An efficient virus-induced gene silencing (VIGS) system for functional genomics in Brassicas using a cabbage leaf curl virus (CaLCuV)-based vector

Zhiliang Xiao1 · Miaomiao Xing1 · Xing Liu1 · Zhiyuan Fang1 · Limei Yang1 · Yangyong Zhang1 · Yong Wang1 · Mu Zhuang1 · Honghao Lv1

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Abstract

Main conclusion CaLCuV-based VIGS effectively works in cabbage and contributes to efficient functional genomics research in Brassica crop species.

Abstract Virus-induced gene silencing (VIGS), a posttranscriptional gene-silencing method, is an effective technique for analysing the functions of genes in plants. However, no VIGS vectors have been available for Brassica oleracea until now. Here, tobacco rattle virus (TRV), pTYs and cabbage leaf curl virus (CaLCuV) gene-silencing vectors (PCVA/PCVB) were chosen to improve the VIGS system in cabbage using the phytoene desaturase (PDS) gene as an efficient visual indicator of VIGS. We successfully silenced the expression of PDS and observed photobleaching phenomena in cabbage in response to pTYs and CaLCuV, with the latter being more easy to operate and less expensive. The parameters potentially affecting the silencing efficiency of VIGS by CaLCuV in cabbage, including the targeting fragment strategy, inoculation method and incubation temperature, were then compared. The optimized CaLCuV-based VIGS system involves the following: an approximately 500 bp insert sequence, an Agrobacterium OD600 of 1.0, use of the vacuum osmosis method applied at the bud stage, and an incubation temperature of 22 °C. Using these parameters, we achieved a stable silencing efficiency of 65%. To further test the effectiveness of the system, we selected the Mg-chelatase H subunit (ChlH) gene in cabbage and knocked down its expression, and we observed yellow leaves, as expected. We successfully applied the CaLCuV-based VIGS system to two other representative Brassica crop species, B. rapa and B. nigra, and thus expanded the application scope of this system. Our VIGS system described here will contribute to efficient functional genomics research in Brassica crop species.

Keywords Brassicas · Cabbage · CaLCuV · Phytoene desaturase (PDS) · pTYs · VIGS system

Abbreviations

CaLCuV Cabbage leaf curl virus
ChlH Chelatase H subunit
PDS Phytoene desaturase
TRV Tobacco rattle virus
VIGS Virus-induced gene silencing

Introduction

Virus-induced gene silencing (VIGS), a posttranscriptional gene-silencing method, plays a role as an antivirus defence system in plants (Ratcliff et al. 1997; Hamilton and Baulcombe 1999; Godge et al. 2008). When the virus with a targeting fragment has entered into plant cells, it begins to replicate and transcribe its DNA or RNA genome.
Double-stranded RNA (dsRNA) intermediates are produced and induce the degradation of target genes. Thus, changes in plant phenotype or physiological indicators can be induced to identify gene function effectively (Godge et al. 2008). VIGS has emerged as a widely functional genomics tool for knocking down the transcript level of genes in plants, especially for genes related to plant disease resistance, stress resistance, growth and development, and metabolic regulation (Hands et al. 2011; Qu et al. 2003; Chen et al. 2013; Hsieh et al. 2013; George et al. 2012). The advantages of VIGS have also emerged in other studies, such as its ease of operation, broad application range, high effectiveness, and independence of genetic transformation (Peng et al. 2002; Qu et al. 2003; Shao et al. 2003; Burch-Smith et al. 2004; Dinesh-Kumar et al. 2011).

In recent years, various virus vectors, such as the tobacco mosaic virus (TMV) (Hiriart et al. 2003), satellite virus-induced silencing system (SVISS) (Gosselé et al. 2003), potato virus X (PVX) (Fauveramant et al. 2004), barley stripe mosaic virus (BSMV) (Holzberg et al. 2010), Cotton leaf crumple virus (CLCrV) (Richard et al. 2012), African cassava mosaic virus (ACMV) (Fauquet et al. 1988; Beyene et al. 2017), tobacco rattle virus (TRV) (Burch-Smith et al. 2006), cabbage leaf crumple virus (CaLCuV) (Tang et al. 2013; Chen et al. 2015), and turnip yellow mosaic virus (TYMV) (Yu et al. 2018), have been successfully used in VIGS. Among them, TRV has the widest host range and induces mild viral symptoms after infecting either dicotyledonous and monocotyledonous plants (Burch-Smith et al. 2004; Chen et al. 2004; Liu et al. 2004; Hidalgo et al. 2012; Fernández-Calvino et al. 2016; Zhang et al. 2017). Each virus vector has a certain host range, and their effects on inducing silencing differ. Several species have been used to establish VIGS systems, e.g., Arabidopsis, apple, lettuce, eggplant, tobacco, strawberry, papaya, and Nicotiana benthamiana (Burch-Smith et al. 2006; Jun et al. 2008; Juenken et al. 2009; Mannathan et al. 2013; Zhao et al. 2015, 2019; Navarro et al. 2017).

