

Research Article



Sugarcane Mosaic Virus-Based Gene Silencing in *Nicotiana benthamiana*

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Received: 29 March 2016; Revised: 14 February 2017; Accepted: 27 July 2017; Published online: 29 December 2017

Background: Potyvirus-based virus-induced gene silencing (VIGS) is used for knocking down the expression of a target gene in numerous plant species. *Sugarcane mosaic virus* (SCMV) is a monopartite, positive single strand RNA virus.

Objectives: pBINTRA6 vector was modified by inserting a gene segment of SCMV in place of *Tobacco rattle virus* (TRV) genome part 1 (TRV1 or RNA1) and the two nonstructural proteins of TRV2(RNA2).

Materials and Methods: SCMV construct was inoculated into 3-4 weeks *Nicotiana benthamiana* plant leaves either by using a needleless syringe or applying pricking with a toothpick.

Results: The construct (SCMV-RNA2) successfully induced post-transcriptional gene silencing (PTGS) of the target genes *GFP* and *ChII* through agroinoculation proving that SCMV is a substitute of the RNA1, which plays a pivotal role in the systemic gene silencing. 2-3-weeks of post inoculation, target genes' silencing was observed in the newly developed non-inoculated leaves.

Conclusions: The newly developed construct expresses the knocked down of the endogenous as well as exogenous genes and only four weeks are required for the transient expression of the gene silencing based on SCMV-VIGS system.

Keywords: *Nicotiana benthamiana*, Post-transcriptional gene silencing, SCMV, *Sugarcane mosaic virus*, VIGS, Virus induced gene silencing.

1. Background

The emergence of sequencing data has helped the research in many model plants in general and in functional genomics in particular. Genome sequencing, next-generation sequencing, bioinformatics tools, shotgun sequencing, RNA-seq, expressed sequence tags, and microarrays have contributed largely in the research area of the biotechnology and molecular biology, respectively (1, 2). However, these tools lack in providing a comprehensive understanding of the functional consequence of the genes' sequences. The function of a gene could be understood through silencing gene expression experiments and stable mutant plant lines. Conversely, there is a limited cloning friendly insertional mutant resources, and what is available, is particularly available for *Arabidopsis* (3) as well as a small number of other plant species (Supplementary

Tables A and C). In addition, the identification of the mutant plant species can be achieved by targeting the induced local lesions in the genomes (TILLING) (4) in which the nucleotide sequences are well annotated and available (5). Gene overexpression and RNA interference (RNAi) studies are presently restricted to the plant species that are acquiescent to the genetic transformation, while, they are laborious and time-consuming (6,7). Micro-RNA-induced gene silencing (8,9) and artificial micro-RNA-mediated gene silencing are used in plants for a targeted gene silencing, but these approaches involve a stable plant transformation, which is laborious, time consuming, and inappropriate with high-throughput studies (10-12). Similarly, a specific gene knockout in the plants can be used by clustered regularly interspaced short palindromic repeats (CRISPR) associated (Cas) nuclease (13), zinc-finger

nucleases (ZFNs) (14), and transcription activator like effector nucleases (TALENs) (15). But, these methods require time consuming stable plant transformation and are mainly suitable as 'reverse genetics' tools.

Virus-induced silencing is a post transcriptional gene silencing method (16, 17) adopted by plants as a shield against viral attacks (18, 19). RNA dependent RNA polymerase produces double stranded RNA during the virus replication leading to PTGS (20-22). siRNA is produced by a process which involves Dicer-like enzyme-mediated cleavage of the dsRNA (23). RNA-induced silencing complex (RISC) chops the viral RNA in a homology-dependent manner (23) as a result of binding and activation of siRNA to RISC (Supplementary Fig. 1). This plant defense mechanism has been used for the development of a process for silencing of the endogenous plant genes (24). To silence a specific plant gene, a fragment of the target gene is cloned into a modified virus vector and delivered to the plant (25-27). The virus replication mechanism multiplies the target gene fragment introduced in the virus vector in the plant cell, and the transcripts spread throughout the plant systemically (28). The induced PTGS triggers the production of siRNA homologs, which will lead to the endogenous plant gene silencing (29).

Since the first report of VIGS in the late 1990s, it has been extensively used by many scientists for understanding and exploiting the role of plant genes in numerous biological processes (24, 30, 31). The viral induced gene silencing has overcome many constraints. It favors to study gene functions when mutations are lethal to the embryonic stages and resulting into deformation of the plants (26, 32). It is also good for high-throughput genetic analysis (33-35), as it can be performed in a short time without any alteration in the plant genome (36, 37).

Various virus-induced gene silencing vectors have been developed which cause gene silencing of the many target genes in numerous plant species (38). Twenty-two VIGS vectors have been reported which are capable of multiple genes silencing in the multiple species (Supplementary Table B). *Tobacco rattle virus* (TRV) based VIGS vectors have been used in many plant species, particularly in *Solanaceae* family such as tomato (*Solanum lycopersicum*) and *N. benthamiana* (37, 39). *Apple latent spherical virus* (ALSV), *Barley stripe mosaic virus* (BSMV), *Tobacco mosaic virus* (TMV), *Potato virus-X* (PVX), and *Tomato golden mosaic virus* (TGMV) have been reported as VIGS vectors capable of gene silencing in *N. benthamiana* (39, 40). PVX and TRV-based VIGS vectors express strong gene silencing

in *N. benthamiana*. TRV has ability to infect meristem tissues (41), while others cannot.

