Simultaneous Silencing of Two Different Arabidopsis Genes With A Novel Virus-Induced Gene Silencing Vector

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Research

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Abstract

**Background:** Virus-induced gene silencing (VIGS) is a useful tool for functional characterizations of plant genes. However, the penetrance of VIGS varies depending on the genes to be silenced, and has to be evaluated by examining the transcript levels of target genes.

**Results:** In this report, we report the development of a novel VIGS vector that permits a preliminary assessment of the silencing penetrance. This new vector is based on an attenuated variant of *Turnip crinkle virus* (TCV) known as CPB that can be readily used in *Arabidopsis thaliana* to interrogate genes of this model plant. A CPB derivative, designated CPB1B, was produced by inserting a 46 nucleotide section of the *Arabidopsis PHYTOENE DESATURASE* (*PDS*) gene into CPB, in antisense orientation. CPB1B induced robust PDS silencing, causing easily visible photobleaching in systemically infected *Arabidopsis* leaves. More importantly, CPB1B can accommodate additional inserts, derived from other *Arabidopsis* genes, causing the silencing of two or more genes simultaneously. With photobleaching as a visual marker, we adopted the CPB1B vector to evaluate the relative importance of several known RNA silencing pathway genes in *PDS* VIGS. This approach allowed us to validate the involvement of *DICER-LIKE 4* (*DCL4*) and *ARGONAUTE 2* (*AGO2*) in *PDS* silencing. Notably, *double-stranded RNA-binding protein 4* (*DRB4*), whose protein product (DRB4) commonly partners with DCL4 in the antiviral silencing pathway, was dispensable for *PDS* silencing induced by CPB1B.

**Conclusions:** The CPB1B-based vector developed in this work is a valuable tool with tracable and visualizable indicator of the silencing penetrance for interrogating *Arabidopsis* genes, especially those involved in the RNA silencing pathways.

**Background**

RNA silencing is a generic term covering a number of mechanistically related phenomena occurring in eukaryotic organisms ranging from fungi to humans. Briefly, silencing is triggered by double-stranded RNA (dsRNA) or partially double-stranded hairpin RNA, which is digested by a dsRNA-specific RNase (Dicer or Dicer-like in plants) into small interfering (siRNAs) or micro (miRNAs) RNAs with length of 21–24 nucleotide (nt) [1, 2]. In addition to Dicer-likes (DCLs), this dsRNA processing step frequently requires one member of the dsRNA-binding protein (DRB) family [3]. Once produced, siRNAs and miRNAs are recruited by Argonaute proteins (AGOs) into RNA-induced silencing complexes for the direct degradation or the translational repression of other homologous RNAs or the modification of homologous chromatin DNA [1, 2].

The virus-induced gene silencing (VIGS) system takes advantage of this defense system to silence endogenous RNA sequences that are homologous to a sequence engineered into the viral genome, which generates the dsRNA that mediates silencing. As a tool, VIGS has many advantages over conventional techniques, such as independent genetic transformation, easy manipulation, high effectiveness, and suitability for the large-scale functional analysis of genes and for analyzing genes that cause lethal
phenotype. In addition, the effect of silencing can be monitored within a short time after inoculating plants with the virus. Given these features, VIGS is an attractive reverse genetic tool for functional genomics in plants [4–8]. In the past decade, a number of viral genomes has been modified as a powerful reverse genetic tool for the functional characterization of genes in plants, such as Tobacco rattle virus [9], Apple latent spherical virus [10, 11], African cassava mosaic virus [12], and Cucumber mosaic virus [13]. However, most of the reported VIGS vectors only silence a single gene, and the VIGS vectors with traceable and visualizable indicator to predict positive gene silencing plants for reverse genetics are lacking.

This work aims to develop an efficient and stable viral vector with visualizable indicator to predict positive gene silencing plants efficiently for reverse functional genomics. The attenuated variant of Turnip crinkle virus (TCV) known as CPB is chosen for testing and further modifications. The CPB, which has compromised ability of TCV capsid protein (CP) to suppress RNA silencing, causes systemic infection with no evident symptom in Arabidopsis. Our previous work has shown that the CPB-CC-PDS generated by fusing 90 nt PDS inserted to CPB can induce modest PDS silencing in Arabidopsis, thereby providing a visual indicator for the silencing inducing capability of this viral mutant [14]. Compared with other VIGS vectors, CPB has a number of advantages. First, the TCV genome consists of just one positive (+) sense RNA, which is merely 4054 nt long and requires neither a 5’ cap nor a 3’ poly A tail, making the in vitro synthesis of infectious RNA extremely simple and cost-effective [15]. Second, the TCV replicates in the model plant Arabidopsis to produce extremely high levels of viral RNA, dsRNA, and viral siRNAs (vsiRNAs), simplifying the detection and purification processes [14, 16–18].

