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Efficient virus-induced gene silencing in *Brassica rapa* using a turnip yellow mosaic virus vector

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Abstract

Virus-induced gene silencing (VIGS) is a post-transcriptional gene silencing method used for unraveling gene functions. As an attractive alternative to mutant collections or stable transgenic plants, it has been widely used in reverse-genetics studies owing to its ease use and quick turnaround time. Turnip yellow mosaic virus (TYMV) has the ability to induce VIGS in *Arabidopsis thaliana*. However, the conventional vector construction is difficult and the efficiencies of the infection methods are low. Here, we improved the vector construction and viral infection methods, inserted an inverted-repeat fragment of the phytoene desaturase gene into a TYMV-derived vector by homologous recombination and transformed *Brassica rapa* with plasmid DNA harboring a cDNA copy of the TYMV genome through particle bombardment. An apparent photobleaching phenotype was detected and efficient VIGS was induced. An 80-bp fragment was sufficient to produce VIGS in leaves, stems, roots, flowers, siliques, and stalks of *B. rapa*. Because TYMV has a wide host range in *Brassica*, the VIGS system described here will contribute to the improvement of high-throughput technology and efficient functional research in *B. rapa* and other *Brassicaceae* crops.

Additional key words: particle bombardment, photobleaching, phylogenetic tree, phytoene desaturase, vector construction.

Introduction

Virus-induced gene silencing (VIGS) is an RNA-mediated reverse genetics technology that has been used in many plant species to study gene functions, either individually or on a large-scale in a high-throughput manner. In the past decades, remarkable advancements have occurred in many high-throughput techniques used to infer gene functions, and various tools for knocking out target genes have been developed, such as the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nuclease system, RNA interference technology, agroinoculation by T-DNA insertion, and transposable element- or chemical-based mutations (Tijsterman and Plasterk 2004, Liu *et al*. 2016). However, these methods have many limitations, such as the silencing of off-targets, functional redundancy, embryonic lethality, and multiple insertions (Pflieger *et al*. 2008).

As an attractive alternative, VIGS is a simple, quick, and cost-effective method for the high-throughput study of gene functions independent of mutant collections or stable transgenic plants. It exploits the innate plant defense system of post-transcriptional gene silencing

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Abbreviations: CaLCuV - cabbage leaf curl virus; CP - coat protein; FPKM - reads per kilobase of exon model per million mapped reads; GADPH - glyceraldehyde-3-phosphate dehydrogenase; PDS - phytoene desaturase; PTGS - post-transcriptional gene silencing; TGMV - tomato golden mosaic virus; TMV - tobacco mosaic virus; TRV - tobacco rattle virus; TYMV - turnip yellow mosaic virus; VIGS - virus-induced gene silencing.
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(PTGS) against intracellular viral proliferation and extracellular viral movement (Baulcombe 1999). With the recombinant virus carrying part of target gene transformed into plant, RNA interference-mediated antiviral mechanisms induce the silencing of the corresponding host gene (Liu *et al*. 2002, Dinesh-Kumar *et al*. 2003). During VIGS, double stranded RNAs corresponding to the host gene are produced and then cleaved by ribonucleases into small interference RNAs that are incorporated into the RNA-induced silencing complex, eventually triggering the degradation of target mRNAs (Schuck *et al*. 2013). VIGS was first established in the model plant *Nicotiana benthamiana*, and it has now been widely reported in tomato, pepper, *Arabidopsis*, barley, soybean, strawberry, grasses and cotton (Gao *et al*. 2011, Juvale *et al*. 2012). To date, VIGS has been employed to unravel gene functions in whole plants and specific plant organs, like fruit and roots (Jia and Shen 2013, Fantini and Giuliano 2016).

Several plant viruses, such as potato virus X, turnip yellow mosaic virus (TYMV), tobacco mosaic virus (TMV), tomato golden mosaic virus (TGMV), tobacco rattle virus (TRV), and cabbage leaf curl virus (CaLCuV), have been adopted as VIGS vectors to silence endogenous plant target genes (Liu *et al*. 2000, Burch-Smith *et al*. 2004). TYMV is a spherical plant virus with a positive-strand RNA genome, and it can infect many members of *Brassicaceae*. Three open reading frames are encoded by its genomic RNA. They produce a 69-kDa protein, which serves as a viral movement protein and RNA suppressor, a precursor protein, which is necessary for viral replication, and a 20-kDa viral coat protein (CP) (Pflieger *et al*. 2008, Fantini and Giuliano 2016).