Tobacco rattle virus (TRV) possesses bipartite genome: TRV1 (RNA1) and TRV2 (RNA2) (42). RNA 1 has a pivotal role in viral systemic movement (43). RNA1 encodes replicase proteins 134 and 194 kDa. RNA 2 has genes responsible for nonstructural and coat proteins (CP) and its genome shows diversity among its various isolates (42). The nonstructural proteins are associated with transmission to the nematodes (44), but these proteins have no role in plant infection. In this study, SCMV based VIGS vector was developed in order to trigger gene silencing of the both exogenous and endogenous genes in *Nicotiana benthamiana*.

2. Objectives

In this study, SCMV based VIGS vector was developed in order to trigger gene silencing of both exogenous and endogenous genes in *Nicotiana benthamiana*.

3. Materials and Methods

3.1. Growth Conditions and Plant Material

Plants of *N. benthamiana* were maintained in a restrained climatic condition in growth room at 20-25 °C with a 14/10 h light (~75 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$)/dark photoperiod. Seedlings were grown in a greenhouse until they attained the two leaf-stage and were ready for inoculation with the SCMV-VIGS construct. The inoculated plants were transferred to the growth room at 23-25 °C until assessed.

3.2. Construction of SCMV-VIGS Vectors

For the development of SCMV based VIGS vector, RNA1 and the two nonstructural proteins of the RNA2 in pBINTRA6 (AF314165) (45) were substituted by *Sugarcane mosaic virus* CP gene segment. Moreover, multiple cloning sites for inserting fragments of the target gene to be silenced were also introduced (46) (Fig. 1). The SCMV-RNA2 derived construct was then cloned into an *Agrobacterium*-mediated binary vector (pCAMBIA1300) for plant inoculation (46, 47) (Fig. 2). VIGS vector was constructed by using LBA4404 *Agrobacterium* strain. 313 bp cDNA was amplified from SCMV (Accession # KC200152) using *Pfu* DNA polymerase applying the primer pair: SCMV-313-F (5'-TGCAGGAGCCCAAGGAGGGG-3') and SCMV-313-R (5'-GGTGCTGCTGCACTCCCAACA-3'), respectively. The cDNA clone was inserted into the *Xma*I site of pBINTRA6 (AF314165)(45) and the resulting clones were confirmed by sequencing, and designated as the SCMV-RNA2 construct (Fig. 2).

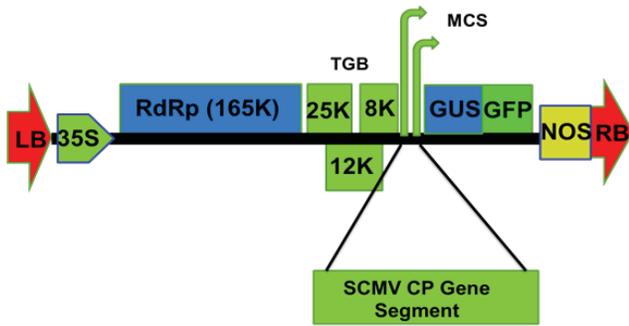


Figure 1. Details of the SCMV-RNA2 construct. The SCMV CP gene segment (313 nucleotides) was inserted into the XmaI site of the pBINTRA6 which enhances its systemic silencing of the both exogenous and endogenous genes of interest. RNA2 is the source of RdRp.

The same cloning strategy was adopted to clone a series of *GFP* and magnesium chelatase subunit I (*ChII*) fragments. The selected sequences were amplified with primer pairs, GFP-F (5'-ATCGATGGTGATGTTAATGGGCAC-3')

and GFP-R (5'-ATCGATGTCATGCCGTTTCATATG-3'). PCR products were purified, treated with T4 DNA polymerase at room temperature to generate sticky ends, followed by a heat treatment at 75 °C for 10 min to deactivate the polymerase. The XmaI linearized pBINTRA6 vector was subjected to the same treatment to create complementary sticky ends. The pBINTRA6 vector (20 ng) and PCR products (~200 ng) were mixed, incubated for 2 minutes at 66 °C followed by a slow cooling to room temperature for annealing. Ten µl aliquots were used for cloning into *Escherichia coli* DH5-α, and transformants were confirmed by endonuclease restriction digestions and colony PCR.

The *N. benthamiana* 200 bp *ChII* gene fragment was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) (48) using the primer pair *ChII*-F (5'-CTGAGGGTGTCAAGGCATTT-3') and *ChII*-R (5'-TTCCGAATCGATCAAGAAGC-3'). The resulting fragment was integrated into pBINTRA6 in the sense orientations to generate *ChII* silencing construct (SCMV-*ChII*).