The silencing efficiency of VIGS is influenced by many parameters, including the vector targeting fragment (insertion direction and the size of the insert sequence) (Liu and Page 2008; Beyene et al. 2017), infection pattern (Fu et al. 2005), culture environment (Tuttle et al. 2008; Xu et al. 2018) and plant growth stage (Cai et al. 2006). Therefore, additional research and exploration are needed for to develop more-efficient VIGS systems.

Cabbage (Brassica oleracea L. var. capitata), an important Brassicaceae crop species, is reported to be recalcitrant to transformation by many genotypes. VIGS has received increasing amounts of attention recently because of its ability to evaluate gene function rapidly and inexpensively. Due to the ability to evaluate gene functions rapidly and inexpensively, VIGS finds increasing attention and has also been established for some Brassicaceae species to analyse critical roles of some genes. In the model plant Arabidopsis thaliana, several VIGS vectors have been successfully established and assist in gene function verification, including TRV, CaLCuV, and pTYs (derived from turnip yellow mosaic virus, TYMV) (Hericourt and Jupin 1993; Nooduan et al. 2004; Cai et al. 2006; Zheng et al. 2010). Using particle bombardment, Yu et al. (2018) established an improved VIGS system based on a TYMV-derived vector that efficiently silenced the phytoene desaturase (PDS) gene in B. rapa. Zheng et al. (2010) applied the VIGS vector TRV to knock down endogenous PDS expression in three plant species (A. thaliana, B. nigra and N. benthamiana). These VIGS studies laid foundations for VIGS studies in cabbage and other Brassicaceae crop species. However, there have been no reports of VIGS in cabbage, and it is unknown whether any VIGS vectors can be applied to reveal the functions of genes in cabbage. Therefore, it is necessary to explore and develop the VIGS system in cabbage.

In this study, we tested TRV, pTYs and CaLCuV to develop a VIGS system in cabbage using the PDS gene as an efficient control for VIGS. We efficiently downregulated the PDS gene in cabbage via pTYs and CaLCuV, and the latter method could also be applied to other Brassicaceae species. This VIGS system will have an important way for analysing gene function among Brassicaceae species.

Materials and methods

Plant materials and growth conditions

Brassica oleracea accession 87-534, B. rapa cultivar Zhongbai 76, B. nigra accession Juntus and B. campestris cultivar 49 Caixin, provided by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS), were used. The plants were incubated in a compost:vermiculite mixture (1:1) in a growth chamber with a 16 h photoperiod and 50% relative humidity. Roots, stems and leaves exhibiting a photobleaching phenotype were harvested and stored at – 80 °C for follow-up analyses. TRV, pTYs and PCVA/PCVB vectors kindly provided by Dr Su Xiaomei (Shandong Academy of Agricultural Sciences, Jinan, China), Prof Zhang Changwei (Nanjing Agricultural University, Nanjing, China) and Prof Liu Yule (Tsinghua University, Beijing, China), respectively, were used.