Brassica rapa, an important economic crop belonging to *Brassicacea*e family, is recalcitrant to transformation. The aim of this research was to established a VIGS system based on a virus vector pTY-S, derived from TYMV, which could efficiently knock down the phytoene desaturase (*PDS*) genes in *B. rapa*.

Materials and methods

Plants and inoculation: After germination in a tray with holes, individual turnip (*Brassica rapa* L. cv. Sijiucaixin) plants were grown in a mixture of soil and *Vermiculite* (3:1) in a climate chamber at a temperature of 22 °C, a 16-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, and a 50 % relative humidity. Two weeks later, the plasmids pTY-S and pTY-PDS were transformed into the seedlings by particle bombardment. Plants inoculated with water were used as the controls. Leaves, roots, and stems were harvest at 15 d post-inoculation, and flowers, siliques, and stalks were harvested at 5 weeks post-inoculation. All tissues were kept in liquid nitrogen for further analyses.

Gene cloning and plasmid construction: The sequences of *BrPDS* were downloaded from the *Brassica* database (http://brassicadb.org/brad/). The open reading frames of the *BrPDS* genes were amplified by PCR, using first-strand cDNA as the template and gene-specific primers, which are listed in Table 1 Suppl. The PCR products were analyzed by agarose gel electrophoresis and purified using an *AxyPrep* DNA gel extraction kit (*AxyPrep,* Beijing, China). Then, they were cloned into the pMD19-T vector (*TaKaRa,* Dalian, China) and confirmed by sequencing. The pTY-S was digested with Eco105I (*SnaBI*), and an appropriate fragment of 40 nt, identical to the two *BrPDS* genes, was selected for hairpin construction. A palindromic DNA fragment of 80 nt corresponding to *BrPDS* genes was designed and synthesized, and 15-nt homologous sequences corresponding to the linearized vector were introduced to both 3′ and 5′ ends. Then, the synthesized 110-nt DNA fragment was ligated to the prepared vector using an in-fusion enzyme at 3:1 ratio. The recombinant plasmid was transformed into *Escherichia coli* strain DH5α cells and subjected to sequencing. The empty vector pTY-S and recombinant vector pTY-PDS were isolated using *maxiprep*-quality plasmid (*Tiangen*, Beijing, China) and concentrated to 2 mg cm^{-3} for particle bombardment.

Particle bombardment: Prior to bombardment, 1 μg of DNA plasmid was coated onto 1-μm gold particles and mixed with 50 mm^3 of 2.5 M $CaCl_2$ and 20 mm^3 of 0.1 M spermidine sequentially. The DNA plasmid was stored on the ice for 20 min and shaken with an oscillator for 30 min. Then, the particles were washed with 70 % (v/v) ethanol once and absolute ethanol twice, leaving 10 mm^3 of particle suspension. Particle bombardment was implemented using a *PDS 1000/He* biolistic gun (*Bio-Rad*, Hercules, USA) according to the manufacturer's instructions. Optimal bombardment device parameters, such as bombardment distance of 9 cm, gas pressures of 8.96 MPa and vacuum of 0.36 MPa were adopted (Cheng *et al*. 2010). After bombardment, the plants were promptly transferred into nutrient substrate, kept in the dark for 24 h, and then grown in a greenhouse.

Sequences of *PDS* genes downloaded from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) in the *FASTA* format were entered into the *ClustalX 2.1* program and aligned using the *ClustalX* algorithm (Larkin *et al.* 2007). The phylogenetic analyses were performed by the *MEGA5* program (http://www.megasoftware.net/) using the neighbor-joining method with 1 000 bootstrap trials (Tamura *et al*. 2013). Multiple expectation maximum for motif elicitation was used to detect conserved motifs (http://meme.nbcr.net/meme/).

RNA isolation and cDNA synthesis: Total RNA was extracted from the samples using an RNA extraction kit (*TaKaRa*), and gel electrophoresis was used to assess RNA quality and quantity. Subsequently, 1 μg of total RNA and oligo dT primers were used to synthesize first-strand cDNA using the *PrimeScript* 1st strand cDNA synthesis kit (*TaKaRa*) according to the manufacturer's instructions.