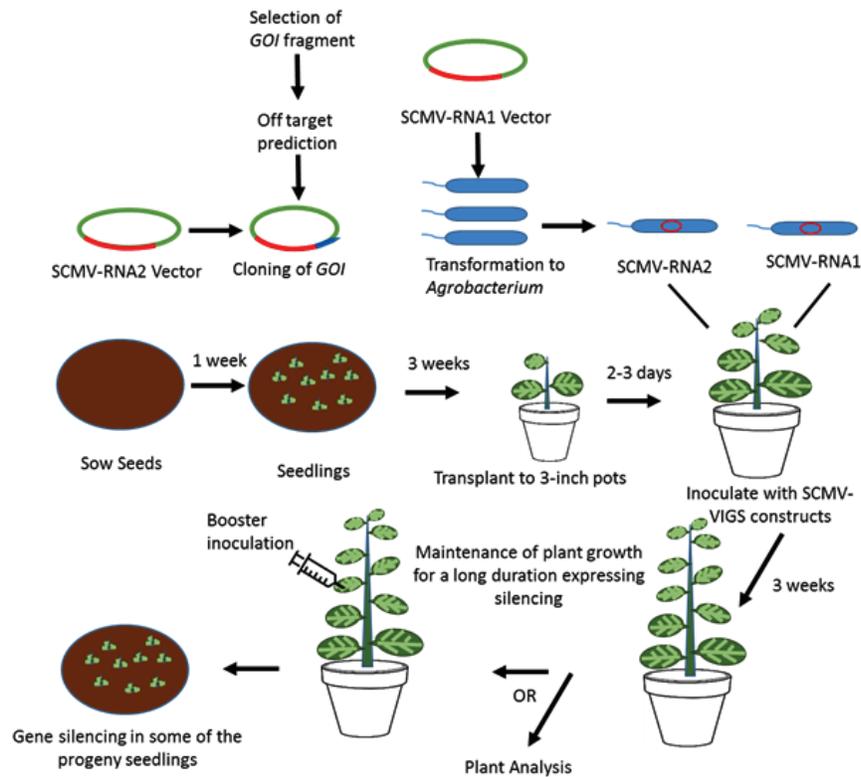


Figure 2. An overview of the SCMV based VIGS protocol in *N. benthamiana*. A GOI is cloned into the SCMV-RNA2 vector by either the gateway-based method or ligation-independent cloning depending on the vector. Transformation of the construct into *Agrobacterium* carried out and co-inoculation of the culture along with *Agrobacterium* carrying the construct into the 3-week-old plants accomplished. Silencing usually starts from the week 2 to the week 3. The silenced plants can be used for analysis from 3 weeks after inoculation. Silencing can be maintained for longer periods by booster inoculations with SCMV-RNA2::GOI.

3.3. Agro-Infiltration of *N. benthamiana*.

The SCMV based VIGS constructs were transformed into *A. tumefaciens* strain LBA4404 by electroporation method. Cultures were grown ready to use for agroinfiltration. Single colonies were incubated overnight at 28 °C while shaking in 5 mL of LB in the presence of antibiotic kanamycin (100 µg.mL⁻¹). One mL of the grown cultures was subcultured in 50 mL LB under the same growth conditions for 12 hrs. The bacterial cells were centrifuged for 10 min at 3000 ×g and were mixed with infiltration buffer [10 mM MgCl₂, 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.2, and 0.1 mM acetosyringone]. The infiltration buffer was incubated for three hours at room temperature until ~0.7 OD₆₀₀ was obtained.

SCMV-VIGS constructs were infiltrated into four to eight *N. benthamiana* leaves immediately above the cotyledons with a 1-mL needleless syringe (Fig. 3). The infiltrated leaves were evaluated for silencing expression after maintenance in a growth chamber for 5 to 12 days post infiltration (dpi). Inoculation was also performed with either of the two methods: the agro-drench method and by pricking with a toothpick

(Fig. 3, supplementary Table 4).

3.4. Semi-quantitative RT-PCR analysis

TRIzol reagent was used to extract total RNA as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). RNA was treated with DNase, and first-strand cDNA synthesis was carried out with M-MLV reverse transcriptase (Promega, Madison, WI, USA). The expression level of the target gene was monitored by semi-quantitative RT-PCR using gene-specific primers (Table 1) that anneal outside the region targeted for silencing.

4. Results

Plant phenotype may change due to silencing of a target gene; here *GFP* silencing changed to red under UV (Figs. 4B, D-E). Similarly, *ChlI* gene silencing resulted in a photobleaching in the plants (Figs. 4G-I). The *ChlI* gene is responsible for the biosynthesis of carotenoids (31). When this gene is silenced carotenoids level is reduced which protects photosystem. Hence, photo-oxidation is initiated resulting in photobleaching under ordinary light intensity (31, 46).