Analysis of PDS in cabbage and vector construction

To test whether TRV, pTYs and CaLCuV could effectively downregulate the expression of target genes in cabbage, the PDS gene, which is strongly associated with the photobleaching phenotype, was used as a reporter gene. The
sequences of BoPDS were obtained from the TO1000 Brassica reference genome (https://plants.ensembl.org/Brassica_oleracea/). The primers for BoPDS (PrimerPDS) were designed via Premier 5 (Premier Biosoft International, Palo Alto, CA, USA). Total RNA was extracted from the leaves of cabbage line 87-534 using an EasyPure™ Plant RNA Kit (Vazyme, Nanjing, China). The coding sequences of BoPDS were amplified by polymerase chain reaction (PCR), and the primers used are shown in Table 1. The PCR profile was as follows: 95 °C for 3 min; 36 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 150 s; and a final cycle of 72 °C for 5 min. The PCR-amplified products were checked by 1% agarose gel electrophoresis (150 V) and were subjected to sequencing. The conserved regions of BoPDS were then selected, and VIGS vectors were constructed. The vector construction of the three vectors was different, and the steps are detailed below. A schematic vector map for each vector is shown in Figs. S1–S5. The vector construction for CaLCuV was as follows: (i) The optimal 500 bp conserved sequence of BoPDS was selected. According to the XbaI restriction sites on PCVA and the homologous arms on both sides, primers containing restriction sites and homologous arms (PCVAP500) were designed (Table S1). (ii) The target sequences were amplified using first-strand cDNA, and PCVA was digested with XbaI (NEB, Ipswich, MA, USA) at 37 °C overnight. The digested PCVA and the target sequence were ligated at 50 °C for 15 min via a ClonExpress Entry One Step Cloning Kit (Vazyme). (iii) The ligation mix from the previous step was transformed into competent DH5α cells (Invitrogen, Darmstadt, Germany), which were then plated on LB plates containing antibiotics (kanamycin at 50 mg/ml) at 37 °C overnight. Single colonies were selected, shaken in liquid LB media (supplemented with kanamycin at 50 mg/ml) and subjected to sequencing by a specific primer—primerA (for PCVA). Empty PCVA, PCAB and PCVAP500 were isolated using a FastPure EndoFree Plasmid Maxi Kit (Vazyme). (iv) The plasmids were separately transformed into Agrobacterium tumefaciens GV3101 by the freeze–thaw transformation method. Single colonies were then selected and shaken in liquid LB media (supplemented with kanamycin at 50 mg/ml and rifampicin at 50 mg/ml), after which the mixture was shaken at 28 °C for 10–12 h. (ii) 200 ml of Agrobacterium induction culture solution was then added to 5 ml of LB liquid media (supplemented with kanamycin at 50 mg/ml and rifampicin at 50 mg/ml), after which the mixture was incubated at 28 °C for 6–10 h to a final OD_{600} of 0.8–1.2. (iii) 2 ml of the above bacterial mixture was then collected and centrifuged for 8 min at 6000 g at 4 °C, after which the supernatant was discarded. (iv) 2 ml of infection buffer (5 mg/ml d-glucose, 10 mM MES, 100 µM acetosyringone, 2 mM Na_{2}PO_{4}·12H_{2}O_{2}) was then added to suspend the bacteria; the mixture was centrifuged for 8 min at 6000 g at 4 °C, after which the supernatant was discarded. (v) 2 ml of infection buffer was added to suspend the bacteria, after which the bacterial cultures were incubated at room temperature for 3–4 h under dark conditions. (vi) With respect to Agrobacterium vacuum infiltration at the bud stage, the Agrobacterium culture suspension of PCVB and either PCVA or PCVAP500 were mixed together at a 1:1 ratio, after which the germinated seeds (1 cm long radical) were soaked in the infection
mixture. A centrifuge tube containing the suspension was placed into a vacuum dryer, and the tube was evacuated using a vacuum pump at −80 kPa for 10 min. With respect to Agrobacterium syringe infiltration at the leaf stage, the culture mixture was infiltrated into the leaves of four- to six-leaf-stage plants by a 1 ml syringe, and the plants were kept in darkness 24 h. (vii) The seeds were then sown in a compost: vermiculite mixture, and the phenotypes were evaluated after 2–3 weeks.

For inoculation of pTYs, the following protocol was used: (i) With respect to preparation of the gene gun ‘bullets’, 1.5 μl of DNA plasmid, gold particles at 1 μM (Bio-Rad, Hercules, CA, USA), 50 mm<sup>3</sup> of 2.5 M CaCl<sub>2</sub> and 20 mm<sup>3</sup> of 0.1 M spermidine were added to a 1.5 ml cryotube on ice. (ii) The particle mixture was then shaken with an oscillator for 4 min. (iii) Afterwards, the particle mixture was centrifuged for 1 min at 10,000 g, after which the particles were washed with 200 μl of absolute ethanol twice, leaving 10 μl of particle suspension. (iv) The particle suspension as added to the holder (Bio-Rad) quickly with a pipette gun. (v) The particles were implemented using a PDS 1000/He biolistics gun (Bio-Rad) at 1300 psi according to the manufacturer’s instructions. (vi) After bombardment, the plants were incubated in the dark for 1 day and then grown in a growth chamber at 21–28 °C. (vii) The seeds were then sown in a compost: vermiculite mixture, and the phenotypes were evaluated after 2–3 weeks.

