Construction of monopartite geminivirus-based virus-induced gene silencing (VIGS) vectors using a two-component strategy

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Abstract

Virus-induced gene silencing (VIGS) is a rapid and efficient tool to elucidate plant gene functions by inserting a target gene fragment into a viral genome and downregulating the expression of the target gene. Geminiviruses have been considered a promising platform for VIGS, but geminiviral VIGS vectors larger than native viral DNA are often unstable, due to the strict size limitation of their genomes. Here, we present a novel VIGS strategy using two copies of a monopartite geminivirus beet curly top virus (genus *Curtovirus*) genome that encodes viral genes in the viral and complementary strands. In the first copy, viral strand-encoded genes (V-genes) were replaced by target gene sequences, leaving complementary strand-encoded genes (C-genes) intact, and in the second copy, C-genes were replaced by other target gene sequences, leaving V-genes intact. Co-inoculation of plants with both VIGS vectors supplied all viral proteins necessary for infection, and they systemically spread and induced VIGS. This two-component approach may be used to establish a VIGS system using various species of monopartite geminiviruses.

Keywords Virus-induced gene silencing · Beet curly top virus · Silencing · Post-transcriptional gene silencing

Introduction

Virus-induced gene silencing (VIGS) uses a viral vector carrying a target gene fragment as a gene knockdown tool for the analysis of plant gene functions. When the vector is introduced into a plant, the target gene fragment produces corresponding double-stranded RNA (dsRNA) and triggers post-transcriptional gene silencing (PTGS), which degrades viral RNA containing the fragment and mRNA of the corresponding endogenous target gene, which then downregulates its expression (Purkayastha and Dasgupta 2009; Robertson 2004). The advantage of this approach over other reverse genetics tools such as genome editing is the short duration of the silencing. In this approach, the effect is transient, and thus, even essential genes for survival, that cause a lethal phenotype when inactivated permanently, can be silenced

and studied. Moreover, because VIGS is dependent on a nucleotide sequence, it can silence a set of genes sharing a particular sequence.

The family *Geminiviridae* is a large group of plant DNA viruses that comprises nine genera including *Begomovirus* and *Curtovirus*. Geminiviruses, viruses belonging to the family, are packaged as twinned icosahedral particles, composed of coat protein (CP) and a single-stranded (ss) circular DNA genome of approximately 3 kb. The genomic DNA enters the plant nucleus with the help of the CP, becomes a circular double-stranded replicative intermediate, expresses genes encoded by the viral and complementary strands, and replicates via rolling-circle replication (Zerbini et al. 2017). During the replication cycle, geminiviral ssDNA genomes do not form dsRNA genomes, although their bi-directional transcripts become partially double-stranded at their overlapping 3′ termini and intramolecular secondary structures. Once these dsRNA regions produce small interfering RNAs (siRNAs), they are used as primers to form double-stranded viral transcripts by host RNA-dependent RNA polymerases (RdRps) (Vanitharani et al. 2005). Consequently, geminiviruses can induce PTGS, and several geminivirus species have been successfully used as VIGS vectors (Carrillo-Tripp et al. 2006).

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Currently, many VIGS systems have been reported, but many important crop, industrial, or ornamental plants still do not have a suitable VIGS system (Dommes et al. 2019). Geminiviruses have the following advantages for developing a VIGS system for a plant of interest (Carrillo-Tripp et al. 2006). (i) The *Geminiviridae* family contains nine genera and more than 480 species (Zerbini et al. 2017), with a wide host range and a broad variety of symptoms (from asymptomatic to severe), allowing us to choose a geminivirus species as a suitable VIGS platform from a wide variety of virus species. (ii) Their small DNA genomes facilitate their cloning and sequencing and the construction of infectious clones and VIGS vectors. (iii) To construct a VIGS system using an RNA virus, the cloned RNA genome requires extensive modification allowing the in vitro or in vivo transcription of infectious viral RNA. For geminiviruses, cloned DNA can be directly used as an inoculum, although *Agrobacterium*-mediated inoculation is also often used. (iv) *Geminiviridae* family members do not encode DNA polymerases; rather, their genome replication depends completely on the host plant's DNA synthesis machinery, which has higher fidelity than RNA virus-encoded RdRps.

On the other hand, geminiviruses have a clear disadvantage as VIGS vectors. There is a strict limitation on the length of their genome (up to their original size of approximately 3 kb) that can be packaged into a viral particle or that can move systemically within the host plant. As a result, artificially elongated viral genomes undergo various deletions or recombinations and revert to their original length during the systemic spread of the virus (Elmer and Rogers 1990; Gilbertson et al. 2003). This strict genome size limitation makes it difficult to insert target gene fragments into geminiviral genomes.

To date, two predominant strategies using geminiviruses as VIGS vectors have been reported, both of which replace a dispensable geminiviral gene with a target gene fragment (Carrillo-Tripp et al. 2006). Both strategies utilize begomoviruses that are classified into monopartite (having one genome) and bipartite (having two genomes) species. One strategy employs a bipartite begomovirus and replaces its AV1 (CP) gene with a target gene fragment (Fig. 1a). The CP gene removal is not deleterious to bipartite begomovirus vectors because particle formation is not necessary for bipartite begomoviruses to systemically infect plants. In addition, one function of the CP that is essential for virus infection—transporting a viral genome into the nucleus where the genome replicates and transporting replicated genomes out of the nucleus (often called the nuclear shuttling function)—can be complemented by a similar function of the nuclear shuttle protein (NSP), which the bipartite viruses encode. The second strategy employs a monopartite begomovirus accompanied by a betasatellite (a subviral DNA), where the βC1 gene of the satellite is replaced with a target gene
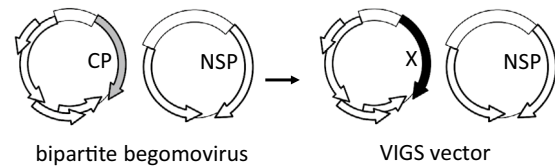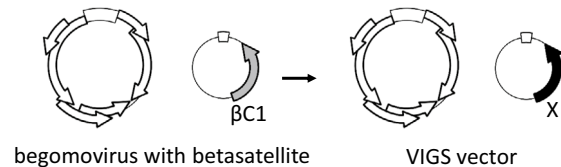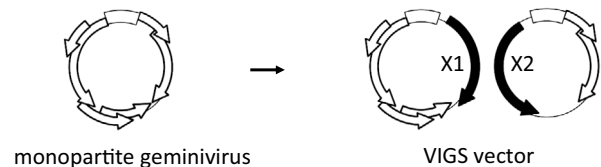