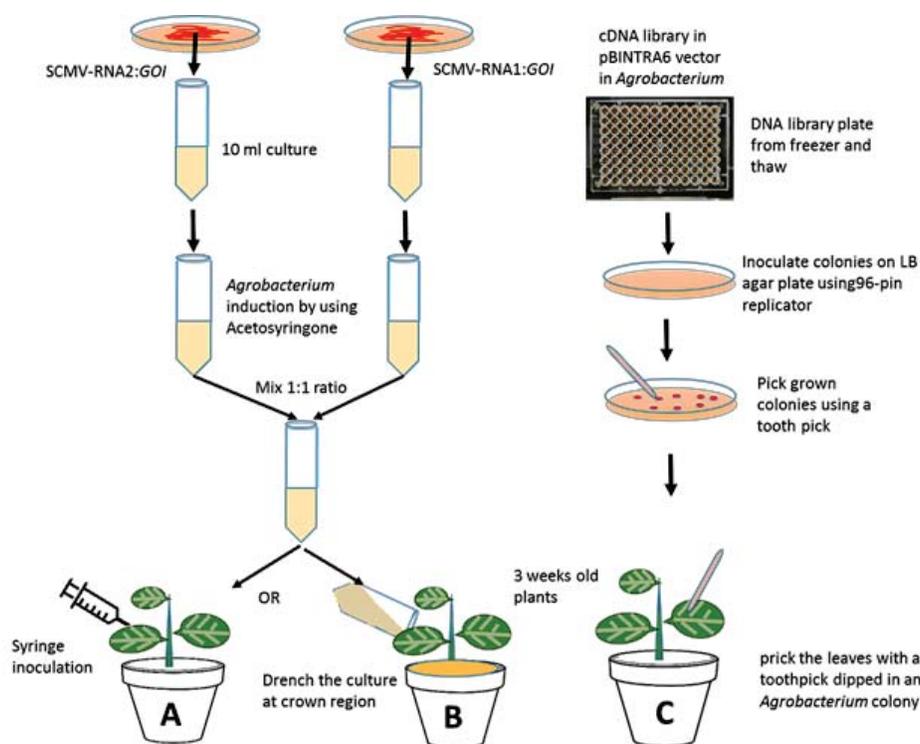


Figure 3. The different inoculation methods of the SCMV-RNA2 into *N. benthamiana* plants. (A) *Agrobacterium* cultures carrying SCMV-RNA2 containing target gene fragment (SCMV-RNA2::GOI) was inoculated into the leaves with a needleless syringe. (B) Agro-drench method that involves drenching of the crown region of the plant with the culture. (C) Another method is pricking the leaves with a toothpick dipped in an *Agrobacterium* colony carrying SCMV-RNA2::GOI.

Table 1. The sequences of the primers used in this study.

Name	Primer Sequence (5'-3')	Length (bp)	GC%	Tm (°C)
SCMV-313-F	TGCAGGAGCCCAAGGAGGGG	20	70	60
SCMV-313-R	GGTGCTGCTGCACTCCCAACA	21	62	58
<i>GFP</i> -F	ATCGATGGTGATGTTAATGGGCAC	24	46	55
<i>GFP</i> -R	ATCGATGTCATGCCGTTTCATATG	24	42	55
<i>ChlI</i> -F	CTGAGGGTGTCAAGGCATTT	20	50	52
<i>ChlI</i> -R	TTCCGAATCGATCAAGAAGC	20	45	52

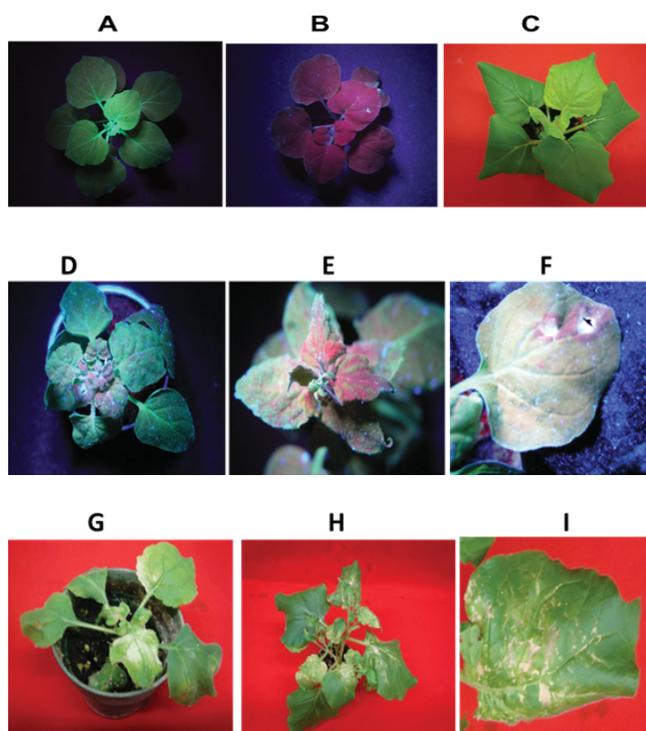


Figure 4. Silencing the *GFP* and *ChlI* genes in *N. benthamiana* plants by SCMV-RNA2 based VIGS. (A) *GFP* transgenic plants of *N. benthamiana* under UV illumination. (B) Control (non-transgenic) *N. benthamiana*. (C) Control (non-transgenic) *N. benthamiana* under visible light. (D-F) The *GFP* gene (exogenous) silencing in 16-c transgenic *N. benthamiana* inoculated with the SCMV-RNA2 construct harboring *GFP* gene segment. The *GFP* sequence does not have any homology with the plant DNA therefore, it will not cause silencing. The plant was photographed at ~2 weeks after inoculation. (G-I) *ChlI* gene (endogenous) silencing (photobleaching) in *N. benthamiana* inoculated with the SCMV-RNA2 construct harboring *ChlI* gene segment, respectively.