Analysis of fragment inserts and gene expression in treated plants

Confirmation of whether the inoculated plant is infected with the virus and whether the virus vectors successfully silence the expression of the target gene is an essential item in the VIGS experiment. In our study, to confirm whether the virus vector successfully infects plants, the treated seedlings were subjected to sequencing in conjunction with the following specific primers: primer A/B (for PCVA/PCVB), CP (for pTYs), and primer 1/2 (for TRV1/2). Quantitative real-time RT-PCR (qRT-PCR) was performed using ChamQ SYBR qPCR Master Mix (Vazyme) to confirm whether the photobleaching phenomenon was induced by BoPDS. The total RNA of the stems and roots was extracted to analyse the gene expression of PDS in other tissues. The gene-specific primers for qRT-PCR were designed on the basis of the PDS sequences (Table 1), and primers for B. oleracea actin (GenBank accession number XM_013731369.1) were used as controls (Hàn et al. 2018). The PCR-amplified products were checked by agarose gel electrophoresis (150 V). Three technical replicates were performed for each gene, and three biological replicates were included for each sample. The relative transcript levels of BoPDS were analysed using the 2<sup>−ΔΔCT</sup> method (Livak and Schmittgen 2001). The error bars represent the SEs from three independent experiments. The data were analysed by ANOVA using SAS software.

Optimization of the VIGS system in cabbage

VIGS is currently applied effectively in many plant species, and successful gene-silencing depends upon the dynamic interaction between the virus and plant, both of which can be affected by various experimental factors. To explore the efficiency of the VIGS system in cabbage, we compared different post-treatment approaches in our study, as described here. The length of the insert sequence of PDS should be 300–800 bp (Liu and Page 2008; Yu et al. 2018), and different lengths of the insert sequence (approximately 300 bp, 500 bp, 700 bp, 800 bp) of PDS were inserted into PCVA. Some studies have indicated that the silencing efficiency of reverse insertion is better than that of forward insertion (Burch-Smith et al. 2004), so both forward and reverse insertions are acceptable for VIGS. Different infection modes and plant growth periods were used (the vacuum osmosis method at the bud stage and injection during the leaf period), with different OD<sub>600</sub> values of the Agrobacterium infection solution (OD<sub>600</sub> = 0.8, 1.0, 1.2, 1.4). We also successively cultivated plants at different temperatures (18 °C, 22 °C, 26 °C, 30 °C). The number of seedlings subjected to each treatment was 20, and each experiment was repeated three times. The infection ratio (the ratio of the number of infected seedlings to the number of inoculated seedlings) and silencing efficiency (the ratio of the number of seedlings exhibiting a silenced phenotype to the number of inoculated seedlings) for each experiment were calculated. The error bars represent the SEs from three independent experiments. The data were analysed by ANOVA using SAS software. Finally, we integrated the best conditions to obtain high and stable silencing efficacy.

Verification of gene function in cabbage and application of VIGS in other Brassicaceae species

To further test our VIGS system for cabbage, we chose several reported or homologous cabbage genes to verify their genetic function by VIGS. Given that cabbage is a leafy vegetable species, the leaf traits of cabbage and other Brassicaceae species have attracted much attention. The Mg-chelatase H subunit (ChlH) gene encodes a subunit of Mg-chelatase, which is involved in chlorophyll biosynthesis (Hiriart et al. 2003). The homologous gene in cabbage (Bo3g009280) was queried via BLAST against the TO1000 genome. The genes were then used for the construction of CaLCuV-based VIGS vectors, and VIGS...
experiments were performed to verify the gene functions, with a PDS vector used as a control. We observed whether gene silencing caused phenotypic changes, and qRT-PCR was also performed to confirm whether the expression of the target genes was knocked down. The function of these genes in cabbage was confirmed based on the expression levels and their corresponding phenotypes.