Fig. 1 Strategies to construct virus-induced gene silencing (VIGS) vectors from geminiviruses. **a** Coat protein (CP)-replacement strategy. The *CP* gene of a bipartite geminivirus is replaced with a target gene fragment (X). The nuclear shuttling function of CP is complemented by that of the encoded nuclear shuttle protein (NSP). **b** βC1-replacement strategy. The βC1 gene of a monopartite begomovirus betasatellite is replaced with a target gene fragment (X). **c** Two-component strategy. Two copies of a monopartite geminivirus genome are used. Viral strand-encoded genes of one copy are replaced with one target gene fragment (X1) and complementary strand-encoded genes of the other copy are replaced with the second target gene fragment (X2). Plants are co-inoculated with both constructs

fragment (Fig. 1b). The βC1 gene removal is not detrimental to the vector because βC1 is a suppressor of PTGS and is dispensable for systemic infection by the virus. However, these two strategies can only be used with a limited range of geminiviruses. The CP-replacement strategy is applicable only to bipartite begomoviruses and cannot be applied to other geminiviruses, and the βC1-replacement strategy is applicable only to geminiviruses associated with betasatellites and cannot be applied to those without the satellite.

Here, we present a novel two-component strategy to construct a set of VIGS vectors by using one monopartite geminivirus. This strategy uses two copies of a monopartite geminivirus genome that encode viral genes in the viral and complementary strands. Viral strand-encoded genes (V-genes) of one copy are replaced by one target gene fragment, leaving complementary strand-encoded genes (C-genes) intact, and C-genes of the other copy are replaced by the second target gene fragment, leaving its V-genes

intact. Plants are then co-inoculated with both constructs, with the expectation that viral proteins necessary for systemic infection will be supplied *in trans* from both constructs (Fig. 1c). To demonstrate this strategy, we constructed two-component geminivirus VIGS vectors from beet curly top virus (BCTV), which belongs to the monopartite genus *Curtovirus* (*Geminiviridae*) and infects a wide variety of plants from at least 44 families (Jeger et al. 2017). BCTV is localized in the phloem and induces hyperplasia of the phloem and a dwarf and distorted plant phenotype (Bennett 1971; Chen et al. 2011). Thus, we selected the BCTV-based vectors to show the practicability of the two-component strategy through clear observation of the phloem-limited severe phenotype. We inoculated *Nicotiana benthamiana* with several sets of vectors and showed that an endogenous plant gene and a transgene can be silenced by these vectors.

Materials and methods

Plant material

Wild-type *N. benthamiana* plants and *N. benthamiana* plants transgenic for the green fluorescent protein (*GFP*) gene, line 16c (Ruiz et al. 1998), were grown under a 16-h light/8-h dark photoperiod at 25 °C.

Construction of infectious BCTV clone

Dried plant leaves infected with BCTV were obtained from the American Type Culture Collection (ATCC No. PV-70). Total DNA was extracted as described by Dellaporta et al. (1983). A 1.6-kb fragment of the BCTV genome, containing the intergenic region (IR) and V-genes, and a 1.7-kb fragment, containing C-genes and IR, were amplified by PCR using primers BCTV-CR-V-5/BCTV-CR-V-3 and BCTV-C-CR-5/BCTV-C-CR-3 (Table 1), respectively, and KOD -Plus- DNA polymerase (Toyobo, Tokyo, Japan) with the recommended conditions. The amplified fragments were subsequently digested with HindIII/SalI and SalI/SacI, respectively, and then cloned together into HindIII/SacI-digested pUC18 plasmid to produce an infectious plasmid clone with two IRs, pUC18-BCTV1.1. The cloned BCTV genome was sequenced by appropriate primers using an Applied Biosystems 3500 Genetic Analyzer and re-cloned into the HindIII/SacI-digested *Agrobacterium* binary vector pCAMBIA-2300 to produce an infectious clone pCAMBIA-BCTV1.1.

Cloning of phytoene desaturase gene

Total RNA was extracted from a *N. benthamiana* plant using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). The RNA extract was treated with RNase-free DNase (Takara, Kusatsu, Japan) for 1 h at 37 °C and purified with phenol–chloroform extraction and ethanol precipitation. A 5-μg sample of RNA was then reverse-transcribed using Superscript Reverse Transcriptase (Promega, Madison, WI, USA) with Oligo(dT)15 and random hexamers as primers. The cDNA was diluted in 100 μl of pure water. Then, 2 μl of the diluted cDNA, 0.25 μm each of primers NbPDS-R2-Mlu and either NbPDS-F2-Nco or NbPDS-F3-Nco (Table 1), 1 μl of KOD -Plus- DNA polymerase (Toyobo), and 2 μl of 10 × buffer in a total volume of 20 μl were mixed together for the PCR, which was run using the recommended conditions. The PCR products, a 612-bp or 201-bp fragment of *N. benthamiana* phytoene desaturase (*PDS*) gene, were digested with NcoI plus MluI, cloned into a plasmid vector pSL1180, and sequenced to produce pSL1180-PDF1 or pSL1180-PDF2, respectively.

Construction of VIGS vectors

BCTV-based VIGS vectors were constructed by recombinant PCR using KOD -Plus- DNA polymerase (Toyobo) as an enzyme and pUC18-BCTV1.1, pSL1180-PDS1, pSL1180-PDS2, or pP35SGFPTnos (Niwa et al. 1999) as templates using the recommended conditions. Two or three overlapping first PCR products were mixed and connected by the second PCR using outermost primers and digested by restriction enzymes as listed below. The digested second PCR products were used to replace the corresponding region of pUC18-BCTV1.1. The PCR-amplified region was sequenced by appropriate primers to confirm correct sequences. The primers used are listed in Table 1. Constructed infectious clones were introduced into the HindIII/SacI-digested *Agrobacterium* binary vector pCAMBIA-2300.

A V1-stop was constructed, using pUC18-BCTV1.1 as a template, in which the codons for the 57th and 58th amino acids (Leu and Gly) of the V1 open reading frame (ORF) were replaced by two termination codons. The two first PCR products, (1) one using primers BCTVIRleftHind/BCTV-CPstopR and (2) one using primers BCTV-CPstopF/BCT-VSalR, were joined in the second PCR using primers BCTVIRleftHind/BCTVSalR and digested by HindIII and SpeI.