RT-qPCR was used to evaluate the silencing levels of the target endogenous genes using gene-specific primers (Fig. 5). The degree of gene silencing depends on many factors i.e. intensity of the viral infection, time of VIGS construct inoculation and the expression of the target genes. *N. benthamiana* plants inoculated with SCMV-RNA2:*ChlI* construct showed >95% reduction in the transcript levels (Fig. 5), while, silencing *ChlI* gene by which phenotype photobleaching occurs, has noticed ~15 dpi (Figs. 4G-I). The silencing of the target gene is directly related to the degree of target gene function and silencing. Sometimes target genes are silenced, but they do not exhibit visual phenotype changes as compared with the control plants inoculated with the control vectors. The virus-induced gene silencing is assumed to be effective if ~80% or greater downregulation of the endogenous target gene has occurred but it is governed by the nature and function of the genes (49). It has been observed that the inoculated plants can maintain the gene silencing for a couple of years and even in the next progeny, as well (50).

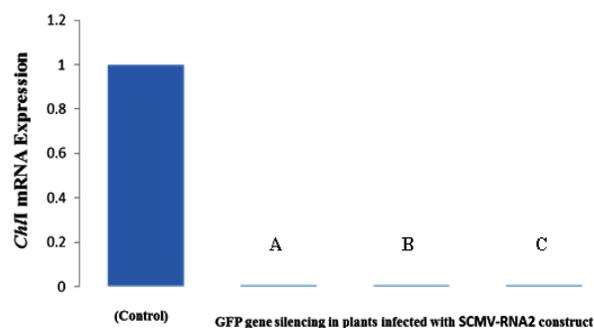


Figure 5. The endogenous *ChlI* transcript levels in the treated plants using different inoculation methods; A) syringe inoculation, B) agrodrenching, and C) picking compared with control plant, quantified using RT-qPCR. The inoculated plants show strong mRNA degradation.

GFP gene (exogenous) silencing in transgenic *N. benthamiana* 16-c line, infected with SCMV-RNA2 construct harboring *GFP* gene segment, appeared as red under UV (Figs. 4D-F). Since *GFP* sequences do not possess homology with the plant DNA, and therefore any other gene(s) of the plant, it will not be silenced.

5. Discussion

The initial investigation on adapting SCMV-CPVIGS construct as a silencing vector was based on the well-established *GFP* silencing system (use of pBINTRA6 vector) in *N. benthamiana* line 16c (Ruiz *et al.*, 1998). The construct cassette was restricted to *GFP* transgene expression under the control of the constitutive promoter 35S derived from *Cauliflower mosaic virus* (CaMV) and NOS terminator (Fig. 1). The *N. benthamiana* 16c (*GFP* transgenic) plants normally appear to be green under UV illumination due to *GFP* fluorescence (Fig. 4A). In contrast, the non-transgenic *N. benthamiana* appears red (Fig. 4B) under UV illumination due to chlorophyll autofluorescence (30, 51). The results presented here indicate that a modified pBINTRA6 VIGS vector has efficiently worked as a silencer and as an expression vector in *N. benthamiana*. SCMV-RNA2 construct carrying the fragment of the *GFP* was capable of silencing *GFP* expression in *N. benthamiana* 16c when inoculated using potyvirus SCMV (Figs. 4 D-F) for the systemic silencing of the target gene(s). Further validation of the VIGS construct was done by cloning a fragment of endogenous gene *ChII* into SCMV-RNA2 construct and inoculating it to the *N. benthamiana*. The inoculation yielded bleaching in the systemic leaves which is the characteristic of the loss of chlorophyll, providing a visible marker for gene silencing (Figs. 4G-I). The results confirmed that the vector is capable of initiating silencing of an endogenous gene in *N. benthamiana* (49).

The VIGS is a powerful technique for down-regulating gene expression in the plants (52-54). It is now widely used as a means of investigating gene function and has been adapted for high-throughput studies (33). Such an analysis would have been far more difficult and time consuming without the availability of VIGS.

GFP has been used extensively to study the systemic movement of the viruses in the plants e.g. CP replacement vectors have been used to study the movement of *Bean dwarf mosaic virus* (BDMV) (55). These studies have shown that DNA viruses do not require the CP for systemic infection of the host plants (56, 57). However, for RNA viruses, the CP is essential for the infectivity as it is responsible for providing some essential movement

functions, either as virions or as CP-RNA complexes (58-61). In pBINTRA6 (62) RNA1 and nonstructural protein genes of the RNA2 are involved in the systemic movement of the virus, but, silencing of the target genes was evident when they were replaced with the *SCMV-CP* gene. Under UV illumination, silencing at the site of inoculation was evident. The silencing appeared as the red area around the site of infiltration. As well, a number of silencing patches were present in the developing leaves at the time of inoculation. It was evident from the results that silencing of the *GFP* along the veins has been initiated. The leaves above this (i.e. developing leaves after inoculation) initially showed silencing along the veins and later spread to the whole leaf surface systemically. All subsequently developing tissues were entirely silenced, thus appearing red under UV illumination. The silencing was persistent and remained until plants' senescence. Our results clearly have shown down regulation of *GFP* and *ChII* in the *N. benthamiana* using our newly developed construct, named SCMV-RNA2.