To apply this technique to other Brassicaceae species, we applied the CaLCuV-based VIGS system to B. rapa and B. nigra, two other basic Brassica species. Among related species, many genes are highly homologous. Zheng et al. (2010) reported that TRV vectors carrying target sequences from cabbage can silence their orthologues in species related to Brassica, such as B. nigra, as well as in A. thaliana and even N. benthamiana. This indicates that silencing vectors have certain universality among related species. Thus, we further infected other Brassicaceae species with PCVAP500/PCVB.

Results

Analysis of the PDS gene and vector construction

Based on the ‘TO1000’ reference genome, we identified two homologous genes of PDS (Bo3g149210, Bo4g127210) in cabbage. The Bo3g149210 and Bo4g127210 sequences of 87-534 were then amplified, and the sequencing and alignment results showed that Bo4g127210 (1614 bp) and Bo3g149210 (1257 bp) were highly evolutionarily conserved (Fig. S6). Thus, we chose a conserved fragment between these two genes as a target sequence to ensure the silencing effect (Fig. 1).

Approximately 500 bp of BoPDS was amplified and ligated to the digested PCVA. Additionally, the same strategy was applied to 300 bp, 600 bp, and 800 bp sequences as well as 500 bp (reverse insertion) sequences. The amplification results showed that the PCVAP500 (500 bp), PCVAPR500 (reverse insertion), PCVAP300 (300 bp), PCVAP600 (600 bp), and PCVAP800 (800 bp) vectors were accurately constructed, as shown in Fig. 2. Moreover, the sequencing results indicate 300, 500, 600, 800 bp fragments derived from Bo4g127210, which was shown at Table S2. The constructed vectors were subsequently transformed into Agrobacterium GV3101. The pTYsBoPDS construct was then transformed into competent DH5α cells and subjected to sequencing, and 40 bp region for pTYs 100% homologous between the two genes (Fig. 2). The same 500 bp sequence (TRV2P500) was used for construction of a TRV2 vector based on the construction strategy for TRV. Our results demonstrate that we successfully constructed three VIGS vectors for cabbage.

PDS silencing in cabbage

After the seedlings were treated, photobleaching phenomenon was observed in the leaves of cabbage treated with PCVAP500/PCVB at 15–30 days post infiltration (dpi) but not in those injected with empty PCVA/PCVB vectors (Fig. 3). The photobleaching phenotype first appeared for the second true leaf (Fig. 3). With the growth of the seedlings, the photobleaching phenotype occurred in newly emerging leaves at 1–3 months (Fig. 3b, c). The photobleaching phenomenon involved a greenish–whitish variegated phenotype rather than a completely white phenotype. Moreover, the photobleaching phenotype was accompanied by leaf shrinkage, which is a typical symptom of virus infection in plants. However, we observed no visible photobleaching phenotype in the stems and roots. The pTYsBoPDS vector was infected with 1000/He biolistic gun; a photobleaching phenotype was also observed for new leaves at 15–30 days post infiltration with this method (Fig. 3d). The photobleaching phenomenon induced by pTYsBoPDS was different from that induced by PCVAP500: the photobleaching spots were spread over the newly developed leaves and occurred along the leaf vein, indicating that different VIGS vectors may show different silencing phenomena. However, seedlings injected with TRV2P500 did not show any photobleaching phenomenon across repeated experiments, even if we tried to modify the
system, such as using the infection buffer for PCVA/PCVB, constructing new TRV2 vectors with other positions and lengths of BoPDS, and so on. To ensure the accuracy of our experimental process, we also constructed TRV2 vectors with the PDS of tomato and successfully observed the photobleaching phenomenon in tomato (Fig. S7).

**Evaluation of PCVA/PCVB fragments and gene expression in infected plants**

To investigate whether the photobleaching phenotypes correlated with reduced expression of the PDS gene by VIGS, the transcript levels of PDS were measured by RT-qPCR. The RT-qPCR results showed that the transcript levels of BoPDS were substantially reduced in the photobleached leaves by PCVAP500/PCVAP700, pTYsBoPDS, or TRV2 vectors with the PDS of tomato and successfully observed the photobleaching phenomenon in tomato (Fig. S7).