Verification by real-time quantitative PCR: To confirm VIGS at the molecular level, real-time qPCR was performed using gene-specific primers and *SYBR Green* (*TaKaRa*) on an *ABI PrismR 7900HT* (*Applied Biosystems*, Carlsbad, CA, USA) according to the manufacturer's instructions. The amplification procedure was as follows: pre-denaturation at 94 °C for 10 s, followed by 40 cycles of 94 °C for 30 s and 60 °C for 30 s, and finally a melting curve was performed (61 cycles at 65 °C for 10 s). Three biological and three technical replicates were carried out for the quantification analyses. Gene-specific primers were designed according to the gene sequences using *Becon Designer v. 7.9* and they are listed in Table 2 Suppl. The *Actin* gene (*Bra028615*) and *glyceraldehyde-3-phosphate-dehydrogenase* gene (*Bra002352*) were used as internal references (Dheda *et al*. 2004, Tang *et al*. 2013). The relative expressions of *BrPDS* genes were calculated using the $2^{-\Delta\Delta CT}$ method as described by Schmittgen and Livak (2008). The *BrPDS* expression patterns in various tissues were analyzed using *Cluster v. 3.0* (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and were calculated as FPKM (reads per kilobase of exon model per million mapped reads). The heat maps of the hierarchical clustering were established using *Tree View v. 3.0* (http://jtreeview.sourceforge.net/) based on the log2-converted FPKM values.

Results

Phytoene desaturase (*PDS*) genes serves as a convenient visual indicator for VIGS. In this study we identified two *PDS* genes from *B. rapa*, and using the *BrPDS* genes as the query in a *BLAST* algorithm-based search, we identified several *PDS* genes in other species. Gene structure and phylogenetic analyses revealed a high homology with *Brassica napus*, *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicon*, and *Zea mays* (Fig. 1). Conserved motif predictions indicated that a similar motif existed among genes encoding PDS. Ten motifs were used to identify the *PDS* gene structures, and the motifs further demonstrated that the *PDS* genes were highly evolutionarily conserved (Fig. 2).

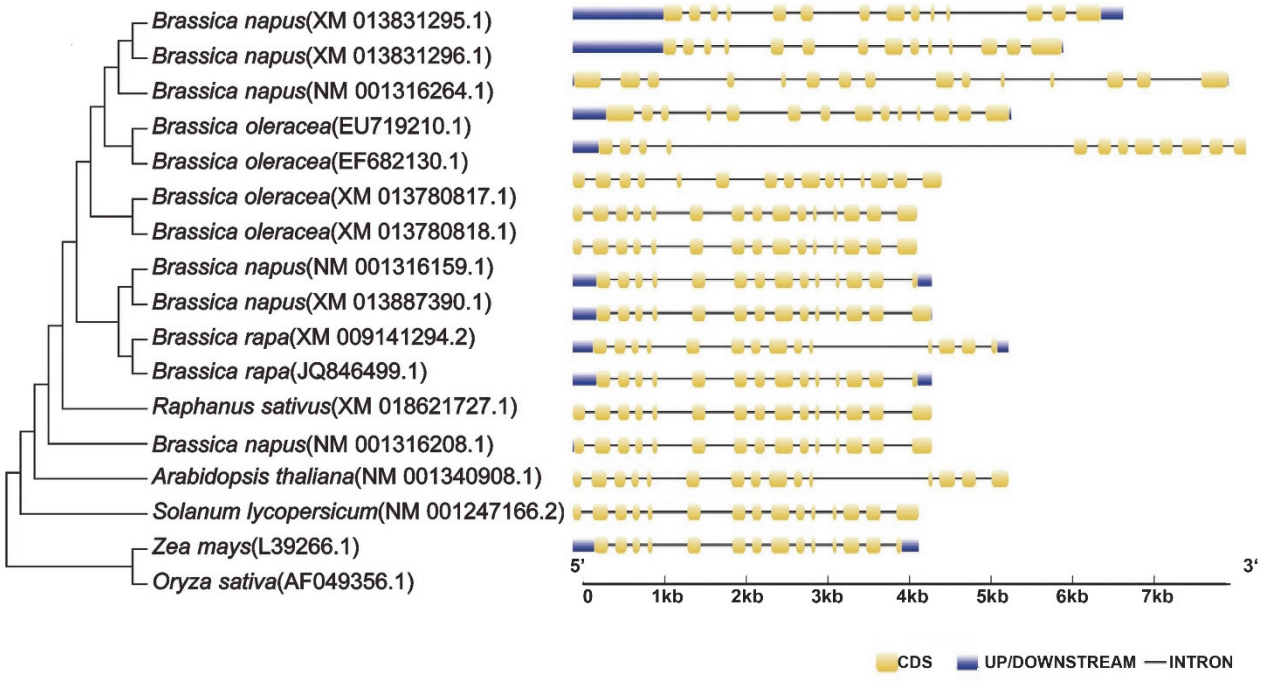

Fig. 1. Phylogenetic relationship and structural characteristics of the *PDS* gene families in *Brassica napus*, *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicon*, and *Zea mays.* The *blue* and *yellow bars* represent the exon and intron structures, respectively. The DNA sequences of the *PDS* genes were used to generate the tree. The unrooted phylogenetic tree was generated by the neighbor-joining method using the *MEGA5* program with 1 000 bootstraps. Relevant *GenBank* accession numbers are given to the right.