Acknowledgments

We acknowledge the Higher Education Commission, Islamabad, Pakistan and professor Baulcombe's laboratory, Department of Plant Sciences, University of Cambridge, UK for providing us with the pBINTRA6 vector under a material transfer agreement.

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Supplementary Table A. Plant species having publicly available mutant resources conducive for cloning the gene of interest.

Sr. No.	Plant species	Name of mutant resource	Reference
1	Arabidopsis	T-DNA insertion mutants	(Belfield <i>et al.</i> , 2012; Enders <i>et al.</i> , 2015; Jia <i>et al.</i> , 2012; O'Malley <i>et al.</i> , 2015)
2	Soybean	<i>Tnt1</i> insertion mutants	(Cui <i>et al.</i> , 2013; Mathieu <i>et al.</i> , 2009)
3	Tomato	T-DNA activation tagged lines and Ds-tagged lines	(Matsukura <i>et al.</i> , 2008)
4	<i>Medicago truncatula</i>	<i>Tnt1</i> insertion mutants	(Tadege <i>et al.</i> , 2009; Tadege <i>et al.</i> , 2008)
5	<i>Lotus japonicus</i>	Transposon mutants	(Urbański <i>et al.</i> , 2012; Urbański <i>et al.</i> , 2013)
6	Rice	T-DNA or <i>Tos17</i> insertion mutants	(Krishnan <i>et al.</i> , 2009; Kuromori <i>et al.</i> , 2009)
7	<i>Brachypodium distachyon</i>	T-DNA insertion mutants	(Bragg <i>et al.</i> , 2012; Thole <i>et al.</i> , 2012)
8	Maize	Transposon tagged mutants	(Hanley <i>et al.</i> , 2000; Settles <i>et al.</i> , 2007)

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Supplementary Table B. Currently available predominantly used VIGS vectors and their target plant species*

Sl. No.	VIGS vector	Examples for target plant species or family or common name	Reference
1.	<i>Apple latent spherical virus (ALSV)</i>	<i>Nicotiana benthamiana</i> , <i>Arabidopsis thaliana</i> , apple, cucurbits and pears	(Igarashi <i>et al.</i> , 2009)
2.	<i>Alternanthera mosaic virus (AltMV)</i>	<i>N. benthamiana</i> and <i>A. thaliana</i>	(Lim <i>et al.</i> , 2010)
3.	<i>Brome mosaic virus (BMV)</i>	Rice, maize, sorghum and foxtail millet	(Ding <i>et al.</i> , 2006)
4.	<i>Barley stripe mosaic virus (BSMV)</i>	Wheat and barley	(Panwar <i>et al.</i> , 2013; Scofield <i>et al.</i> , 2005)
5.	<i>Bamboo mosaic virus</i> and its satellite RNA (SatBaMV)	<i>N. benthamiana</i> and <i>Brachypodium distachyon</i>	(Liou <i>et al.</i> , 2014)
6.	<i>Bean pod mottle virus (BPMV)</i>	Common bean, soybean	(Díaz-Camino <i>et al.</i> , 2011; Zhang <i>et al.</i> , 2010)
7.	<i>Cucumber mosaic virus (CMV)</i>	<i>N. benthamiana</i> and soybean	(Matsuo and Matsumura, 2011; Nagamatsu <i>et al.</i> , 2007)
8.	<i>Cymbidium mosaic virus (CymMV)</i>	Phalaenopsis orchid	(Hsieh <i>et al.</i> , 2013a; Hsieh <i>et al.</i> , 2013b)
9.	<i>Potato virus X (PVX)</i>	<i>N. benthamiana</i> and potato	(Faivre-Rampant <i>et al.</i> , 2004)
10.	<i>Pea early browning virus (PEBV)</i>	<i>Pea</i> , <i>barrel medic</i> and <i>lotus</i>	(Constantin <i>et al.</i> , 2004; Grønlund <i>et al.</i> , 2008)
11.	<i>Tobacco rattle virus (TRV)</i>	<i>This vector has wide host range (a few examples include many members of Solanaceae family, Arabidopsis, California poppy and cotton.</i>	(Burch-Smith <i>et al.</i> , 2006; Gould and Kramer, 2007; Liu <i>et al.</i> , 2002; Xiquan <i>et al.</i> , 2011)
12.	<i>Turnip yellow mosaic virus (TYMV)</i>	<i>N. benthamiana</i> and <i>A. thaliana</i>	(Pflieger <i>et al.</i> , 2008)
13.	<i>African cassava mosaic virus (ACMV)</i>	<i>N. benthamiana</i> and <i>cassava</i>	(Fofana <i>et al.</i> , 2004)
14.	<i>Beet curly top virus (BCTV)</i>	<i>Spinach</i> and <i>tomato</i>	(Golenberg <i>et al.</i> , 2009)
15.	<i>Cabbage leaf curl virus (CaLCuV)</i>	<i>N. benthamiana</i> and <i>A. thaliana</i>	(Muangsan <i>et al.</i> , 2004)
16.	<i>Cotton leaf crumple virus (CLCV)</i>	<i>N. benthamiana</i> and <i>cotton</i>	(Tuttle <i>et al.</i> , 2012; Tuttle <i>et al.</i> , 2008)
17.	<i>Grapevine virus A (GVA)</i>	<i>N. benthamiana</i> and <i>grapevine</i>	(Muruganatham <i>et al.</i> , 2009)
18.	<i>Grapevine leaf roll-associated virus-2 (GLRaV2)</i>	<i>N. benthamiana</i> and <i>grapevine</i>	(Kurth <i>et al.</i> , 2012)
19.	<i>Pepper huasteco yellow vein virus (PHYVV)</i>	<i>Capsicum sp.</i>	(del Rosario Abraham-Juárez <i>et al.</i> , 2008)
20.	<i>Rice tungro bacilliform virus (RTBV)</i>	<i>Rice (Indica and Japonica)</i>	(Purkayastha <i>et al.</i> , 2010)
21.	<i>Tobacco curly shoot virus (TCSV)</i>	<i>Tobacco</i> and <i>tomato</i>	(Huang <i>et al.</i> , 2009)
22.	<i>Tomato yellow leaf curl China virus (TYLCCNV)</i>	<i>Tobacco</i> and <i>tomato</i>	(Cai <i>et al.</i> , 2007; Tao and Zhou, 2004)