ΔV1V3-PDS was constructed, whereby 653 nt of V1 ORF including a part of the overlapping V3 ORF was replaced by a 612-bp fragment (from the 445th to 1056th nucleotide [nt]) of the *PDS* gene (GenBank EU165355) plus the 33-bp region of the pSL1180 multicloning site. The three first PCR products, (1) one using primers BCTVIRleftHind/BCTV-CPPDS-FR and pUC18-BCTV1.1 as a template, (2) one using primers BCTV-CPPDS-FF/BCTV-CPPDS-RR and pSL1180-PDS1 as a template, and (3) one using primers BCTV-CPPDS-RF/BCTVSalR and pUC18-BCTV1.1 as a template, were joined in the second PCR using primers

Table 1 Primers used in this study

Name of a primer	Sequence (5'—> 3')*
BCTV-CR-V-5	acgtaagcttATTGAATCGGGCTCTCTTCAAATCTC
BCTV-CR-V-3	CCAAGTCGACAGGGAAATCAAAAAAC
BCTV-C-CR-5	CCCTGTCGACTTGGTTCTTCA
BCTV-C-CR-3	acgtgagctcATTTATAAGTACATATACATGTAAAAATAACGTATATAAAACGAC
BCTV-CPstopF	gtatgatgatatctagtgaGCTGGTGGTATAGGATCTACCATT
BCTV-CPstopR	caccagctcactagatatCATCATACATCTTTAACTTCAGAGTCC
BCTV-RepstopF	gttctaaggcgatatcactaGTCTTCTTTGGTTACTGAACATTGAGG
BCTV-RepstopR	ccaaagaagactagtgatatcGCCTTAGAACAGCTCCTCGC
BCTV-VGFP-FF	tatatgtacttataaATGGTGAGCAAGGGCGAGGAG
BCTV-VGFP-FR	cgcccttgctcaccatTTATAAGTACATATACATGTAAAAATAACG
BCTV-VGFP-RF	ctgtacaagtaaTTCCCGATAACGGTCAGGC
BCTV-VGFP-RR	gaccgttatcgggaaTTACTTGTACAGCTCGTCCATGC
BCTV-CGFP-FF	gagcccgattcaatATGGTGAGCAAGGGCGAGGAG
BCTV-CGFP-FR	ttgctcaccatATTGAATCGGGCTCTCTTCAAATC
BCTV-CGFP-RF	cgagctgtacaagtaaGTTCTGTATAACGTCATTGATGACGTAG
BCTV-CGFP-RR	acgttatacagaacTTACTTGTACAGCTCGTCCATGCC
BCTV-CPPDS-FF	ggaagtgttcgttgtCCATGGGTAGCTGCATGGAAAGAT
BCTV-CPPDS-FR	gcagctacccatggACAACGAACACTTCCTATGAAAATTAAC
BCTV-CPPDS-RF	cgaccgcggctcgagGTATGAAGACGTGAAGGAGAATGCTTTACTCTATG
BCTV-CPPDS-RR	ttcacgtcttcatacCTCGAGCCGCGGTCGCGA
BCTV-C2PDS-FF	agacaaacttaaagtCCATGGGCACTTAACTTCATAAACCCTG
BCTV-C2PDS-FR	gttaagtgcccatggACTTTAAGTTTGTCTGCCACTCTCTTTGTGC
BCTV-C2PDS-RF	atttctattacaatgCTCGAGCCGCGGTCGCGA
BCTV-C2PDS-RR	cgaccgcggctcgagCATTGTAATAGAAATATTAAATCATGTATTGTATTCTGTCTTC
BCTV-SalF	GAGAGAAGATCGTTTTTTGATTTCC
BCTV-SalR	TTTTGAGAGTCTTGAAGAACCA
BCTVIRleftHind	tttttAAGCTTATTGAATCGGGCTCTCTTC
BCTVIRrightSac	tttttGAGCTCATTTATAAGTACATATACATGTAAAAATAACG
BSCTVSeqC1	ATTCAGGGAGCTAAATCCAG
BSCTVSeqV1	AAGGCGAGGTCTTTCATCA
BSCTV-C3F	GAACATGAGGACTATTTGGAAAG
BSCTV-V2F	ACGAACTTTTATGGGACC
BSCTV-CGFP-R2	ACCCTCGTGACCACCTTC
NbPDS-F2-Nco	catgccatggGTAGCTGCATGGAAAGA
NbPDS-F3-Nco	gcccatggGCACTTAACTTCATAAACC
NbPDS-R2-Mlu	actgacgcgtCTCGATCTTTTTTATTCGTGAGT
qpt1-F	ATAGTGCACCCTCATGCAAT
qpt1-R	GTGCTGGTGGCTTCACTAATA
GFP qrt F1	TCATCTTTTCACTTCTCCTATCATTATCCT
GFP qrt F2	TCACCTTCACCCTCTCCACT
PDS qrt F1	TAAACCCTGACGAGCTTTCGA
PDS qrt R1	TCCACTGGAGTGGCAAACACAA
EF-1 F	GATTGGTGGTATTGGAACTGTC
EF-1 R	AGCTTCGTGGTGCATCTC

*Nucleotides in capital letters are complementary to the expected template

BCTVIRleftHind/BCTVSalR and digested by HindIII and SpeI.

ΔV1V2V3-GFP was constructed, whereby a 720-bp region covering the V2 and V3 ORFs and a part of the V1 ORF was replaced by the *GFP* gene (720 bp). The three first PCR products, (1) one using primers BCTVIRleftHind/BCTV-VGFP-FR and pUC18-BCTV1.1 as a template, (2) one using primers BCTV-VGFP-FF/BCTV-VGFP-RR and

pP35SGFPTnos as a template, and (3) one using primers BCTV-VGFP-RF/BCTVSalR and pUC18-BCTV1.1 as a template, were joined in the second PCR using primers BCTVIRleftHind/BCTVSalR and digested by HindIII and SpeI.

C1-stop was constructed using pUC18-BCTV1.1 as a template, in which two termination codons were inserted between the codons for the 23rd and 24th amino acids (Asp and Ala) of the C1 ORF. The two first PCR products, (1) one using primers BCTVIRrightSac/BCTV-RepstopR and (2) one using primers BCTV-RepstopF/BCTV-SalF, were joined in the second PCR using primers BCTVIRrightSac/BCTV-SalF and digested by SacI and KpnI.

ΔC1C2C3-PDS was constructed, in which the C2 ORF (543 bp) including a part of overlapping C1 and C3 ORFs was replaced by a 201-bp fragment (from the 855th to 1055th nt) of the PDS gene plus the 33-bp region of the pSL1180 multicloning site. The three first PCR products, (1) one using primers BCTVIRrightSac/BCTV-C2PDS-FR and pUC18-BCTV1.1 as a template, (2) one using primers BCTV-C2PDS-FF/BCTV-C2PDS-RR and pSL1180-PDS2 as a template, and (3) one using primers BCTV-C2PDS-RF/BCTVSalR and pUC18-BCTV1.1 as a template, were joined in the second PCR using primers BCTVIRrightSac/BCTVSalR and digested by SacI and KpnI.