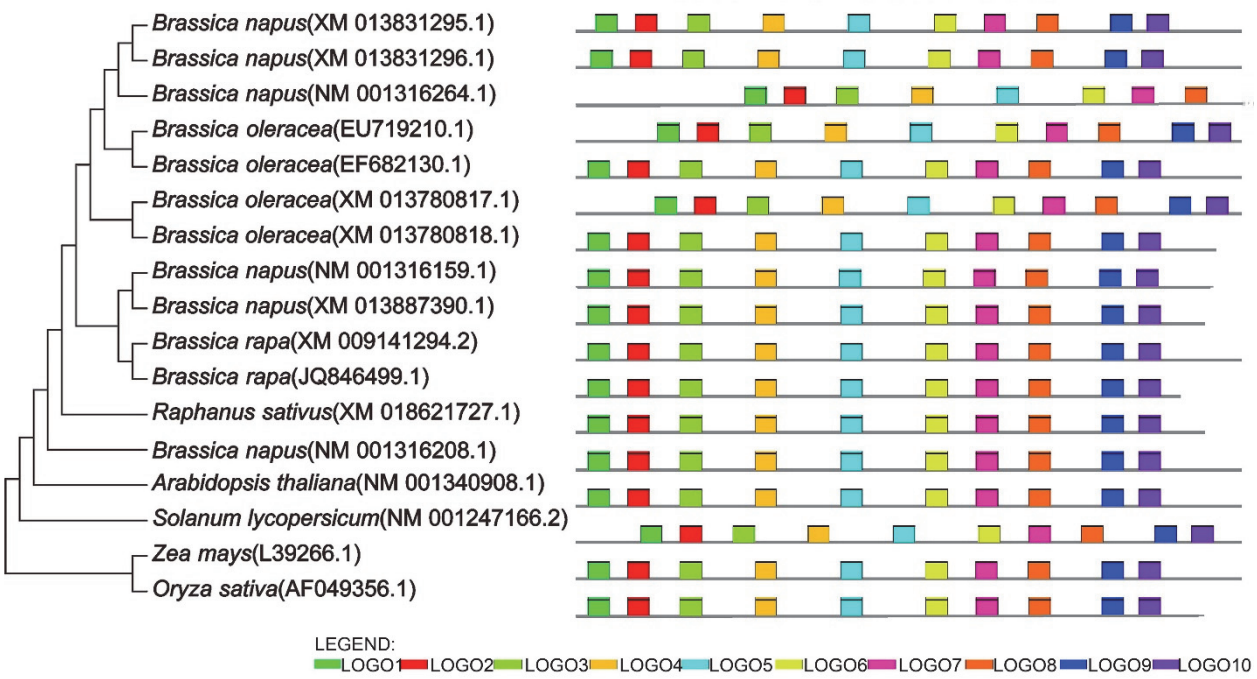

Fig. 2. Schematic diagram of conserved motifs in *PDS* gene families. Different motif types are represented by different *color blocks* as indicated at the bottom of the figure. The same color in different proteins indicates the same group or motif.

