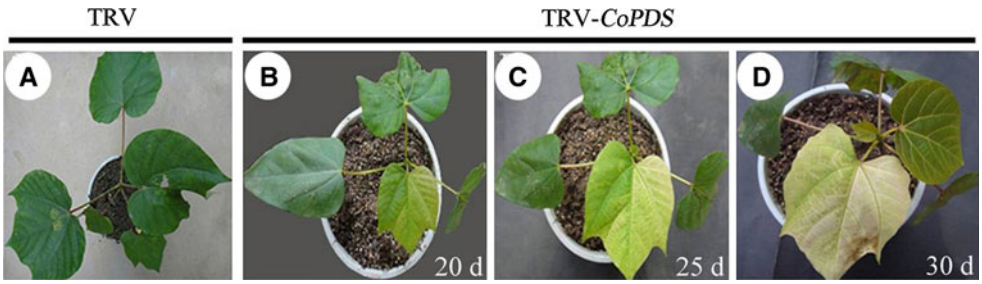

Fig. 5 *PDS* silenced by TRV-*CoPDS* in seedling of tung tree. **a** Plant treated with TRV vector (*left*) remained green. **b**, **c** and **d** shown the development of photobleached phenotype for 20, 25 and 30 days after agro-infiltration, respectively

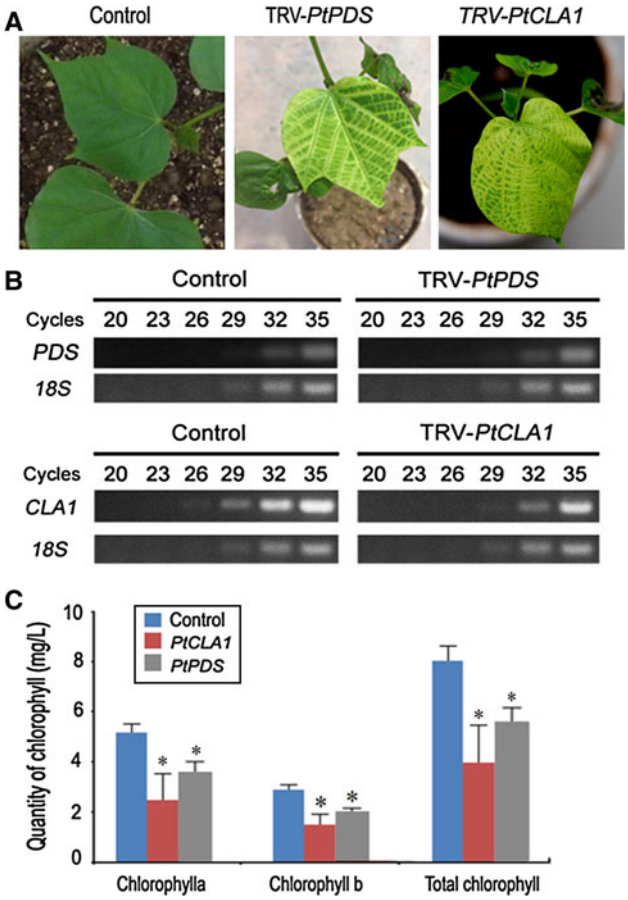

Fig. 6 Silencing of *VfPDS* and *VfCLA1* by a heterologous gene fragment from *Populus tomentosa.* **a** TRV-*PtoPDS*- and TRV-*PtoCLA1*-treated plants 20 days after infiltration. Photobleached phenotype was observed in the plant infiltrated with TRV-*PtoPDS* and TRV-*PtoCLA1* while the control remained green. **b** RT-PCR analysis in *PDS* and *CLA1* silenced plant, respectively. PCRs with cycles of 22, 24, 26, 28 and 30 were performed by *PtoPDS*- or *PtoCLA1*-specific primers. **c** Comparison of the content of chlorophyll a, chlorophyll b and total chlorophyll between silenced and control plants. *Error bars* represent SE of three replications. Statistical differences were determined using the Student's *t* test; * $P < 0.05$

plants, in an initial experiment, we infiltrated young leaves of three-week-old tung tree with *CoPDS* from tea-oil tree whose identity is 88.15 % aligned with *VfPDS*. The first effects of the silencing became visible approximately 20 days after *Agrobacterium* inoculation as discoloration of newly emerging leaves (Fig. 5b). Five days later, some leaves showed almost complete discoloration (Fig. 5c). In most of infected plants, *CoPDS* silencing remained easily detectable throughout subsequent developmental stages. The *CoPDS* silencing response did not seem to move below the node of leaf infection, many plants infiltrated with TRV-VIGS vector showed complete discoloration of all newly emerging leaves. In some cases, the silencing response disappeared so that normal green leaves developed again above photobleaching leaves (Fig. 5d). While plants infiltrated with the pTRV2 empty vector grew normally and did not exhibit any photobleaching phenotype (Fig. 5a). These results indicated that heterologous gene sequences could effectively silence their respective orthologs by TRV-mediated VIGS in tung tree.