In the current study, a CPB derivative, designated CPB1B, was produced by inserting a 46 nucleotide section of the Arabidopsis PDS gene into CPB, in antisense orientation. We have demonstrated that CPB1B induced robust PDS silencing, causing easily visible photobleaching in systemically infected Arabidopsis leaves. More importantly, CPB1B can accommodate additional inserts, derived from other Arabidopsis genes, causing the silencing of two or more genes simultaneously. With photobleaching as a visualizable indicator of the penetrance of VIGS, we adopted the CPB1B vector to evaluate the relative importance of several known RNA silencing pathway genes in antiviral defense against TCV. Firstly, we validate the involvement of DICER-LIKE 4 (DCL4) in antiviral defense against TCV. Furthermore, we have revealed the AGO2 gene involved in the DRB4-independent DCL4-mediated PDS silencing. Notably, double-stranded RNA-binding protein 4 (DRB4), whose protein product (DRB4) commonly partners with DCL4 in the antiviral silencing pathway, was dispensable for PDS silencing induced by CPB1B. These results demonstrated the CPB1B-based VIGS system as a valuable tool for interrogating Arabidopsis genes, especially those involved in the RNA silencing pathways. At the same time, this work opens a potential avenue for the development of VIGS vector through the synthesis of short fragments, which include several predicted siRNA sequences to silence two or more functional genes at the same time.

Methods And Materials

Plant materials
The sources of dcl4, dcl2, and dcl2drb4 mutant plants were described previously [17,18]. Uninfected Arabidopsis plants were reared in a growth room with 14 h of daylight and temperature of 22 °C. The Arabidopsis plants (3 to 4 weeks old) were inoculated using the *in vitro* transcription of viral RNAs. Usually, six plants were inoculated with each transcript. After inoculation using the *in vitro* transcription of viral RNAs, the infected plants were moved into versatile environmental test chamber (SANYO).

**Generation of VIGS constructs**

The original TCV VIGS vector plasmid, CPB, was constructed in Dr. Qu's lab at Ohio state University, as described in a previous study [14]. Briefly, CPB contained an arginine (R) to threonine (T) mutation at position no. 130 of the TCV CP that substantially compromised the ability of TCV CP to suppress RNA silencing [16]. CPB was further modified by changing the AT dinucleotide at nt 3807 to 3808 of the TCV genome to CC, creating CPB-CC with a new KpnI site immediately downstream of the TCV CP coding region (Fig. 1B) [14].

To determine the optimal orientation of the foreign insert in CPB-CC-based VIGS vector, the *PDS* fragment inserted in CPB-CC-PDS was subjected to the software [https://www.genscript.com/tools/sirna-target-finder](https://www.genscript.com/tools/sirna-target-finder) to predict the siRNAs. In accordance with the results, one set of short complementary oligos containing the predicted siRNA sequence were synthesized. The complementary oligos in 1 X T4 DNA ligase buffer were annealed in the Thermocycler with the following cycling conditions: 95 °C, 5 min; 65 °C, 5 min; 35 °C, 5 min; and 25 °C, 5 min. The resulting annealed dsDNA oligo was ligated into the CPB-CC digested and dephosphorylated using KpnI. The ligated plasmid was transformed into *Escherichia coli* (DH5α strain)-competent cells. The primers TCV-3334F and TCV-4000R (Table S3) were used for colony screening. The orientation of the insertion was determined using the restriction enzymes EheI and KpnI. The resulting two CPB-CC-derived constructs were named CPB1F and CPB1B, which were inserted with *PDS* fragment in the sense (F) or antisense (B) orientations, respectively.

The primers CPB1B-F, CPB1B-102R, CPB1B-139R, CPB1B-215R (Table S2) were designed to amplify 102-, 139-, and 215 nt *PDS* fragments to generate CPB1B-based VIGS vectors with different sizes of insert (Fig. S2). The resulting fragments were ligated into KpnI-treated CPB1B through the Gibson assembly master mix (New England BioLabs) in accordance with the manufacturer's description. The primers TCV-3334F and TCV-4000R (Table S3) were used for colony screening.

For the construction of CPB-CC vectors carrying the insert of *Arabidopsis* *DCL4* and *AGO2* gene respectively, the sequences of *DCL4* (NM_122039.5), and *AGO2* (NM_102866.3) were retrieved from NCBI. The reverse complement sequence of each gene was subjected to the software [https://www.genscript.com/tools/sirna-target-finder](https://www.genscript.com/tools/sirna-target-finder) to predict the siRNAs. The fragments with size of 100 ± 2 nt, which included at least one of the top five predicted siRNA sequences, was selected (Table S1). The selected fragments were amplified from the *Arabidopsis* cDNA by using the primer sets AtDCL4A-F/AtDCL4A-R, AtDCL4B-F/AtDCL4B-R, AtAGO2A-F/AtAGO2A-R, and AtAGO2B-F/AtAGO2B-R (Table S2). The resulting PCR products were cloned into KpnI-cut CPB1B through the Gibson assembly master mix mentioned above. Positive colonies were screened using primers TCV-3334F and TCV-4000R
(Table S3). Similarly, one 100 nt GUS fragment was selected (Table S1) and amplified from the pCAMBIA1302 with primer set GUS-F/GUS-R (Table S2) to generate a nontarget VIGS control. All constructs were sequenced to verify their identity.