Different vectors may exhibit the same silencing efficiency (Fig. 4). PDS expression was most significantly reduced in the leaves, which is consistent with the phenomenon that photobleaching is most obvious in leaves compared with other organs. The reduced expression levels of BoPDS in the stems and roots indicated that gene silencing can spread throughout whole cabbage plants, suggesting that the VIGS system can be used to knock down the expression of all genes in cabbage plants or an entire gene family in multiple tissues.

To test whether the photobleached phenotype was due to the presence of the corresponding vectors, the presence of transcripts was also determined by RT-PCR. The photobleached plants showed the presence of the PCVA and PCVB bands. Similarly, pTYs bands were detected from the photobleached plants treated with pTYs. These results indicated that silencing of the PDS gene was induced by the presence of the PCVA/PCVB or pTYsBoPDS virus vector. The above bands of each photobleached plant were the same.
as the lines shown in Fig. 2. However, the TRV1 and TRV2 bands were not detected in plants treated with the TRV vector, suggesting that this virus vector may not be able to infect cabbage. For the silenced phenotypes that cannot result in visible phenotypes do not display visible signs, RT-PCR assessments and qRT-PCR can be used as important tools to confirm whether inoculated plants can be infected and to knock down the expression of target genes.

**Optimal conditions for CaLCuV-based VIGS in cabbage**

After successful application of CaLCuV-based VIGS and pTY-based VIGS in cabbage, we chose the former for further study because particle bombardment adopted for pTY-based VIGS was expensive and complex. In contrast, CaLCuV-based VIGS via *Agrobacterium* vacuum infiltration at the bud stage is easy to perform and inexpensive. Thus, different post-treatment approaches were used to optimize the CaLCuV-based VIGS system to achieve the highest silencing efficiency. Each method was performed in three repeated experiments, each involving 20 seedlings. For different cDNA insert lengths, the infection rates were similar, while the silencing rates of the 300 bp, 500 bp, 700 bp, and 800 bp fragments were 15%, 50%, 35%, and 4%, respectively. These data showed that the insert lengths for the silencing vectors did not affect the infection efficiency but did affect the silencing efficiency, and 500 bp was the optimum length for PCVA. Both the forward and reverse insert directions of the 500 bp insert yielded similar silencing rates, which indicated that the insertion direction had no effect on silencing efficiency. For the different infection modes and plant growth periods, the vacuum osmosis method at the bud stage showed more advantages, including increased infection efficiency, batch production, and easy operation. Moreover, the infection rates for different OD$_{600}$ values and growth temperatures showed that these factors directly affect the infection process and that non-optimal conditions can severely interfere with the infection process. Different OD$_{600}$ values of the *Agrobacterium* infection solution also affected the efficiency of silencing, with high or low OD$_{600}$ values reducing the silencing efficiency, of which an OD$_{600}$ of 0.8–1.0 of the *Agrobacterium* infection solution was notable in terms of silencing efficiency. Infected plants at 22 °C presented the highest rate. Thus, both relatively high and relatively low growth temperatures decreased the silencing efficiency, and VIGS at high temperatures did not work. We found that high temperatures accelerated the absence of the photobleaching phenotype.

The silencing efficiency using different post-treatment approaches is shown in Fig. 5, and the raw data of the infection rates and silencing efficiency are shown in Table S3. Based on the above results, we selected the following optimal components and conditions to carry out VIGS experiments: use of PCVAP500/PCVB VIGS vectors, use of the vacuum osmosis method at the bud stage, an OD$_{600}$ of 1.0 for *Agrobacterium* infection, and a temperature of 22 °C. The silencing efficiency of *PDS* was approximately 65%, which indicated a highly stable and efficient VIGS system in cabbage by PCVA/PCVB.

**Verification of the functions of BoChlH in cabbage**

Based on the optimized VIGS system, PCVABoChlH silencing vectors were constructed and infected into cabbage, with control plants treated with water only. The seedlings were also infected by PCVAP500/PCVB to ensure that our operation was correct in this experiment. The treated plants exhibited a yellowing phenotype on the newly developed leaves, with no changes after treatment with water (Fig. 6a). The RT-PCR assessment and qRT-PCR results showed that the PCVABoChlH silencing vector successfully infected the plants, and the gene expression was significantly reduced in the leaves (Fig. 6b, c). Using CaLCuV-based VIGS in cabbage, we successfully verified that *BoChlH* is involved in chlorophyll biosynthesis, and reducing the expression of this gene in cabbage causes corresponding phenotypic changes.