Silencing of *VfPDS* and *VfCLA1* by heterologous gene fragments from *P. tomentosa* Carr

To examine whether a heterologous gene sequence from a distantly related species can trigger VIGS in tung tree, we infiltrated seedlings of tung tree with *PtoPDS* from *P. tomentosa* Carr. Photobleaching phenotype in the upper new leaves of tung tree was initially observed 20 days after infiltration with *PtoPDS*-expressing Agrobacteria, but the discoloration was limited to the main veins of newly formed leaves (Fig. 6a), while the control plants infected with TRV vector alone showed normal growth (Fig. 6a). These results indicated that the heterologous *PDS* gene sequence from *Populus* in a TRV-based VIGS vector was capable of suppressing expression of its ortholog in tung tree.

In plants, *CLA1* encodes 1-deoxyxylulose 5-phosphate synthase which is the first enzyme of the 2-C-methyl-D-erythritol-4-phosphate pathway involved in chloroplast development (Estevez et al. 2000). The *CLA1* gene is highly conserved in various plant species (Mandel et al. 1996). Silencing of *CLA1* in *Arabidopsis* and *S. melongena* plants led to an albino phenotype on true leaves (Mandel et al. 1996; Estevez et al. 2000, 2001). We obtained a 507 bp fragment of the *PtoCLA1* gene from *P. tomentosa* Carr. by RT-PCR. Sequencing results showed that a poplar *CLA1* gene (*PtoCLA1*) shared 91.05 % identity with tung

tree *VfCLA1* at the nucleotide level (Supplementary Material Fig. S3). The *PtoCLA1* fragment was inserted into TRV2 and seedlings of tung tree were infiltrated with *Agrobacterium* cultures containing the TRV1 and TRV2 constructs. Photobleaching phenotype was visible in the upper new leaves of all treated plants about 20 days post infiltration (Fig. 6a).

In order to confirm that observed photobleaching was due to silencing of the *VfPDS* and *VfCLA1* genes at the molecular level, we performed semi-quantitative RT-PCR on all treatment plants. Compared to the untreated plants, the target mRNAs were reduced to approximate 75 % in the targeted gene silenced plants (Fig. 6b). PDS and CLA1 enzymes are involved in chlorophyll synthesis. The contents of the chlorophyll a, chlorophyll b and total chlorophyll in *PtoPDS*-silenced plants were reduced by 35 % compared to that of the controls infected with TRV alone. Meanwhile, the content of the total chlorophyll in *PtoCLA1* silenced plants was also decreased greatly compared to that of controls (Fig. 6c). The results suggested that heterologous VIGS could be efficiently implemented to silence *PDS* and *CLA1* in tung tree.

Discussion

Virus-induced gene silencing (VIGS) as one of the reverse genetic tools has been used routinely for analysis of gene function in many plant species (Baulcombe 1999; Burch-Smith et al. 2004), mainly because VIGS is a very simple and robust method that does not require stable transformation which is a laborious, time-consuming and small-scale work. More importantly, this method can use heterologous gene sequences to silence orthologs in plants that do not have extensive gene sequence information. In this study, we developed a protocol for VIGS in tung tree (*V. fordii*) using a TRV vector. The TRV vectors carrying *PDS* gene fragment of tung tree induced a high-efficiency silencing by *Agrobacterium*-mediated VIGS (Fig. 2). We also carried out a systematic study to determine the feasibility of using heterologous gene sequences from various wood species to silence their orthologs in *N. benthamiana*. As measured by suppression of target transcript levels, the VIGS efficiency of the *PDS* gene in silenced tobacco plants was 46–63 % (Fig. 4b), suggesting that VIGS by TRV vector in tobacco was not effectively induced when using heterologous gene fragments from wood plant species. But we found that *PtoPDS* and *PtoCLA1* gene sequences from *P. tomentosa* Carr. as well as *CoPDS* sequence from *C. oleifera* could be used to silence their respective orthologs in tung tree (Figs. 5, 6). These data indicated that the TRV-based VIGS should have broad applications for functional genomics studies in heterologous wood species.