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Supplementary Table C. Plant species suitable for TRV-VIGS for silencing target genes.

Sr. No.	Plant species	Suitable genotype or cultivar	Method of inoculation	Preferred age of plant	Environmental temperature (day)	Reference
1	<i>Nicotiana benthamiana</i>	-	Syringe-mediated inoculation; Agrodrench	3 week old plants	21±2 °C	(Ryu <i>et al.</i> , 2004; Senthil-Kumar <i>et al.</i> , 2013; Senthil-Kumar and Mysore, 2011)
2	Tomato (<i>Solanum lycopersicum</i>)	Glamour & micro-tom	Syringe-mediated inoculation at cotyledonary leaves; vacuum infiltration	10 day old plants	21±2 °C	(Burch-Smith <i>et al.</i> , 2006; Liu <i>et al.</i> , 2002; Senthil-Kumar and Mysore, 2011)
3	<i>Arabidopsis thaliana</i>	Columbia	- Multiply the virus constructs in <i>N. benthamiana</i> and inoculate virus containing sap* - TRV1 and TRV2 mixed culture is delivered into apoplast of leaf cells by using needleless syringe	Two week old plants	23±2 °C	(Burch-Smith <i>et al.</i> , 2006; Lu <i>et al.</i> , 2003; Rojas <i>et al.</i> , 2012)
4	Cotton (<i>Gossypium hirsutum</i>)	Coker 312	Agroinoculation by vacuum infiltration, infiltration by needleless syringe	Two week old plants	23-25 °C	(Qu <i>et al.</i> , 2012; Xiquan <i>et al.</i> , 2011)
5	<i>Aquilegia vulgaris</i> (also applicable to opium or California poppy)	-	Agroinoculation by vacuum infiltration	Varies (about 12-15 weeks)	Growth condition varies (20 °C suitable for VIGS)	(Gould and Kramer, 2007; Hileman <i>et al.</i> , 2005; Wege <i>et al.</i> , 2007)
6	<i>Jatropha curcas</i>	-	Agroinoculation by needle-less syringe or vacuum infiltration	Varies (2-3 leaf stage)	25 °C	(Ye <i>et al.</i> , 2009)
7	Tobacco (<i>Nicotiana tabacum</i>)	Xanthi nc	Agrodrench	2-3 week old plants	21±2 °C	(Ryu <i>et al.</i> , 2004)
8	Petunia (<i>Petunia hybrida</i>)	-	Agroinoculation by needle-less syringe	-	25 °C	(Chen <i>et al.</i> , 2004)
9	Pepper (<i>Capsicum spp.</i>)	-	Agroinoculation by needle-less syringe; Agrodrench	2 week old plants	25 °C	(Chung <i>et al.</i> , 2006; Ryu <i>et al.</i> , 2004)
10	Several other plants of Solanaceae	-	Agroinoculation by needle-less syringe	-	-	(Senthil-Kumar <i>et al.</i> , 2007)
11	<i>N. benthamiana</i> , tomato and several other Solanaceae plants	-	Sprout vacuum inoculation	Immediately after germination	-	(Yan <i>et al.</i> , 2012)