A hairpin sequence (76 - 80 nt) identical to the target sequence is sufficient to trigger the silencing of endogenous genes by the TYMV-derived vector pTY-S in *Arabidopsis* (Pflieger *et al*. 2008). Here, we utilized pTY-S to construct a plasmid carrying an 80-nt palindromic DNA fragment corresponding to *BrPDS* genes. The pTY-S contains a full-length cDNA copy of TYMV, and a unique *SnaBI* site was inserted into its coat protein (*CP*) gene (Fig. 3). To specifically and efficiently knock down the *PDS* gene, a highly conserved fragment was cloned into the *SnaBI* site. To reconstitute the TYMV CP stop codon, 2 - 4 nt of the fragment should be TGA, TAG, or TAA, and to avoid reconstituting the *SnaBI* site, the fragment of 1 - 3 nt cannot be GTA. Sequences (T/A/G/C)TGA, (T/A/C)TAG, or (T/A/C)TAA were identified in the *BrPDS* genes and then, 40 nt were counted from the first residue. The candidates were entered into the *Brassica* database and queried using a *BLAST* algorithm-based search. A sequence unique to *BrPDS* genes was selected, *BraPDS*-5′-TTGAGGAA CAACGAGATGCTGACATGGCCAGAGAAAATAA-3′ was self-hybridized and cloned into pTY-S. The VIGS using this recombinant plasmid was then performed in *B. rapa* plants using particle bombardment. A 100 % infection rate can be achieved with as little as 1 µg of DNA per plant using VIGS. Plants infected with the empty vector pTY-S were used as controls. At 3 weeks after inoculation, photobleaching was observed in leaves, and occurred in newly emerging tissues, like stems, roots, siliques, and stalks as the plants developed and persisted for the plant life. This did not occur in control plants. The plants inoculated with pTY-S and pTY-PDS displayed slower growth rates, and were, therefore, smaller than the untreated plants (Fig. 4). They also had slight viral infection symptoms, such as leaf distortion and mosaicism, and reduced sizes of leaf, flower, and fruit, which were gradually alleviated as the plants developed. The result demonstrated the plants were infected by pTY-S successfully and the pTY-S had the ability to silence the two homologous *BrPDS* genes simultaneously.

To confirm the silencing effect at the molecular level, real-time semi-quantitative PCR was performed to assess

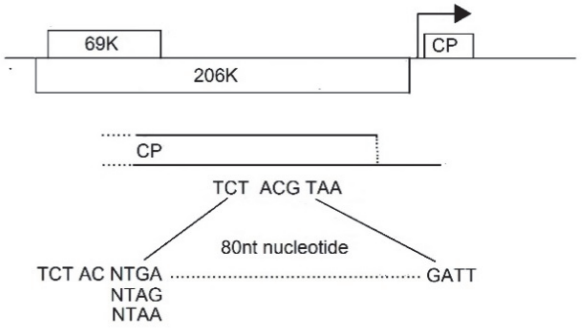

Fig. 3. Genomic organization of pTY-S and details for vector construction. PTY-S encodes a 69-kDa viral movement protein, a precursor protein and a 20-kDa viral coat protein (CP). The *CP* gene from the pTY-S vector was engineered with a unique SnaBI restriction site and an 80-nt DNA fragment of target was inserted into the SnaBI site.The target sequence beginning with either (T/A/G/C)TGA, (T/A/C)TAG, or (T/A/C)TAA should be selected for vector construction.

the accumulation of *CP* derived from pTY-S. The *CP* mRNA was significantly abundant in pTY-S and pTY-PDS plants, while it could not be detected in untreated plants (Fig. 5). The PCR products were sequenced and the TYMV CP was present in various tissues (Fig. 3 Suppl.). To determine the effectiveness and specificity of knocking down the *BrPDS* gene family by VIGS in *B. rapa*, the amount of *PDS* gene transcripts in different tissues were investigated by real-time qPCR (Table 1). The gene-specific primers were designed to anneal outside the region targeted for silencing so that only the endogenous mRNAs were detected (Table 2 Suppl.). The relative expression of the *BrPDS* genes are shown in Fig. 5. It suggests that PDS mRNA was 50 - 76 % less abundant in the photobleached tissues of plants infected with pTY-PDS than in pTY-S tissues. The VIGS efficiency varied in different tissues, although the *BrPDS* expression in flowers was nearly unchanged owing to its low expression level. Thus, the TYMV-derived vector has the ability to silence a gene family in multiple tissues.