To date, there have been minimal effective methods for gene silencing in tree species except for the use of transgenic procedures. However, successful transformation is confined to a limited species and cultivars in trees. It is also time-consuming and labor intensive due to low transformation efficiency in most of woody plants. VIGS vector systems offer an alternative for characterization of gene functions without plant transformation (Ratcliff et al. 2001; Holzberg et al. 2002; Liu et al. 2002; Gronlund et al. 2008). In these VIGS systems previously reported, the most widely used VIGS vectors are derived from the tobacco rattle virus, which has been commonly used in many herbaceous plants (Burch-Smith et al. 2004). The TRV invades a wide range of hosts and is able to spread vigorously throughout the entire plant (Senthil-Kumar et al. 2007). Recently, a TRV-based VIGS assay was successfully developed in cotton, which was a shrub native to tropical and subtropical regions around the world, and the genes of interest were readily silenced with an efficiency of 100 % after inoculation at the seedling stage (Gao et al. 2011). Qu et al. (2012) further reported that the TRV-VIGS system could be used for rapid functional analysis of genes involved in cotton fiber development. In addition, it has also been well demonstrated that TRV vector could trigger VIGS in *J. curcas*, a small woody plant belonging to Euphorbiaceae (Ye et al. 2009). Tung tree (*V. fordii*) is another species of the Euphorbiaceae family, native to southern China and northern Vietnam. It is valuable for tung oil, which sources from the oilseeds of the tree and commonly used in formulations of inks, dyes, coatings, and resins. A recent study has demonstrated that tung oil is a raw material for biodiesel production after blending with other biodiesel (Chen et al. 2010). However, less is known about functions of these genes involved in oil biosynthetic pathway in tung seeds. In addition, there is no report of a method for genetic transformation of tung tree so far. VIGS is a rapid, simple and robust method for determining and studying the function of plant genes and does not require stable transformation. In this paper, we demonstrated for the first time that TRV system could effectively induce endogenous gene silencing in seedlings of tung tree (Fig. 2). Therefore, TRV-mediated VIGS provides a convenient and effective method to achieve functional gene data in tung tree.

The rapid innovation of sequencing technologies is vastly expanding the sequence database for important plant species, the majority of which are difficult to manipulate for functional genomics studies. For example, *Populus* is one of favorable model tree because of its small genome and its genome sequencing has been completed and released in 2006 (Tuskan et al. 2006). Therefore, the study on functional genomics in *Populus* has become a major task. *Agrobacterium*-mediated genetic transformation is a

powerful tool and an efficient technique for the study of plant functional genomics, however, it is both time-consuming and labour-intensive. VIGS provides a rapid and powerful tool to dissect gene functions in species that are not amenable to stable genetic transformation. Large-scale VIGS experiments have been adopted as a fast-forward genetics approach to screen for interesting phenotypes (Baulcombe 1999; Burch-Smith et al. 2004). More importantly, this technique can use heterologous gene sequences to silence orthologs in plants (Senthil-Kumar et al. 2007). But reliability and effectiveness of VIGS systems depends on both plant species and virus vectors. In this study, we demonstrated that the VIGS system using TRV vector only induced moderate reduction of *PtoPDS*-mRNA levels in *Populus* (Fig. 3), and even *C. oleifera* could not be infected by TRV vector (Supplementary material Fig. S2). Fortunately, the TRV-VIGS system was shown to be effective in tung tree, when a marker gene *VfPDS* was silenced, resulting in a photobleaching phenotype (Fig. 2). In order to develop a high throughput loss-of-function assay for functional genomic studies in *Populus*, we successfully silenced *PDS* and *CLA1* in tung tree using heterologous gene sequences from *P. tomentosa* Carr. (Fig. 6). Based on these results, we believe that the TRV-mediated VIGS could be applied broadly for functional genomics studies in tung tree using heterologous gene sequences from *Populus*, indicating that heterologous VIGS is a promising approach in functional genomics of woody plants.

In conclusion, we have demonstrated that TRV-mediated VIGS can be used in a wide range of Euphorbiaceae species, and this adds to the increasing list of wood species that are able to be used for VIGS-mediated studies. We have also developed the TRV-VIGS system to silence effectively their respective orthologs in tung tree using heterologous gene sequences from even distantly related species such as *Populus* and *C. oleifera*. The lose-of-function assay by TRV-mediated VIGS developed in this study provides an alternative tool for functional genomics studies of *Populus* genes.

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