**The CaLCuV-based VIGS system can be used in other Brassica plant species**

The sequences of the *PDS* gene in *B. oleracea*, *B. nigra*, *B. rapa*, and *B. campestris* were compared, and the results showed that the *PDS* gene is highly conserved in the above crop species. The PCVAP500/PCVB silencing vector can be used to knock down endogenous *PDS* expression in these Brassicaceae species, and the photobleaching
phenomenon was observed in these species (Fig. 7a–c). The infection phenotype and duration were consistent with those of cabbage. However, only a narrow area bordering the veins appeared bleached in *B. rapa*, *B. rapa* (AA), *B. nigra* (CC) and *B. oleracea* (BB) constitute the three basic species of the Brassicaceae family. Together, these results indicate that the CaLCuV-based VIGS system may be applicable to additional Brassicaceae species.

**Discussion**

Cabbage is an important vegetable crop species due to its broad adaptability, strong resistance, healthcare-related value, and enjoyable taste (Fang et al. 1997). Many important traits of cabbage have been reported, including petal colour, yellow–green leaves, green glossiness, hybrid...
lethality, resistance to *Fusarium* wilt and self-incompatibility (Han et al. 2015; Liu et al. 2016, 2017; Xiao et al. 2017). Transgenic technology and new clustered regularly interspaced short palindromic repeats (CRISPRs) have recently received attention because of the stable inheritance that results; these techniques are important for analysing gene function, and several optimized transformation systems in various Brassica crop species have been reported (Lee et al. 2004; Lawrenson et al. 2015; Thomas et al. 2017; Ayako et al. 2018; Chen et al. 2019; Xiong et al. 2019). However, low transformation efficiency and time consumption limit their effective and widespread application. Thus, developing a low-cost and rapid high-throughput method to evaluate and validate gene function in cabbage is necessary. VIGS is widely used for plant functional genomics because of its high efficiency and high speed, so we attempted to use this technique in cabbage and ultimately successfully established a VIGS system in this species.

Various viruses have been successfully applied to VIGS to reveal gene functions. In our study, the *PDS* gene was successfully silenced by pTYs and CaLCuV, whereas the TRV vector was unsuccessful, suggesting that each virus vector can effectively silence genes of only some species, even related ones. pTY-based VIGS and CaLCuV-based VIGS can achieve the same effect according to silencing phenomenon and qRT-PCR results. pTY-based VIGS needs particle bombardment to transform plants, which involves the use of an expensive and complex instrument. In contrast, CaLCuV-based VIGS by *Agrobacterium* infection was simple and inexpensive, so we chose this method for further study. This is the first report that applies the CaLCuV vector to cabbage. CaLCuV is a member of the genus *Begomovirus* in the family *Geminiviridae* and has a broad host range (Tang et al. 2013; Nooduan et al. 2014; Chen et al. 2015). The CaLCuV-based vector can use miRNAs to knock down RNA-mediated silencing of genes in *Nicotiana benthamiana* (Tang et al. 2013). We also successfully applied the CaLCuV-based VIGS vector to *B. rapa, B. nigra* and *B. campestris*. Our results indicate that the CaLCuV-based VIGS system may be applicable to additional Brassicaceae species.

Optimizing the silencing efficiency of VIGS constructs is crucial for successful silencing experiments. It is necessary to coordinate the dynamic balance between the replication and transmission of viruses in plant hosts and the growth and development of plants to ensure that silent signalling molecules can spread and propagate systematically in plants, ensuring the high efficiency and persistence of systematic silencing (Burch-Smith et al. 2004). According to previous studies, an insertion fragment causing target gene silencing of 23 bp to ~ 1.5 Mb and 300–800 bp inserts may be the optimum length for silencing effects (Lacomme et al. 2003; Burch-Smith et al. 2006; Thomas et al. 2010). In our study, the 500 bp insert sequence exhibited a higher silencing efficiency, and insert sequences longer than 800 bp were of little use, causing off-target silencing. How to effectively transfer virus vectors into plant cells is the key to the replication and transfer of viruses in plants. At present, most transformation vectors are transformed using the *Agrobacterium* mediated method, including the *Agrobacterium* toothpick stabbing method (Naylor et al. 2005), plasmid mechanical friction inoculation (Igarashi et al. 2009), host virus juice inoculation (Fofana et al. 2014), particle bombardment (Jeyabarathy et al. 2015), *Agrobacterium* infection by injection (Szittya et al. 2003; Pandey et al. 2009; Kang et al. 2010), and *Agrobacterium* vacuum infiltration (Lacomme et al. 2003). Both infection methods used in our study successfully transferred the virus vector into the plants; *Agrobacterium* vacuum infiltration at the bud stage can be used to carry out batch experiments and confirm the phenotypes at the seedling stage. Formulation of the infection solution and the concentration of the bacterial solution also affect the silencing efficiency. The results showed that the *Agrobacterium* solution at an OD_{600} of 1.0 used for infection had the highest silencing efficiency.