**Infection of *Arabidopsis* with *in vitro* transcripts**

The *in vitro* transcripts of TCV variants were produced using the TranscriptAid T7 HighYield Transcription Kit (Fermentas, Glen Burnie, MD) in accordance with the manufacturer’s instruction. The integrity and the concentration of the transcripts were examined using agarose gel electrophoresis. The inoculum was prepared by diluting the transcripts to 10 ng/μL by using the inoculation buffer (pH 9.2) containing 50 mM glycine, 30 mM K$_2$HPO$_4$, 1% bentonite, and 1% celite. The inoculum (10 μL, 10 ng/μL) was used for mechanical inoculation with a gloved finger on each *Arabidopsis* leaf with three leaves for each plant.

**Reverse transcription-PCR (RT-PCR)**

Total RNAs were isolated from the upper uninfected *Arabidopsis* leaves at 14 days postinoculation (dpi) using the TRIzol (Tiangen Biotech Beijing Co., Ltd) following the manufacturers protocol. Leaves from six different plants (one leaf per plant) were pooled before RNA extraction to minimize sampling errors. The first-strand cDNA was generated using reverse-transcription reactions through the FastQuant RT kit (KR106-01, Tiangen Biotech Beijing Co., Ltd) in accordance with the manufacturer’s instruction. PCR was carried out with the primer sets TCV-3334F and TCV-4000R to detect the genetic stability of the foreign inserts of CPB1B-based vectors in *Arabidopsis* (Table S3).

**Semiquantitative RT-PCR**

The semiquantitative RT-PCR was used to analyze the mRNA expression levels of the endogenous *PDS* genes in either *dcl2* or *dcldrb* plants inoculated with CPB-CC or CPB1B-based vectors at 14 dpi. The first-strand cDNAs were obtained as described above. The expression level of the *PDS* gene was determined using primer sets AtPDS-1200F/AtPDS-1600R (Table S3). The expression of the *Actin* gene determined using the primer set AtActin1-F/AtActin1-R (Table S3) was referred as an internal control to normalize cDNA concentrations. PCR amplifications were performed for 28 cycles. Each PCR was replicated three times by using the cDNAs from independent experiments. The results of amplification were checked using 1.5% agarose gel electrophoresis. All PCR amplifications were performed using the EasyTaq PCR SuperMix (AS111, TRANS, Beijing, China).

**Quantitative real-time PCR (qPCR)**

The transcription levels of *PDS, DCL4*, or *AGO2* genes in the upper leaves inoculated with CPB1B-based vectors at 14 dpi were determined using qPCR with primer sets AtPDS-604F/AtPDS-757R, DCL4-363F/DCL4-539R, or AGO2-1575F/AGO2-1754R, respectively (Table S3). The expression of the *Actin* gene determined using the primer set AtActin1-443F/AtActin1-618R (Table S1) was referred as an internal control to normalize cDNA concentrations. All qPCRs were carried out in triplicate for each cDNA sample.
by using the SYBR ®Premix Ex Taq™ II Kit (Takara) on the StepOne™ Real-Time PCR system (Applied Biosystems), and the comparative ΔΔCT method was used to evaluate amplified product quantities in the samples.

**Detection of viral RNAs by using RNA blot hybridization**

The Northern blot assay was used to detect TCV viral RNAs. Total RNA was extracted as described above. Each total RNA sample (0.8 µg) was separated on 1% agarose gels containing 2% formaldehyde before being transferred to the Hybond-N + membranes (GE Healthcare Life Science). The membranes were incubated with the DIG-labeled DNA probe, which was prepared as follows. A 667 nt fragment of TCV was obtained using PCR with primers TCV-3334F and TCV-4000R (Table S3). The fragment was used to generate a DIG-dUTP-labeled probe by using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). The images of the Northern blot results were taken using an ImageQuant LAS4000mini (GE Healthcare Life Science). The total RNA on the agarose gel was stained with ethidium bromide as a loading control.