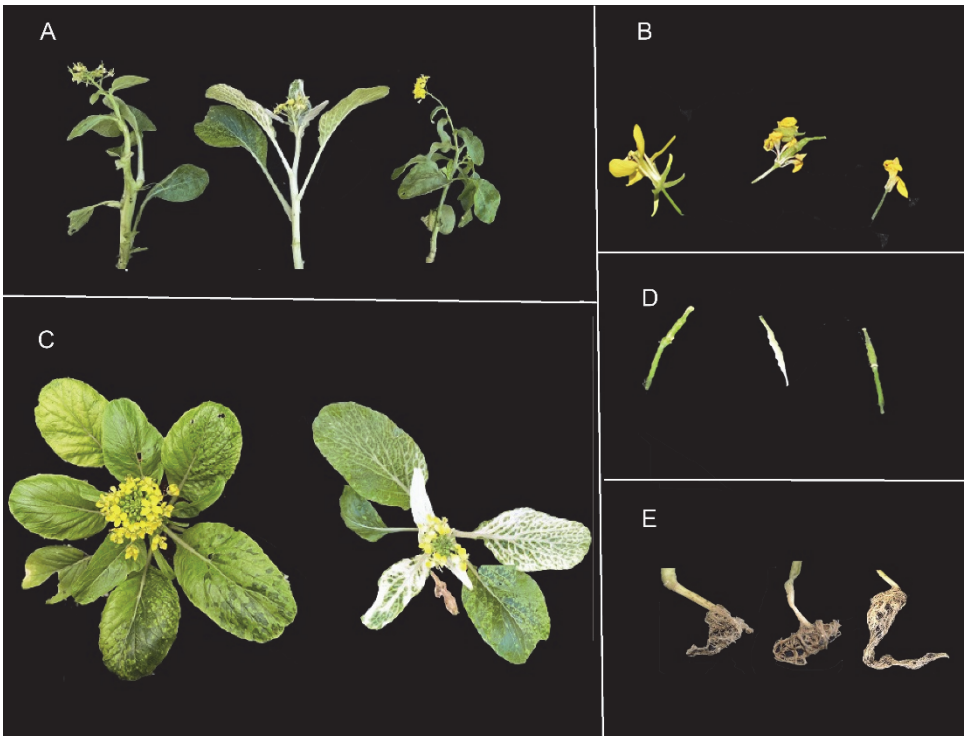

Fig. 4. Viral induced silencing of the *PDS* genes in various *Brassica rapa* tissues. *B. rapa* plants were inoculated with pTY-S, pTY-PDS, or water (*from left to right*). Photographs of stems (*A*), flowers and stalks (*B*), leaves (*C*), silique (*D*), and roots (*E*) were taken at 5 weeks post-inoculation.

Table 1. The relative expression of *BrPDS* genes in *B. rapa* plants inoculated with PTY-S and PTY-PDS verified by real-time qPCR. The expressions were present as log2 transformed values. A negative value represents upregulation in pTY-PDS plants when compared with pTY-S plants. Means ± SEs were calculated from three independent repeats.

Gene	Chromosome position	Gene ID	Log2 (fold change) leaf	stem	root	flower	silique	stalk	*A. thaliana* homolog
BrPDS1	A08:15480213-15483394	Bra010751	-2.0376	-0.9249	-0.9885	0.00918	-0.9821	-0.9936	AT4G14210
BrPDS2	A04:5072704-5076300	Bra032770	-2.0066	-1.0015	-0.9986	0.01301	-1.0156	-1.0632	AT4G14210

Discussion

Phytoene desaturase genes are crucial in the upstream processes that regulate the carotenoid biosynthetic pathway (Campisi *et al*. 2006), which interacts with multiple metabolic pathways, like biosynthesis of chlorophyll, light-harvesting pigments, abscisic acid, and gibberellic acid (Lindgren *et al*. 2003). The VIGS has been used in many plant species as an efficient tool for knocking down gene expression (Senthil-Kumar and Mysore 2011, Pflieger *et al*. 2013). Unlike other functional genomic tools, VIGS has many advantages,

including its ease and quickness, independence of stable plant transformations, as well as knowledge of only partial sequence of a gene is sufficient to produce VIGS (Senthil-Kumar and Mysore 2011). Multiple members of a gene family can be knocked down simultaneously by targeting a highly conserved sequence shared by the members. VIGS can also be employed in the functional validation of genes required for special developmental stages because plants can be inoculated at a chosen growth stage (Peele *et al*. 2001). Thus, VIGS technology is an important tool for reverse genetics studies of a variety of processes occurring in plants (Baulcombe 1999, Benedito *et al*. 2004, Burch-Smith *et al*. 2004).