Environmental factors also affect the accumulation, transmission and growth of viruses in plants. The optimum temperature for effective silencing varies with the virus vector and host plant (Tuttle et al. 2008). A high-temperature environment can lead to a significant reduction in viral content and gene-silencing efficiency in plants. Moreover, viral content and gene-silencing efficiency increased significantly under low-temperature conditions. Tuttle et al. (2008) discovered that the ambient temperature needed to be lower than 21 °C to obtain a good silencing phenotype and that TRV-mediated VIGS was nearly completely inhibited when the ambient temperature was higher than 28 °C. Xu et al. (2018) tested the silencing efficiency by TRV under different growth temperatures (18 °C, 25 °C and 30 °C), and both the speed of the response and the efficiency of infection were...
optimal at 25 °C. A temperature of 22 °C was the most suitable temperature for PCVA/PVCB in cabbage, suggesting that an appropriate low temperature can improve silencing efficiency. We found that the silencing phenotypes of the PDS genes did not occur for whole leaves but rather as spots or patches on leaves, which indicated that the diffusion and proliferation of virus vectors are not homogenous. However, on the basis of our results, this did not affect our judgement of the silencing phenotypes. We found that the silencing spots described above also appeared in other VIGS experiments: those involving S. pseudocapsicum, cotton and pepper silencing by TRV; N. benthamiana silencing by CaL-CuV; and so on (Tuttle et al. 2008; Tang et al. 2013; Kim et al. 2017; Xu et al. 2018). The adjustment of factors potentially affecting the silencing efficiency failed to change the situation. We hypothesize that this situation may result from plant resistance to the virus.

In summary, we achieved strong, stable silencing efficacy by CaLCuV in cabbage. Of course, a higher silencing efficacy may be achieved by testing more factors related to the VIGS system. A 65% silencing efficacy is within the standards of popularization and application. The silencing phenotypes of ChlH verified our gene-silencing system. In our experiments, we chose genes related to easily observable phenotypes, leaf phenotypes and seedling-stage phenotypes. We did not develop VIGS experiments for use at the flowering stage or fruiting stage because of the complex phenotypes, leaf phenotypes and seedling-stage phenotypes. Fortunately, VIGS systems have been developed for flowers, fruits and trees (Sasaki et al. 2011; Fernandez-Moreno et al. 2013; Spitzerrimon et al. 2013). We believe that we can gradually overcome these difficulties and widely apply VIGS in cabbage.

Conclusion

In this study, we successfully silenced the expression of PDS and observed the photobleaching phenomenon in cabbage caused by pTYs and CaLCuV vectors. The factors potentially affecting the silencing efficiency of VIGS by CaLCuV in cabbage and the optimized VIGS system using the CaLCuV silencing vector were determined to be as follows: an approximately 500 bp insert sequence, an Agrobacterium OD600 of 1.0, use of the vacuum osmosis method at the bud stage, and an incubation temperature of 22 °C. Using these parameters, we achieved a stable silencing efficiency of 65%. Moreover, we successfully knocked down the expression of ChlH and observed the expected yellow colour. We applied the CaLCuV-based VIGS system to two other representative Brassica crop species, B. rapa and B. nigra, thus expanding its scope of application. Our VIGS system described here will pave an important way for efficient functional genomics research in Brassica crop species.

Author contribution statement

ZX designed and developed the VIGS experiments and wrote the manuscript. HL and MZ edited the manuscript. MX, XL, ZF, LY, YZ and YW analysed the data.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

No human subjects were involved in this study.

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