**Results**

**CPB-CC-based vector inserting the 46 nt PDS fragment in the antisense orientation is more efficient in VIGS than that in the sense orientation**

The *PDS* gene encoding phytoene desaturase, a key enzyme in carotenoid biosynthesis, is widely used as a marker for the effectiveness of VIGS because the silencing of *PDS* produces a typical white color due to photobleaching [9, 10, 19, 20]. Previous study showed that the TCV-derived vector CPB-CC-*PDS*, which harboring a 90 nt *PDS* fragment, can induce modest *PDS* silencing in infected plants [14]. PNRSV-based vectors are reported to harbor foreign inserts that can trigger silencing in the sense orientation (11), whereas the BMV VIGS vector inserting the antisense strand of a gene results in a high degree of silencing [21]. In other studies, the sense or the antisense strand of a gene results in a similar level of BSMV-based VIGS in barley and wheat [22, 23]. These findings prompted us to determine the optimal orientation of the foreign insert in the CPB-CC-based VIGS vector. To this end, the 90 nt *PDS* fragment inserted in CPB-CC-*PDS* was subjected to a software to predict the potential siRNAs. One set of short complementary primers, PDS842-887F/PDS883-841R consisting of the predicted siRNA sequence were synthesized (Table S2). Additional nucleotides “TAC” were added to the 3’ of primer PDS883-841R to facilitate the short complementary primers, thereby producing stacked ends capable of ligating into KpnI-digested CPB-CC after the annealing treatment. After ligation, the 5’ proximal (relative to *PDS* fragment) KpnI site was restored, but the 3’ proximal KpnI site was lost. This feature helped the characterization of the orientation of insert and allowed fusing another fragment of the interested target gene in tandem with the *PDS* fragment mentioned above. The resulting CPB1F construct inserted in the sense orientation had unique KpnI site at the 5’ of the inserted *PDS* fragment. By contrast, the resulting CPB1B construct inserted in the antisense orientation had unique KpnI site at the 3’ of the inserted *PDS* fragment.
The *in vitro* transcripts of the resulting constructs, namely, CPB1F and CPB1B, together with CPB-CC were used to infect *dcl4* mutant plants kept at 18 °C. At 11 dpi, the upper uninoculated leaves of CPB1F- or CPB1B-infected plants exhibited the photobleaching phenotype, resulting from the reduction in the expression level of *PDS* (Fig. 1A). The extent of the photobleaching of CPB1B-infected plants was slightly stronger than that of CPB1F-infected plants, suggesting that the *PDS* gene was silenced to a greater extent in CPB1B-infected plants compared with its counterpart. The semiquantitative RT-PCR validated that the *PDS* mRNA levels in CPB1B- and CPB1F-infected plants substantially decreased compared with those in CPB-CC-infected plants (Fig. 1C). These results clearly illustrated that the CPB-CC-based vectors inserting the 46 nt *PDS* fragment in both orientations could effectively trigger *PDS* silencing in *Arabidopsis*, and inserting foreign fragments in the antisense orientation was more effective than that in the sense orientation.

**The optimal insertion size of the CPB1B VIGS vector is around 100 nt**

Given that different viruses can tolerate foreign inserts in a particular range of sizes, a series of CPB1B-derived vectors harboring another *PDS* fragment of varied size in the antisense orientation were further constructed by inserting 102, 139, and 215 nt *PDS* fragment to KpnI-treated CPB1B, resulting in CPB1B-derived vectors with one more *PDS* fragment in size of 102, 139, and 215 nt, designated as CPB1B102, CPB1B139, and CPB1B215, respectively. The *in vitro* transcripts of the resulting constructs were used to infect *dcl2drb4* mutant plants kept at 18 °C. At 11 dpi, the upper leaves of all plants inoculated with CPB1B gradually exhibited photobleaching. The photobleaching was observed with two days delay in CPB1B102 and CPB1B139-infected plants, but this observation was not evident until at 18–20 dpi in the CPB1B215-infected plants. The photobleaching of all plants inoculated with CPB1B215 was observed in the main vein, and only a few lateral veins exhibited albinism (Figs. 2A and 2B). The VIGS persisted throughout the plant growth period in the infected plants and increased with time, as indicated by the photobleaching. As shown in Figs. 2A and 2B, the virus-infected plants generated varying degrees of *PDS* silencing depending on the size of inserts. The virus harboring a long foreign insert induced weak *PDS* silencing. Consistently, as detected by semiquantitative RT-PCR, the mRNA expression levels in CPB1B, CPB1B102, and CPB1B139-infected plants were substantially lower than that in plants infected with CPB-CC virus that did not contain the *PDS*insert, an a mild decrease in the mRNA expression level in plants infected with CPB1B215 was observed compared with that in plants infected with CPB-CC (Fig. 2C). This result confirmed that the photobleaching phenotype was correlated with the silencing of the endogenous *PDS* gene, which served as a visualizable marker to indicate the penetrance of VIGS.