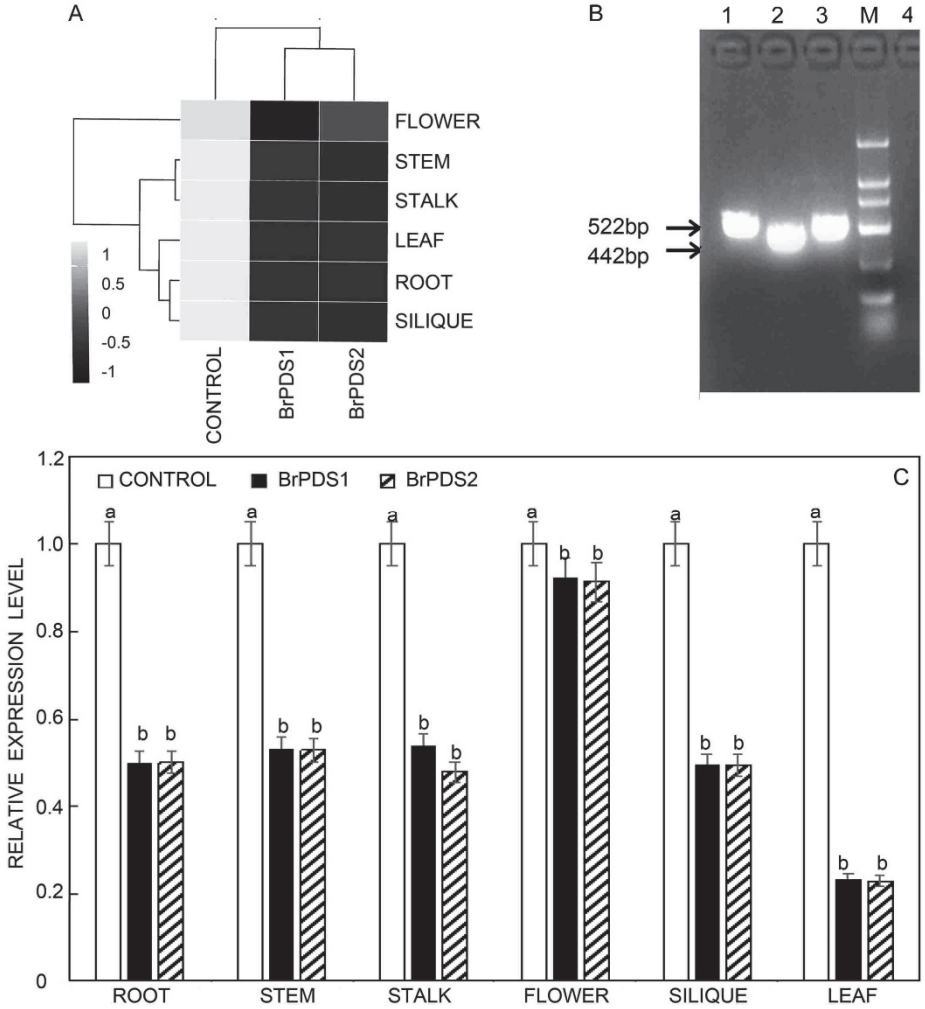

Fig. 5. The expression patterns of *BrPDS* genes in plants inoculated with pTY-S or pTY-PDS determined by real-time qPCR.. *A* - The heat map of *BrPDS* gene expression patterns in various tissues generated by *Cluster 3.0* software. The *bar* represents log2 transformed values from low to high expression. *B* - Quantification of coat protein mRNA by real-time semi-quantitative PCR in plants inoculated with pTY-S (*line 2*), pTY-PDS (*lines 1* and *3*) or water (*line 4*), M - marker. *C* - Relative expressions of *BrPDS* genes in various tissues. *Black*, *stripped*, and *white bars* indicate the expression in pTY-BrPDS1, pTY-BrPDS2, and pTY-S plants, repectively. Means ± SDs, $n = 3$; means with different letters are significantly different at 5 % level. The *actin* and *glyceraldehyde-3-phosphatedehydrogenase* genes were used as internal references.

To date, 15 VIGS vectors derived from plant RNA and DNA viruses have been developed (Benedito *et al*. 2004). However, there are limitations to the VIGS technology currently employed for functional analyses. The CaLCuV and TRV produce VIGS in the model plant *Arabidopsis* (Ratcliff *et al.* 2001, Benedito *et al.* 2004, Burch-Smith *et al.* 2004, Valentine *et al*. 2004). The TRV vector is the most widely-used vector owing to its wide host range, but the introduction of the TRV vector into *Arabidopsis* plants is difficult, and it is necessary to passage TRV vectors through *Nicotiana benthamiana* to produce virions (Robertson 2004). The production of recombinant TRV RNA depends on the *in vitro* transcription of a linearized cDNA clone, which is also expensive (MacFarlane 1999). Not only is this process tedious, but it also requires relatively large culture

volumes for infiltration. Venegas *et al*. (2010) has successfully induced VIGS in *B. napus* utilizing the DNA virus CaLCuV for particle bombardment. The CaLCuV possesses a bipartite genome containing two DNA components (termed A and B). The A component is necessary for the replication and encapsidation of viral DNA, and the B component encoded proteins required for movement (Turnage *et al*. 2002). Sense sequences were inserted into the CaLCuV-A component and transformed into seedlings with CaLCuV-B component by biolistic delivery, which can apparently down-regulate target genes. However, the severe symptoms and expensive cost limit their usefulness, and the viral DNA cannot be transmitted to the next generation (Venegas *et al*. 2010).