ΔC1C4-GFP was constructed, whereby the first 753 nt of the C1 ORF, also containing the C4 ORF, was replaced by the *GFP* gene (720 bp). The three first PCR products, (1) one using primers BCTVIRrightSac/BCTV-CGFP-FR and pUC18-BCTV1.1 as a template, (2) one using primers BCTV-CGFP-FF/BCTV-CGFP-RR and pP35SGFPTnos as a template, and (3) one using primers BCTV-CGFP-RF/BCTVSalR and pUC18-BCTV1.1 as a template, were joined in the second PCR using primers BCTVIRrightSac/BCTVSalR and digested by SacI and KpnI.

Agrobacterium-mediated virus inoculation

Constructed binary vector-based infectious clones were introduced into *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw method (Holsters et al. 1978). A single colony of the transformed bacteria was grown in 5 ml LB broth with 50 µg/ml kanamycin at 28 °C until the optical density at 600 nm (OD_{600}) was 0.5. The bacteria were harvested by centrifugation and suspended in an infiltration medium (10 mm MES, pH 5.7, 10 mm $MgCl_2$, 150 µm acetosyringone) to obtain bacterial suspensions with an OD_{600} of 0.5. After incubation at 28 °C for 12 h, the abaxial side of the leaves were infiltrated with the bacteria using a 1-ml syringe without a needle. The same inoculum was applied to three to five independent plants as replicates. Infiltrated plants were kept under the same growth conditions as mentioned above. At 30 days post-inoculation, the symptoms

were recorded, and upper leaves were collected for further analyses.

GFP imaging

Systemically infected leaves were illuminated with a handheld UV lamp. Images were captured using a digital camera.

Real-time quantitative RT-PCR

The mRNA levels were quantified using real-time quantitative RT-PCR as reported by Kotakis et al. (2010). Total RNA was extracted from the systemically infected leaves of individual plants at 30 days after inoculation using TRI Reagent. The RNA extract was treated with RNase-free DNase (Takara) for 1 h at 37 °C and purified with phenol–chloroform extraction and ethanol precipitation. Then 5 µg of RNA was reverse transcribed using Superscript Reverse Transcriptase (Promega) with Oligo(dT)15 and random hexamers as primers. The cDNA was diluted in 100 µl of pure water. For quantitative RT-PCR, each reaction in a total volume of 20 µl contained 2 µl of diluted cDNAs, 0.25 µm each of primers, and 10 µl of TB Green Fast qPCR mix (Takara). The primer sets for the *N. benthamiana PDS* gene, an internal control elongation factor 1α gene, and transgenic *GFP* gene were PDS qrt F1/PDS qrt R1, EF-1 F EF-1 R, and GFP qrt F1/GFP qrt F2, respectively (Table 1). Reactions were run in a Thermal Cycler Dice Real Time System (Takara) for 30 s at 94 °C, 20 s at 55 °C, and 20 s at 72 °C. The threshold cycle was generated automatically by the Thermal Cycler Dice Real Time System Version 5.0 software. Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The data are presented as means ± SD. Statistical analyses were performed using R (Ver. 4.0.3). The differences between relative gene expression levels of the wild-type BCTV-inoculated plant and VIGS vector-inoculated ones were compared using Dunnett's multiple comparison test (Dunnett 1964).

Detection of VIGS vector DNA in systemic leaves

For detecting virus genomes in systemically infected leaves, the upper leaves from inoculated plants were used. Total plant DNA was isolated using the method of Dellaporta et al. (1983), dissolved in TE buffer (pH 8.0), and quantitated by spectrophotometry. PCR was carried out using 50 ng of total plant DNA as a template and individual VIGS vector-specific primers (Table 2). The quality of the extracted DNA was assessed by amplifying a 365-bp fragment of *N. benthamiana* genomic quinolinate phosphoribosyltransferase 1 (*qpt1*) gene (GenBank HE799295) by primers qpt1-F and qpt1-R (Table 1). The following PCR program was used: initial denaturation at 94 °C for 3 min; 25 cycles of

Table 2 Summary of constructed VIGS vectors

Type*	Name	Inactivated gene(s)	Introduced gene fragment	Primer sets for PCR amplification	Expected size (bp) of the PCR product	Figure
Wild type	BCTV	None	None	BSCTV-C3F/BSCTVSeqC1	1405	3c, 4b
V(-)C(+)	V1-stop	V1	None	BSCTV-C3F/BSCTVSeqC1	1405	4b
V(-)C(+)	ΔV1V3-PDS	V1, V3	PDS	NbPDS-F2-Nco/BCTV-SalR	938	3c, 5b
V(-)C(+)	ΔV1V2V3-GFP	V1, V2, V3	GFP	BCTV-VGFP-FF/BCTV-SalR	1253	5b
V(+)C(-)	C1-stop	C1	None	BSCTV-C3F/BSCTVSeqC1	1405	3c
V(+)C(-)	ΔC1C2C3-PDS	C1, C2, C3	PDS	BSCTV-V2F/NbPDS-F3-Nco	1344	4b
				BSCTVSeqV1/NbPDS-F3-Nco	1198	5b
V(+)C(-)	ΔC1C4-GFP	C1, C4	GFP	BCTV-SalF/BSCTV-CGFP-R2	1181	5b

*In V(-)C(+) vectors, one or multiple V-genes were inactivated and all C-genes were left intact. In V(+)C(-) vectors, one or multiple C-genes were inactivated and all V-genes were left intact

denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 1.5 min.