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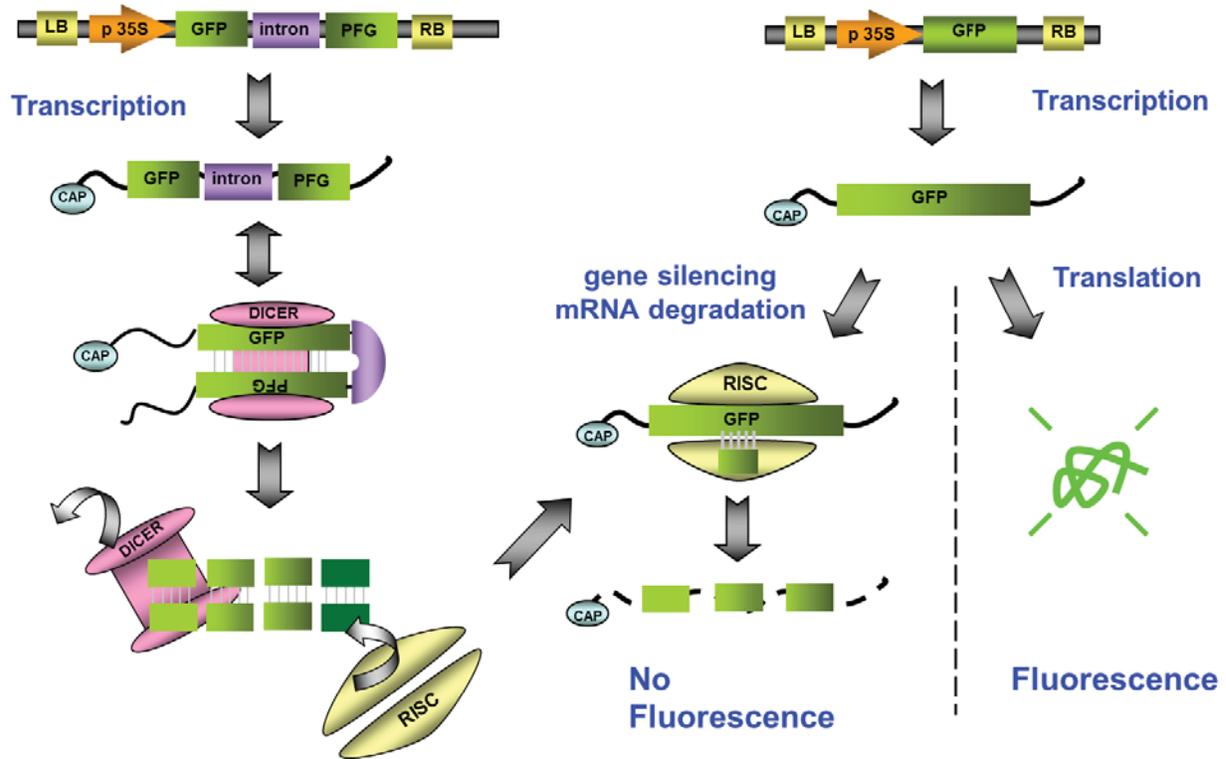
Supplementary Table D. Different methods of TRV inoculation for achieving VIGS.

Sr. No.	Name of the method	Brief protocol	Age of the plant used	Application	Reference
1	Syringe inoculation	TRV1 and TRV2 containing mixed <i>Agrobacterium</i> culture is delivered into apoplast of leaf cells by using needleless syringe	3 weeks old	For silencing one or few genes by a reverse genetics approach	(Velásquez <i>et al.</i> , 2009)
2	Agrodrench	Drench TRV1 and TRV2 containing mixed <i>Agrobacterium</i> culture into the plant growth medium near crown region	1-2 weeks old	For silencing large number of genes, especially for gene silencing in roots	(Ryu <i>et al.</i> , 2004)
3	Pricking leaves using toothpick	TRV1 <i>Agrobacterium</i> culture is inoculated using needleless syringe and at this inoculated site TRV2 colonies are pricked using toothpick	3 weeks old	For large scale gene silencing of genes using cDNA library for a forward genetics screening	(Senthil-Kumar <i>et al.</i> , 2013)
4	Spray inoculation	Leaf surface is dusted with carborundum. TRV1 and TRV2 containing mixed <i>Agrobacterium</i> culture is sprayed using a sprayer.	3 weeks old	Silencing a few genes independently in a large number of plants	(Padmanabhan and Dinesh-Kumar, 2009)
5	Vacuum infiltration of plant	Plant is immersed in TRV1 and TRV2 containing mixed <i>Agrobacterium</i> culture and vacuum is applied	All ages	Silencing genes in young seedlings	(Padmanabhan and Dinesh-Kumar, 2009)
6	Vacuum or syringe inoculation of fruit (examples, tomato & strawberry)	Inoculation of TRV1 and TRV2 containing mixed <i>Agrobacterium</i> cultures into detached fruit by vacuum suction or needle-less syringe	Detached fruit	Understanding fruit development (example, ripening) related genes	(Fu <i>et al.</i> , 2005)
7	Sprout vacuum inoculation	Inoculation of TRV1 and TRV2 containing mixed <i>Agrobacterium</i> in small seedlings by vacuum	Small seedlings immediately after germination	Gene function in the early stages of plant growth	(Yan <i>et al.</i> , 2012)
8	Rub inoculation of virus containing sap or virions on to the target plant leaf	Inoculation of TRV1 and TRV2 in <i>N. benthamiana</i> leaves and then virus particles or sap from the inoculated leaf is collected and inoculated on to the target plant species.	About 3 week old plants	This is an alternate method for Agroinoculation of TRV vectors. This method is efficient in some plant species (for example, <i>Arabidopsis</i>) where the Agroinoculation is not preferred.	(Lu <i>et al.</i> , 2003)

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Supplementary Figure 1. The virus induced gene silencing mechanism within plant body. VIGS is a PTGS method used by plants as a defense mechanism against invading viruses. During viral replication double-stranded RNA (dsRNA) is produced by an RNA dependent RNA polymerase and this triggers PTGS. One of the major aspects of this process involves Dicer-like enzyme-mediated cleavage of the dsRNA to produce siRNA. This siRNA binds to and activates the RNA-induced silencing complex (RISC) which cleaves the viral RNA in a homology-dependent manner.