The VIGS-associated processes required by previous VIGS vectors are difficult and time-consuming. TYMV is an typical RNA virus and can induced VIGS, which is initiated by dsRNA molecules through the formation of dsRNA duplexes between the complementary RNA strands and/or folding into secondary structures of the viral genomic RNA (Molnar et al. 2005, Voinnet 2005). In this paper, we described a TYMV-derived vector, pTY-S, harbouring an insert as short as 80 bp, which produces VIGS by biolistic delivery. The introduction of small inserts can result in more stable genome and, thus, facilitates the reproducibility of the VIGS phenotype compared with other VIGS vectors (Lacomme *et al.* 2003). The insertion of inverted-repeat sequences, which fold into hairpin structures, can strongly enhance VIGS, potentially by increasing the pool of produced dsRNA (Lacomme *et al.* 2003). The pTY-S harbours a viral cDNA copy of TYMV positioned between the CaMV 35S promoter and terminator (Kay *et al*. 1987). Previous studies indicated that inoculation by abrasion with the intact plasmid DNA of a TYMV-derived vector can induce robust and reliable gene silencing in *Arabidopsis* (Pflieger *et al*. 2008). However, the silencing efficiency was weak when employed in *B. rapa*, and the plasmid construction was difficult owing to the low efficiency of blunt-end ligations. Thus, we improved the vector construction using homologous recombination and adopted particle bombardment to transform plants, producing a strong and stable VIGS effect. To efficiently silence the *BrPDS* gene family, we constructed the recombinant plasmid pTY-PDS, which carries a palindromic DNA fragment of 80 nt, to target the highly conserved sequence regions because the TYMV RNA genome can survive the insertion of foreign sequences of 80 nt (Pflieger *et al*. 2008). As little as 1 μg of plasmid DNA can effectively silence genes of interest, and a 50 - 76 % silencing efficiency can be achieved which varied in different tissues. Leaves showed the highest silencing efficiency, followed by stems, stalks, siliques and roots. However, because of its high costs, the use for studying gene functions is still limited. To continue improving the VIGS technology, we optimized the inoculation method using juice of the infected leaves, which were ground in MMA [10 mM 2-(N-morpholino) ethanesulfonic acid, 10 mM $MgCl_2$, and 200 μM aceto-syringone] solution (Bai *et al*. 2017). Apparent photo-bleaching was observed after 3 weeks. Another distinct advantage is the persistent period of VIGS. Here, we observed a photobleaching during the whole life cycle. In some VIGS vectors, like TMV, the silencing signal cannot be detected in the newly merged leaves after 4 weeks (Faivre-Rampant *et al*. 2004).

Despite these benefits, inherent potential limitations, such as the silencing of off-targets and the symptoms of virus infection might mask the silenced phenotype, still exist (Kay *et al*. 1987). To avoid the silencing of off-target genes, a gene fragment that specifically targets genes of interest should be chosen. Furthermore, to eliminate confusion over disease symptoms, plants inoculated with an empty vector pTY-S and a vector carrying the *PDS* fragment pTY-PDS were used as negative controls. Compared with shorter sequences, an 80-nt insertion into the viral genome led to the attenuation of viral symptoms in *Arabidopsis* (Pflieger *et al*. 2008). Additionally, we found that the VIGS efficiency ranged from 50 to 75 % among different tissues when using the TYMV-derived vector, suggesting that viral movement was not as efficient when entering roots and flowers. This requires further improvements. Taken together, we advise that an 80-nt hairpin sequence identical to the target sequences should be used for VIGS to alleviate the interference caused by the virus itself.

Conclusions

In this study, we established an improved VIGS system based on the TYMV-derived vector that efficiently silenced the *BrPDS* gene family. The improved system included the enhancement of the VIGS capability through particle bombardment and the optimization of the vector construct by homologous recombination. The pTY-S was digested, and an 80-nt fragment identical to the genes of interest was synthesized for plasmid construction. The plasmid harbouring the insert was used to transform plants by particle bombardment. Then, the large-scale employment of VIGS technology can be achieved through abrasion. The VIGS system described here may provide an avenue for large-scale functional genomic screens using the particle bombardment of plasmid DNA. This will substantially contribute to the characterization of many *B. rapa* genes.

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