Results

Construction of an infectious BCTV clone

From infected plant material, we amplified and cloned a 1.6-kb fragment containing the IR plus V-genes and a 1.7-kb fragment containing C-genes plus the IR of the BCTV genome and sequenced multiple clones. The assembled BCTV genome had a length of 2926 bp, and its double-stranded replicative intermediate is shown in Fig. 2a (left). Its gene organization was typical of curtoviruses, and the genome contained seven ORFs—ORF V1 (CP gene), V2 (movement protein (MP) gene), and V3 (PTGS suppressor gene) in the viral strand and ORF C1 (replication associated protein (Rep) gene), C2 (TGS suppressor gene), C3 (replication enhancer gene), and C4 (pathogenicity determinant gene) in the complementary strand—as well as an IR between V2 and C1. Its nucleotide sequence (GenBank LC621348) was 96.6% identical to that of a severe strain of BCTV (BCTV-Svr[US-SVR-Cfh]), formerly *Beet severe curly top virus* (GenBank U02311) (Stenger 1994). The virus was identified as a strain of this species because 77% and 94% genome-wide pairwise identity values are the respective species and strain demarcation thresholds for the genus *Curtovirus* (Varsani et al. 2014). The infectious clone, named pCAMBIA-BCTV1.1, that was constructed (Fig. 2a, right) is a 1.1 copies of the BCTV genome having two IRs at both ends cloned into an *Agrobacterium* binary vector. Expressed Rep protein will introduce a single-strand cut (nick) at a replication origin located within both IRs, and circularize the ssDNA in between to produce a viral genome. Via *Agrobacterium*, transferred (T-)DNA of pCAMBIA-BCTV1.1 was introduced into *Nicotiana benthamiana*
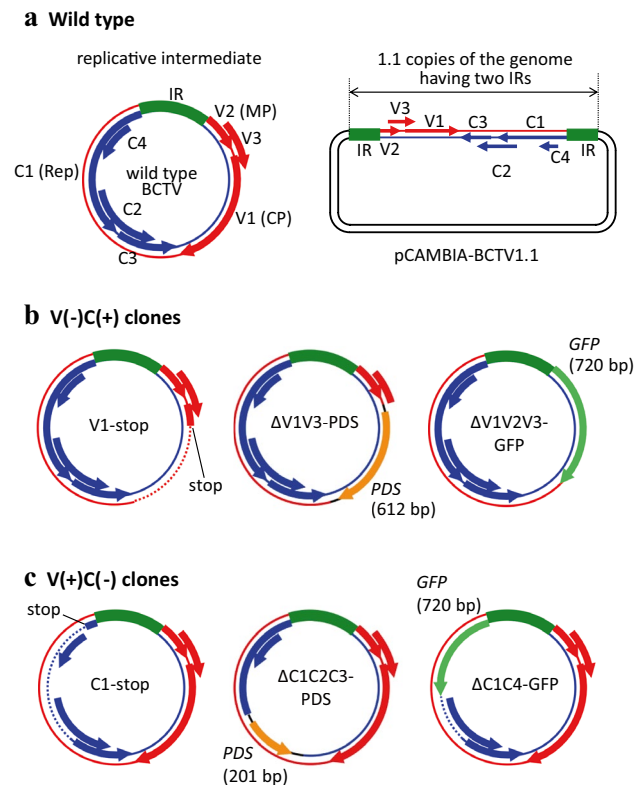

Fig. 2 Gene organization of replicative intermediates expected to be produced in the infected plants after inoculation with constructed BCTV-derived VIGS vectors. **a** Wild-type BCTV (left) with its infectious clone pCAMBIA-BCTV1.1 (right). **b** V(-)C(+) vectors with defective V-genes (Δ denotes inactivated genes) and intact C-genes. **c** V(+)C(-) vectors with intact V-genes and defective C-genes. V1–V4: viral strand-encoded genes (V-genes), C1–C4: complementary strand-encoded genes (C-genes), IR: intergenic region, CP: coat protein gene, MP: movement protein gene. Rep: replication associated protein gene, stop: termination codon, *GFP*: green fluorescent protein gene, *PDS*: phytoene desaturase gene

Fig. 3 Plants inoculated with ΔV1V3-PDS and C1-stop vectors. **a** ▶ Symptoms of *Nicotiana benthamiana* plants 30 days after inoculation with agrobacteria harboring the following binary vectors. Plants received (i) empty binary vector pCAMBIA-2300 (mock), (ii) binary vector with ΔV1V3-PDS construct and that with the C1-stop construct, (iii) binary vector with ΔV1V3-PDS construct and a wild-type BCTV (WT) infectious clone or (iv) binary vector with a WT infectious clone. **b** Close-up views of the upper leaves of plants 30 days after inoculation with the following inocula. (i) Empty vector. (ii) WT BCTV. (iii) ΔV1V3-PDS plus WT. (iv) ΔV1V3-PDS plus C1-stop. Scale bar: 1 cm. **c** PCR analysis of DNA extracted from the upper leaves of the plants. Upper gel: PCR products using primers that amplify the WT virus or C1-stop. Middle gel: PCR products using primers that amplify ΔV1V3-PDS. Lower gel: PCR products derived from a plant *qpt1* gene as DNA quality controls. M; size marker. **d** Mean (±SE) mRNA transcript levels of *PDS* in the upper leaves of the plants. mRNA levels quantified by quantitative RT-PCR with EF-1α mRNA as an internal standard are presented as the relative value compared with the WT-inoculated sample (100%) from three independent experiments. Asterisks indicate samples are significantly different from the WT-inoculated sample (**P* < 0.05) using Dunnett's multiple comparison test

plants. At 2 weeks (14 days) post-inoculation (wpi), any new leaves were slightly curled and smaller than new leaves on control plants, and at 5 wpi (35 days), stunting, vein swelling, and severe upward curling of young leaves were present (Fig. 3a: iv). Mock-inoculated plants that were inoculated with agrobacteria harboring an empty vector pCAMBIA-2300 had no symptoms (Fig. 3a: i). The PCR results demonstrated the wild-type BCTV genome was present in the symptomatic upper leaves of BCTV-inoculated plants but not in asymptomatic upper leaves of mock-inoculated plants (Fig. 3c, upper). The plant *qpt1* gene was amplified from both extracted DNA samples, showing adequate quality of the samples (Fig. 3c, lower).

Construction of VIGS vectors

BCTV-derived VIGS vectors were constructed and their 1.1 copies (with two IRs, one at each end) were cloned into *Agrobacterium* binary vector pCAMBIA-2300. Gene organization maps of the replicative intermediate dsDNAs that were expected to be produced in the nucleus from the vectors are shown in Fig. 2b, c. In these constructs, V-genes or C-genes were inactivated by the introduction of termination codons or by replacement with the plant genes to be silenced. The names of the constructs, inactivated genes, and replacement genes are summarized in Table 2. Because geminivirus genes overlap extensively, replacement of one gene often results in the inactivation of multiple genes.