The genetic stability of foreign inserts in these recombinant viruses was evaluated through conventional RT-PCR by using the primers TCV-3334F/TCV-4000R flanking the foreign insert in CPB genomic RNA. The predicted sizes of RT-PCR amplification products derived from plants infected with CPB-CC, CPB1B, CPB1B102, CPB1B139, and CPB1B215 were 667, 713, 812, 849, and 925 nt, respectively. The predicted RT-PCR products were amplified from all infected samples respectively (Fig. 2D). These data suggested that the CPB1B-based VIGS vector could tolerate foreign inserts with size up to 215 nt, whereas harboring 139 or 215 nt foreign inserts substantially affected the movement and the silencing efficiency of the
virus, apparently indicating delayed appearance and reduced photobleaching (Fig. 2A & 2B). The CPB1B102 fused with 102 foreign insert did not affect the silencing efficiency substantially, but the movement of the CPB1B102 virus (two days delay) was somewhat slower than that of the original CPB1B vector, as indicated by photobleaching. Thus, the optimal insertion size of the VIGS vector was around 100 nt.

**CPB1B permits simultaneous silencing of two different Arabidopsis genes**

The efficiency of CPB1B-VIGS as a novel tool in the reverse genetics studies in *Arabidopsis* was further evaluated by silencing the *DCL4* gene, a primary DCL in *Arabidopsis*. Two pieces of *DCL4* fragments with size of 100 nt consisting of at least one of the top five predicted siRNA were selected for cloning into the KpnI-treated CPB1B. Similarly, CPB1BGUS with the same size as the GUS gene fragment was generated and served as the no-target control. The *in vitro* transcripts of the resulting constructs were then used to infect *dcl2* mutant plants kept at 18 °C. At 13 dpi, the photobleaching, which resulted from the downregulation of the *PDS* gene, was observed in the upper leaves of all plants inoculated with CPB1BGUS, and the rosette of the CPB1BGUS-infected plants was substantially smaller than that of the uninfected plants (Fig. 3A). These results indicated that CPB1BGUS could silence the *PDS* gene effectively. However, the photobleaching was not obvious in the CPB1BDCL4A- and the CPB1BDCL4B-infected plants even though the symptom of virus infection were as severe as those CPB1BGUS-infected plants (Fig. 3A). The phenotypes of the CPB1BDCL4A- and the CPB1BDCL4B-infected plants were similar to that of *dcl2dcl4* double knockout mutant plants, which did not show any photobleaching despite the high levels of viral RNA, when inoculated with CPB-CC-PDS [14](32).

The mRNA expression levels of the *PDS* and the *DCL4* genes were detected using qRT-PCR at 14 dpi. As shown in Fig. 3B, the *PDS* transcript levels were downregulated in CPB1BGUS-, CPB1BDCL4A-, and CPB1BDCL4B-infected plants relative to that of uninfected healthy plants. Consistent with the photobleaching phenotype, the extent of reduction in CPB1BDCL4A- or CPB1BDCL4B-infected plants was less than that in CPB1BGUS-infected plants. qRT-PCR also revealed that the relative amount of *DCL4* transcripts in CPB1BDCL4A- and CPB1BDCL4B-infected plants were substantially lower than those in uninfected plants, whereas the abundance of *DCL4* mRNA in CPB1BGUS-infected plants increased slightly. These results indicated that CPB1B-derived vectors could silence *PDS* and the target gene inserted in tandem simultaneously. Inoculation with CPB1BGUS, the no-target control, could stimulate the expression of *DCL4*, implicating that DCL4 was involved in the antivirus defense against TCV. This finding was consistent with those reported in previous studies [14, 17, 24].

The effect of silencing the *DCL4* gene on virus replication was further evaluated by monitoring the TCV viral RNA. The upper uninoculated leaves were collected from the CPB1BDCL4A-, CPB1BDCL4B-, CPB1BGUS-infected plants and uninfected plants at 21 dpi and subjected to RNA extraction and Northern blot hybridization with TCV-specific probes. As shown in Fig. 3C, compared with CPB1BGUS-infected plants, the CPB1BDCL4A- and the CPB1BDCL4B-infected plants had substantially increased TCV viral RNA levels, in which the *DCL4* gene was downregulated substantially. This result revealed that *DCL4*
knockdown could elevate the replication of TCV, which indicated that DCL4 was involved in the antivirus defense against TCV. This result agreed with those of previous studies, which showed that DCL4 has a critical role in antiviral defense [14, 17, 24]. Thus, CPB1B VIGS can be used as a novel tool for the functional characterization of the target gene in Arabidopsis with visualizable indicator of the penetrance of VIGS.