There were two types of VIGS vectors: (1) V-gene defective/C-gene intact [V(−)C(+)] vectors, which had one or multiple V-genes inactivated and all C-genes remained intact (Fig. 2b), (2) V-gene intact/C-gene defective [V(+)C(−)] vectors, which had one or multiple C-genes inactivated and all V-genes remained intact (Fig. 2c).
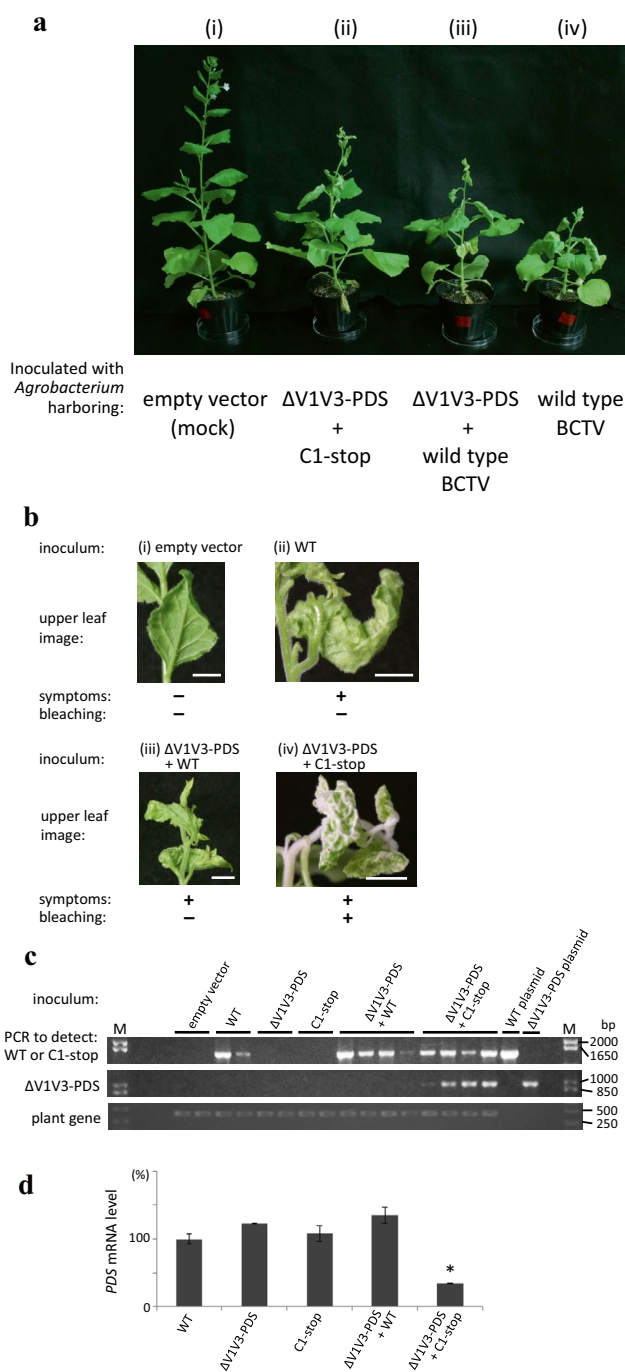
Two plant genes were selected as targets for VIGS: (1) an endogenous gene encoding phytoene desaturase (PDS), a key enzyme for the biosynthesis of carotenoids necessary for chlorophyll accumulation. When this gene is silenced, the green color of leaves is expected to turn white (bleached); and (2) a transgene encoding a green fluorescent protein (GFP) that emits fluorescence under UV light (Ruiz et al. 1998). When this gene is silenced, its fluorescence is expected to disappear.

Silencing of plant genes using VIGS vectors in which V-genes are replaced by target gene fragments

N. benthamiana plants were inoculated with the V(−)C(+) vector ΔV1V3-PDS alone or together with other vectors. Plants inoculated with an empty vector did not develop any symptoms in the upper leaves (Fig. 3b: i). Plants inoculated with wild-type BCTV exhibited typical symptoms (Fig. 3b: ii) and viral DNA was detected (Fig. 3c), whereas those inoculated with ΔV1V3-PDS alone (Supplementary Fig. 1a) or C1-stop alone (Supplementary Fig. 1b) did not develop any symptoms in the upper leaves, and DNA from either vector was not detected (Fig. 3c). Absence of ΔV1V3-PDS nor C1-stop in upper leaves indicated that V(−)C(+) vector (ΔV1V3-PDS) alone or V(+)C(−) vector (C1-stop) alone did not move systemically. Plants co-inoculated with ΔV1V3-PDS plus wild-type BCTV were clearly stunted compared with the control (Fig. 3a: plant iii vs plant i) and severe upward curling of the upper leaves (Fig. 3b: iii). BCTV DNA was detected, but ΔV1V3-PDS DNA was not (Fig. 3c), which indicates the V(−)C(+) vector did not move systemically when co-inoculated with wild-type BCTV. Plants inoculated with the V(−)C(+) vector (ΔV1V3-PDS) plus the V(+)C(−) vector (C1-stop) were also stunted compared with plants that received the negative control empty vector (Fig. 3a: plant ii vs i), but not as severely as those that were inoculated with the wild-type virus (Fig. 3a: plant iv). Upper leaves of plants inoculated with ΔV1V3-PDS plus C1-stop developed BCTV symptoms (Fig. 3b: iv), and DNA of both vectors was detected by PCR (Fig. 3c). The upper leaves had a bleached phenotype, typical of *PDS* silencing, especially in the veins (Fig. 3b: iv). qRT-PCR quantitation demonstrated *PDS* mRNA was significantly reduced in these leaves compared with plants inoculated with wild-type BCTV (Fig. 3d). The presence of both ΔV1V3-PDS and C1-stop and the reduced *PDS* mRNA accumulation in upper leaves indicate that the V(−)C(+) VIGS vector can move systemically in a mixed inoculum with a complementary V(+)C(−) vector and induce VIGS of the endogenous *PDS* gene.

Silencing of plant genes using VIGS vectors in which C-genes are replaced by target gene fragments

Next, we used another set of VIGS vectors in which C-genes were replaced by a target *PDS* gene fragment. When *N. benthamiana* plants were inoculated with ΔC1C2C3-PDS alone or V1-stop alone, no symptoms developed in the upper leaves (Supplementary Fig. c, d), and DNA of neither vector was detected (Fig. 4b). Plants inoculated with the ΔC1C2C3-PDS plus V1-stop developed BCTV symptoms in the upper leaves (Fig. 4a: iii), and both inoculated vectors were detected in the upper leaves (Fig. 4b). The upper leaves
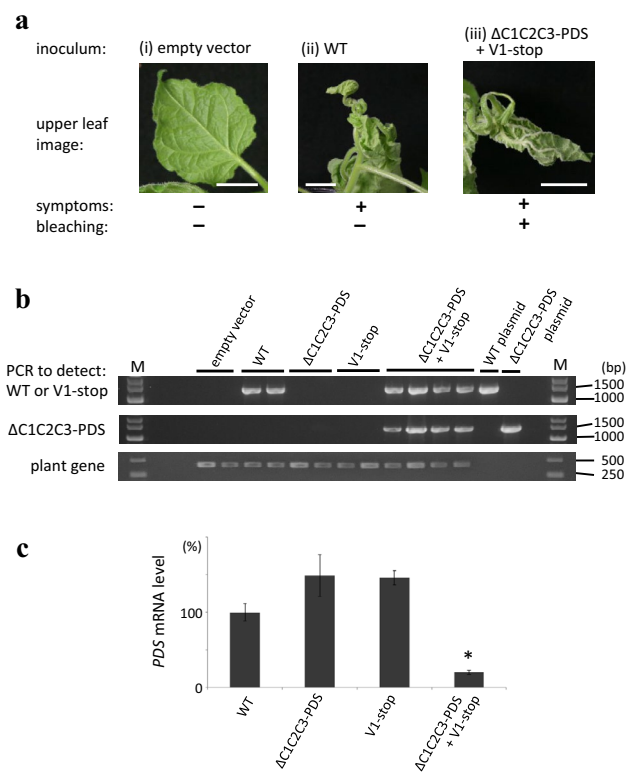