**AGO2 involved in the DRB4-independent DCL4-mediated PDS silencing**

Previous study has shown a substantial subset of the DCL4 antiviral activity, which is DRB4-independent, and that dcl2drlb4 double knockouts have caused a far smaller loss of antiviral silencing than dcl2dcl4 double knockouts. CPB-CC-PDS can induce PDS silencing in dcl2drlb4 plants but not in dcl2dcl4 double knockout [18]. AGOs are the effector proteins in eukaryotic small RNA (sRNA)-based gene silencing pathways controlling gene expression, transposon activity, and antivirus defense [25]. CPB1B-based VIGS vectors which with one piece of the 100 nt AGO2 gene fragment, CPB1BAGO2A and CPB1BAGO2B, were generated using the method mentioned above to investigate whether AGO2 gene involved in this DRB4-independent DCL4-mediated antiviral defense in dcl2drlb4 mutant. The in vitro transcripts of these two CPB1B-derived vectors and CPB1BGUS as well which serving as control were used to infect dcl2drlb4 double knockout mutant plants kept at 18 ℃. Photobleaching was observed at 13 dpi, which indicated that the penetrance of VIGS in virus-inoculated plants (Fig. 4A). qRT-PCR was used to verify the silencing of the PDS gene. As shown in Fig. 4B, the mRNA expression level of PDS in virus-inoculated plants decreased substantially compared with that in uninfected plants. qRT-PCR also revealed that the abundance of AGO2 mRNA in CPB1BAGO2A- and CPB1BAGO2B-infected plants decreased profoundly compared with that in uninoculated or CPB1BGUS-infected plants (Fig. 4B). These results reconfirmed that the CPB1B-derived vector could silence PDS and the targeted gene inserted in tandem simultaneously and that photobleaching could serve as a gene silencing indicator. However, the expression level of the AGO2 gene increased in plants inoculated with CPB1BGUS with 100 nt GUS gene, which was not an Arabidopsis endogenous gene. This result implicated that the AGO2 was involved in the antiviral defense in the dcl2drlb4 double mutant.

The upper uninoculated leaves were collected from the CPB1BAGO2A-, CPB1BAGO2B-, and CPB1BGUS-infected plants and uninfected plants at 21 dpi and subjected to RNA extraction and Northern blot hybridization with TCV-specific probes to corroborate the function of AGO2 in the antiviral defense in dcl2drlb4 double mutant. As shown in Fig. 4C, compared with those in CPB1BGUS-infected plants, the TCV viral RNA levels in CPB1BAGO2A- and CPB1BAGO2B-infected plants increased substantially. These results revealed that the AGO2 knockdown could facilitate the replication of TCV, indicating that the involvement of AGO2 in PDS silencing in the dcl2drlb4 mutant. Collectively, these results demonstrated the CPB1B-based VIGS system as a valuable tool with visualizable indicator of VIGS for interrogating Arabidopsis genes, especially those involved in the RNA silencing pathways.

**Discussion And Conclusions**
VIGS is an attractive reverse genetics tool for functional genomics in plants. Therefore, in the past
decade, tremendous improvements in VIGS have been reported [4–8, 12, 26]. Efforts has been made to
select the efficient reporter gene [10, 19, 27–29]. As far as we know, VIGS vectors with traceable and
visualizable indicator for the prediction of positive gene silencing plants for reverse genetics are still
lacking. In this study, we have developed a novel VIGS vector, designated CPB1B, based on an attenuated
variant of *Turnip crinkle virus* (TCV) known as CPB. CPB1B induced robust PDS silencing, causing easily
visible photobleaching in systemically infected *Arabidopsis* leaves, which provides a preliminary
assessment and traceable marker of the silencing penetrance. More importantly, CPB1B can
accommodate additional insert leading the silencing of two different Arabidopsis genes simultaneously.
The VIGS persists throughout the plant growth period in the infected plants and increases with time, as
indicated by the photobleaching.

TCV is a small icosahedral plant virus with a (+)-strand RNA genome encoding five proteins. CPB-CC-PDS,
a TCV derivative vector bearing 90 nt *PDS* fragment, can induce modest *PDS* silencing, providing a visual
indicator for the gene silencing [14](32). Studies show that the antiviral RNA silencing in plants enlists
DCL2 and DCL4, DCL3 to a lesser extent, to process the dsRNA of virus origin into viral siRNAs (vsiRNAs)
with size of 21–24 nt [2, 14, 17]. Theoretically, the insertion of a 21–24 nt fragment can induce silencing.
To obtain a short PDS fragment as a reporter gene, we have predicted the potential siRNA sequence in the
90 nt *PDS* insert in CPC-CC-PDS [14] and generated two constructs with the same 46 nt *PDS* fragment
while inserted in different orientation. The inoculated plants with *in vitro* transcripts of the resulting
constructs can effectively trigger *PDS* silencing in *Arabidopsis* and the silencing efficiency of CPB1B with
foreign fragment inserted in the antisense orientation is more effective than that of CPB1F with foreign
fragment inserted in the sense orientation. However, constructs possess the left sequences of the 90 nt
*PDS* (Table S1), designated CPB2F and CPB2B, which does not contain any predicted siRNA sequence
cannot induce *PDS* silencing efficiently (Fig. S2). Thus, the selection of the inserting fragment with
predicted siRNA sequence is critical in the development of a VIGS vector with high gene silencing
efficiency.

RNA viruses have optimal genome capacity for efficient replication and virion assembly. Therefore, virus-
based vectors have restrictions in carrying and expressing heterologous sequences in accordance with
their genome capacity [30–32]. As shown in Fig. 2, although the inserted fragments of CPB1B102 and
CPB1B have the same predicted siRNA sequences (Fig. S2), the silencing efficiency induced by
CPB1B102 is lower than that of CPB1B. Furthermore, CPB1B139 triggered less *PDS* silencing than
CPB1B102 did, eventhough its inserting has one more predicted siRNA sequence than that of CPB1B102
(Fig. S2). We have speculated that the increased insert length affects the movement of the virus, thereby
affecting the efficiency of silencing. Thus, to develop efficient VIGS vector minimizing the size of inserting
fragment is important too.In the present work, we have demonstrated that CPB1B and CPB1F with 46 nt
*PDS* fragment could trigger *PDS* silencing efficiently. These results open a potential avenue for the
development of VIGS vector through the synthesis of short fragment, which includes several predicted
siRNA sequences at the same time. Further attempts should be made to develop efficient VIGS vector
through synthetic fragment which could silence two or more functional genes simultaneously.
The RNase III enzyme Dicer is essential for the initiation of RNA silencing [1, 2, 14, 17, 24]. The efficiency of CPB1B-based VIGS as a novel efficient tool for reverse genetics studies in *Arabidopsis* is evaluated by silencing the *DCL4* gene in the *dcl2* knockout mutant. Results show that CPB1B-based VIGS vectors with *DCL4* insert, namely, CPB1BDCL4A and CPB1BDCL4B, can knock down *DCL4* efficiently (Fig. 3B), resulting in the increased accumulation of viral RNA (Fig. 3C). These findings are consistent with those reported in a previous study that *DCL4* is a key factor of *Arabidopsis* in antiviral defense. However, no apparent photobleaching is observed in *DCL4*-downregulated *dcl2* plants despite the high levels of viral RNA, which is similar to the phenotype of *dcl2dcl4* double knockout mutant infected with CPB-CC-PDS. The antiviral RNA silencing in plants enlists *DCL2* and *DCL4*, *DCL3* to a lesser extent, to process the dsRNA of virus origin into vsiRNAs [14, 17, 24]. Thus, the infected *dcl2* plants cannot produce enough vsiRNA to silence *PDS* when *DCL4* is downregulated despite the high levels of viral RNA. Collectively, these results validate the involvement of *DCL4* in *PDS* silencing.

AGO1s are the effector proteins in eukaryotic siRNA-based gene silencing pathways controlling gene expression, transposon activity, and antivirus defense [25]. Among the 10 AGO genes encoded by the *Arabidopsis* genome, AGO1, AGO2, and AGO7 are demonstrated to be involved in antiviral defense against TCV [14, 17]. The application of the CPB1B-based VIGS system developed in the present work shows that the AGO2 gene is involved in *PDS* silencing in *dcl2drb4* double mutant. As illustrated in Fig. 3, qRT-PCR reveals that the abundance of the *AGO2* mRNA decreases profoundly in *dcl2drb4* plants infected with the VIGS vector with the *AGO2* gene insert, namely, CPB1BAGO2A or CPB1BAGO2B, compared with that in uninoculated plants or plants infected with CPB1BGUS, which is a nontarget VIGS control. Northern blot shows that the downregulation of the *AGO2* gene increases the accumulation of the TCV viral RNA, indicating that AGO2 is involved in the DCL4-mediated antiviral defense in *dcl2drb4* plants. Since DRB4 which commonly partners with DCL4 in the antiviral silencing pathway has been knocked out in *dcl2drb4* plants, this results also indicated that DRB4 was dispensable for *PDS* silencing induced by CPB1B.

In summary, the CPB1B-based vector developed in this work is an efficient novel tool for interrogating *Arabidopsis* genes with tracable and visualizable indicator to predict positive gene silencing plants, especially those involved in the RNA silencing pathways. In addition, the development of the VIGS vector by inserting a synthetic fragment consisting of predicted siRNA sequence opens a potential avenue for the development of VIGS vector through the synthesis of short fragments, which include several predicted siRNA sequences to silence two or more functional genes at the same time.

**Declarations**

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Availability of data and materials

The material used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Local, National and International guidelines were followed in this study with virus induced gene silencing in plants.