Fig. 4 Plants inoculated with ΔC1C2C3-PDS and V1-stop vectors. **a** Close-up views of the upper leaves of plants 30 days after inoculation with the following inocula: (i) Empty vector. (ii) Wild-type BCTV (WT). (iii) ΔC1C2C3-PDS plus V1-stop. Scale bar: 1 cm. **b** PCR analysis of DNA extracted from the upper leaves of the plants inoculated with VIGS vectors. Upper gel: PCR products using primers that amplify the WT virus or V1-stop. Middle gel: PCR products using primers that amplify ΔC1C2C3-PDS. Lower gel: PCR products derived from a plant *qpt1* gene as DNA quality controls. M; size marker. **c** Mean (± SE) mRNA transcript levels of the *PDS* gene in upper leaves of plants. mRNA levels, based on quantitative RT-PCR with *EF-1α* mRNA as an internal standard, are presented as the relative value compared with WT-inoculated sample (100%) from three independent experiments. Asterisks indicate samples that are significantly different from the WT-inoculated sample ($P < 0.05$) using Dunnett's multiple comparison test

of all inoculated plants developed a bleached phenotype, especially in their veins (Fig. 4a: iii). *PDS* mRNA transcripts were significantly reduced in these leaves (Fig. 4c), which indicates that the V(+)C(−) VIGS vector (ΔC1C2C3-PDS) can move systemically when present with a V(−)C(+) vector and can induce VIGS of the endogenous *PDS* gene.

Silencing of multiple plant genes using a pair of VIGS vectors

We next co-inoculated plants with a V(−)C(+) vector containing the first target gene fragment and a V(+)C(−) vector containing the second target gene fragment to simultaneously silence two genes. In addition to the *PDS* gene, we

used the *GFP* transgene in the transgenic *N. benthamiana* line 16c as the second target. Line 16c plants inoculated with V(+)C(−) vector (ΔC1C4-GFP) alone or V(−)C(+) vector (ΔV1V3-PDS) alone did not develop symptoms in the upper leaves (Supplementary Fig. 1e, f), and the vector DNA was not detected (Fig. 5b). Plants inoculated with ΔC1C4-GFP plus ΔV1V3-PDS developed BCTV symptoms in the

were significantly reduced in these leaves (Fig. 5c, upper), which indicates that V(+)C(−) VIGS vector (ΔC1C4-GFP) and V(−)C(+) vector (ΔV1V3-PDS) can move systemically when present together and induce the VIGS of both inserted genes.

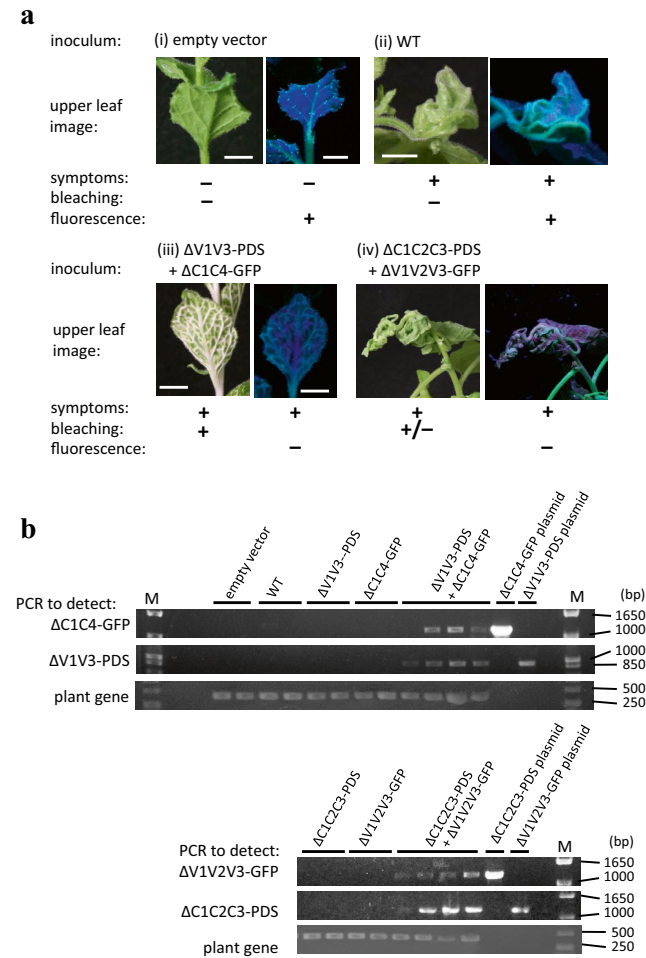When plants were co-inoculated with another combination, V(+)C(−) vector ΔC1C2C3-PDS and V(−)C(+) vec-

Fig. 5 Plants inoculated with ΔV1V3-PDS and ΔC1C4-GFP vectors or ΔC1C2C3-PDS and ΔV1V2V3-GFP vectors. **a** Close-up views under normal light (left) or UV light (right) of the upper leaves of plants 30 days after inoculation with the following inocula. (i) Empty vector. (ii) Wild-type BCTV (WT). (iii) ΔV1V3-PDS plus ΔC1C4-GFP. (iv) ΔC1C2C3-PDS plus ΔV1V2V3-GFP. Scale bar: 1 cm. **b** PCR analysis of DNA extracted from upper leaves of plants. Top gel: PCR products using primers that amplify ΔV1V3-PDS. Middle gel: PCR products using primers that amplify ΔC1C4-GFP. Bottom gel: PCR products derived from a plant *qpt1* gene as DNA quality controls. M; size marker. **c** Mean (± SE) mRNA transcript levels of *PDS* and *GFP* genes in the upper leaves of the plants. mRNA levels, quantified by quantitative RT-PCR with *EF-1α* mRNA as an internal standard, are presented as the relative value compared with the WT-inoculated sample (100%) from three independent experiments. Asterisks indicate samples are significantly different from the WT-inoculated sample ($P < 0.05$) using Dunnett's multiple comparison test.

upper leaves (Fig. 5a: iii), and both inoculated vectors were detected in the upper leaves (Fig. 5b). The upper leaves of these plants had a bleached phenotype and no GFP fluorescence, especially in their veins (Fig. 5a: iii). *PDS* and *GFP* mRNAs were quantitated by RT-PCR, and both mRNAs

tor ΔV1V2V3-GFP, both vectors infected plants systemically, inducing symptoms as expected (Fig. 5a: iv), and DNA of both vectors were detected in systemic leaves (Fig. 5b). However, in this case, bleaching indicating *PDS* downregulation was not clear (Fig. 5a: iv, left), and a decrease in *PDS*

mRNA was not observed (Fig. 5c, lower left), although *GFP* expression had clearly disappeared (Fig. 5a: iv, right) and *GFP* mRNA was significantly lower (Fig. 5c, lower right).