Competing interests

None of the authors have competing interests.

Consent for publication

Not applicable.

Author's contributions

X.Z., Y.W, C. Z., and K.W. performed the experiments. X.Z., and Z. L. conceived the study. X.Z. and K.W. interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

CPB-CC-based vectors harboring 46 nt PDS fragment inserted in both orientations can effectively trigger PDS silencing in Arabidopsis. (A) PDS silencing induced by CPB-CC-based vectors in dcl4 plants. Images are recorded at 25 dpi. At 11 dpi, the upper uninoculated leaves of CPB1F- or CPB1B-infected plants exhibit a photobleaching phenotype in the upper leaves, resulting from the reduction in the expression level of PDS. CPB1F and CPB1B represent a 46 nt PDS fragment inserted in the sense and the antisense orientations, respectively. (B) Diagrams of TCV, CPB-CC, and CPB1B constructs. The CPB-CC construct is produced by changing the AT dinucleotides to CC at nt 3807 to 3808, within the 3’ UTR of CPB, which has R130T mutation denoted by a red star, resulting in a new KpnI site. The CPB1B construct is produced by fusing 46 nt PDS fragment in CPB-CC in the antisense orientation. (C) Downregulation of PDS mRNA levels by CPB1F and CPB1B in dcl4 plants as determined using semiquantitative RT-PCR. The samples are collected at 14 dpi. The Actin1 mRNA is used as a control to ensure that similar amounts of RNA are used in all reactions.
Figure 2

PDS silencing in dcl2drb4 plants inoculated with different CPB1B-based VIGS vectors harboring foreign inserts with varying sizes in the antisense orientation. Images of plants recorded at (A) 21 and (B) 38 dpi. At 11 dpi, the upper leaves of CPB1B-inoculated plants gradually exhibit photobleaching. Photobleaching is observed with two days delay in CPB1B102- and CPB1B139-infected plants, but this observation is not evident at 18–20 dpi in the CPB1B215-infected plants. The photobleaching of all plants inoculated with CPB1B215 is observed in the main vein, and only a few lateral veins have exhibited photobleaching. The VIGS persists throughout the plant growth period in infected plants and increases with time, as indicated by the photobleaching. (C) Downregulation of PDS mRNA levels by using different CPB1B-based VIGS vectors with foreign inserts of varied sizes, as determined using semiquantitative RT-PCR. The samples are collected at 14 dpi. The Actin1 mRNA is used as a control to ensure that similar amounts of RNA are used in all reactions. (D) Conventional RT-PCR of the genetic stability of the foreign insert in the recombinant virus by using primers TCV-3334F/TCV-4000R. The predicted sizes of RT-PCR amplification products derived from plants infected with CPB-CC, CPB1B, CPB1B102, CPB1B139, and CPB1B215 are 667, 713, 812, 849, and 925 nt, respectively. The RT-PCR products are amplified from all infected samples.
Figure 3

Silencing PDS and DCL4 gene in Arabidopsis simultaneously through the CPB1B-based VIGS vectors and its effect on virus replication. (A) Images of plants recorded at 51 dpi. At 13 dpi, the photobleaching is observed in the upper leaves of all plants inoculated with CPB1BGUS, and the rosette of CPB1BGUS-infected plants is substantially smaller than that of uninfected plants. However, the photobleaching is not observed in the CPB1BDCL4A- and CPB1BDCL4B-infected plants, but all infected plants show severe symptoms of virus infection as CPB1BGUS-infected plants. (B) qRT-PCR analysis of PDS and DCL4 expression levels in the upper uninoculated leaves of CPB1B-based VIGS vector-infected plants at 14 dpi. Expression is normalized against AtActin1 gene was used as an internal control. The data were analyzed using Student’s t test and asterisks denote significant differences between treatments ( *P <0.05, ** P <0.01). (C) The viral RNA accumulation levels in the upper uninoculated leaves of CPB1B-based VIGS vector-infected plants at 14 dpi.
Figure 4

AGO2 gene involved in the DRB4-independent DCL4-mediated antiviral defense in Arabidopsis. (A) Images of plants recorded at 25 dpi. At 13 dpi, the photobleaching is observed in the upper leaves of all plants inoculated with CPB1B-based VIGS vectors. (B) qRT-PCR analysis of PDS and AGO2 expression levels in the upper uninoculated leaves of CPB1B-based VIGS vector-infected plants at 14 dpi. Expression is normalized against AtActin1 gene was used as an internal control. The data were analyzed using Student’s t test and asterisks denote significant differences between treatments ( *P <0.05, ** P <0.01). (C) The viral RNA accumulation levels in the upper uninoculated leaves of CPB1B-based VIGS vector-infected plants at 14 dpi.

Supplementary Files

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