Discussion

Geminiviruses are considered a promising platform for VIGS vectors because of their high-fidelity replication, small genome size, the easy inoculation method, and the large number (more than 480) of species that can be infected (Carrillo-Tripp et al. 2006). However, strict limitations regarding their genome size (approximately 3 kb) have impeded their use as VIGS vectors because a fragment of the target plant gene to be silenced must be inserted into them. Artificial geminivirus constructs larger or smaller than the original genome size lead to a reversion to the original size by deletion or recombination in systemically infected leaves (Elmer and Rogers 1990; Etessami et al. 1989; Gilbertson et al. 2003). To date, several strategies have been reported to overcome this problem.

Geminivirus VIGS vectors in current use replace viral genes dispensable for systemic infection with a target gene fragment. One replaceable gene is the CP gene (approximately 0.8 kb) of bipartite begomoviruses (Fig. 1a) (Beyene et al. 2017; Krenz et al. 2010; Lentz et al. 2018; Turnage et al. 2002), which can be replaced with 0.6–1.0-kb inserts. Another dispensable gene is the βC1 gene of the monopartite begomovirus betasatellite (approximately 0.4 kb) (Fig. 1b), which can be replaced with 0.2–0.5-kb inserts (Tao and Zhou 2004). Only a few exceptions to these two strategies include the insertion of an approximately 0.1-kb target gene fragment into the IR of DNA-B of a bipartite begomovirus (Peele et al. 2001). The problem with above two strategies is that, they cannot be applied to monopartite geminiviruses (including monopartite begomoviruses and geminiviruses belonging to the other eight genera) without a betasatellite.

In this study, we developed a novel two-component vector approach to construct VIGS vectors from monopartite geminiviruses and demonstrated that this approach works for *N. benthamiana* using BCTV. We examined the suitability of BCTV-based vectors for a two-component strategy through gene expression analyses and phenotypic analyses in the phloem. In this strategy, two copies of monopartite geminivirus genomic DNA are used. In the first copy, all V-genes and the IR are left intact, but C-genes are dispensable and replaceable by the target gene fragments to be silenced. In the second copy, all C-genes and the IR are left intact, but any V-genes can be replaced (Fig. 1c). Co-inoculation with these two components allows the production of all viral proteins necessary for systemic infection *in trans* from either construct and the systemic infection and VIGS induction of both components.

Interestingly, we found that wild-type viruses did not help these vectors to move systemically, although wild-type viruses can theoretically supply all viral proteins necessary to do so. After co-inoculations with the ΔV1V3-PDS vector and the wild-type virus, only the wild-type virus moved systemically (Fig. 3c), probably because wild-type viruses can replicate more efficiently in the absence of VIGS vectors and, as a result, can move more rapidly within the plant. It is therefore important for our two-component strategy to use VIGS vectors that are defective in systemic infection themselves and depend on each other's presence.

Regarding the length of the target gene fragments, we designed all *PDS* and *GFP* gene fragments so that the total lengths of VIGS vectors were approximately 3 kb. We did not check the efficacy of shorter or longer fragments because artificial geminiviral genomes longer or shorter than the original size by insertion (Elmer and Rogers 1990; Gilbertson et al. 2003) or deletion (Etessami et al. 1989), respectively, were reported to be unstable and to revert to their original size (approximately 3 kb) when they moved systemically. Considering this size limitation, using our strategy, we can theoretically insert target gene fragments into the two component vectors with a total length of 2 kb or longer. In this study, we showed that fragments of at least 612–720 bp were effective for gene silencing and were stably propagated.

An additional advantage of our strategy is that two distinct target genes could be knocked down simultaneously as we did for the *PDS* and *GFP* genes.

Regarding the effectiveness of the silencing, target fragments replaced with V-genes or C-genes were equally effective in inducing VIGS. To drive target gene fragment transcription, V1 gene (ΔV1V3-PDS construct), V2 gene (ΔV1V2V3-GFP construct), and C1 gene promoters (ΔC1C4-GFP construct) were equally effective. However, when the C2 gene promoter was used in the ΔC1C2C3-PDS construct, VIGS was not reliable, as seen in the ΔC1C2C3-PDS/ΔV1V2V3-GFP co-inoculation experiment (Fig. 5a: iv), and *PDS* was not downregulated (Fig. 5c, lower). This result was either because transcription from the C2 promoter was weak or unstable or because the *PDS* gene fragment length in this construct (0.2 kb) was much smaller than that of the other *PDS* construct (0.6–0.7 kb).

We chose BCTV as a VIGS vector platform because this geminivirus can infect an extremely large number of plant hosts. However, it was not the best virus for silencing *N. benthamiana* genes. First, it is strictly phloem-limited, so strong VIGS was induced only around veins. Other geminiviruses, such as African cassava mosaic virus and tomato golden mosaic virus, can move outside the phloem to infect more cell types, including mesophyll cells in *N. benthamiana*. Second, BCTV induces severe symptoms in *N. benthamiana* plants, which might hamper the

elucidation of the functions of the silenced target genes. The optimal virus species for use as a VIGS vector platform will cause no symptoms. Recently, it has become possible to isolate geminiviruses that infect the host plant without symptoms. Because geminiviral and nanoviral genomes and their replicative intermediates in the nucleus are small, circular DNA, they can be amplified by rolling circle amplification (RCA), a technique that amplifies any small circular DNA without needing prior knowledge of the sequence. Overall, 79 novel geminivirus-like complete sequences have been identified from 20 asymptomatic cactus species (Fontenele et al. 2020).

In this study, we developed a novel two-component vector approach to construct VIGS vectors from monopartite geminiviruses and demonstrated that this approach works for *N. benthamiana* and BCTV. This approach eliminates the problem of the size limitation of monopartite geminivirus-based VIGS vectors, and it will be useful for multiple gene silencing and constructing geminivirus-based VIGS systems for specific plant species.

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