ABSTRACT

MUANGSAN, NOODUAN. Analysis of Begomoviruses-Mediated Gene Silencing (Under the direction of Dr. Dominique Robertson)

Viral vectors carrying a host-derived sequence insert induce silencing of the corresponding gene in infected plants. This virus-induced gene silencing (VIGS) is a defense mechanism that is related to post-transcriptional gene silencing (PTGS) in transgenic plants. Here I describe modified vectors derived from two members of the Begomoviruses: Tomato Golden Mosaic Virus (TGMV) and Cabbage Leaf Curl Virus (CbLCV).

In order to gain insight into the geminivirus-induced gene silencing (G-VIGS) mechanism, I tested six known silencing-deficient Arabidopsis mutants: *sgs1, sgs2, sgs3, ago1, som* and *mom*. *Sgs1, sgs2, sgs3 and ago1* mutants are impaired for PTGS of transgenes and endogenous genes, whereas *som* and *mom* mutants release transcriptional gene silencing (TGS). SGS2 and AGO1 encode an RNA-dependent RNA polymerase (RdRP) and an eIF2C-like protein belonging to PAZ/PIWI family, respectively. SGS1 and SGS3 map to genes with unknown function. SOM and MOM are chromatin-associated proteins.

All mutants were bombarded with CbLCV carrying a gene fragment homologous to an endogenous gene required for chlorophyll biosynthesis, magnesium chelatase (CH42). G-VIGS of *CH42* endogenous gene occurred efficiently in the *sgs1, ago1, som* and *mom* mutants, but it was inhibited (not abolished) in *sgs2* and *sgs3* mutants. These results indicate that SGS2 and SGS3 are required for G-VIGS, whereas SGS1, AGO1, SOM and MOM have minimal or no effect. The findings that SOM and MOM are not required for G-VIGS support the ideas that G-VIGS is PTGS-specific or that geminiviruses counteract TGS. Considering

these results, G-VIGS has unique requirements distinct from previously described silencing pathways in plants.

I also showed that TGMV- and CbLCV-derived vectors triggered VIGS of endogenous genes in *Nicotiana benthamiana* and *Arabidopsis thaliana*, respectively. G-VIGS silencing of RdRP affected silencing while G-VIGS of a gene upregulated by AL1 negatively affected geminivirus replication or movement, suggesting that G-VIGS could be used as a preliminary screen for resistance genes. This research has provided an insight into genetic silencing mechanisms mediated by the Begomoviruses, and their potential tools as episomal vectors in reverse genetic studies.

Analysis of Begomoviruses-Mediated Gene Silencing

By

NOODUAN MUANGSAN, B.S.

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APPROVED BY:

Dr. Dominique Robertson Chairman	Dr. Judith Thomas
Dr. William F. Thompson	Dr. Linda Hanley-Bowdoin

DEDICATION

To kids in remote areas who wish to have a better education

BIOGRAPHY

I was born on August 5th, 1974 in a small village far from a city in Nakhonrat-cha-sima province, THAILAND. I grew up with two younger sisters (Noodao and Tunyaruk) and one younger brother (Poramate). At home, there was no electricity until I was in the fourth grade and no running water even now. Once finishing elementary school at home, my teachers took me to a high school, and convinced my parents to pay for tuition during the school year. My parents hesitated to let me go to school at that time because no kids from my village had ever gone to high school before. I did all sorts of work (rice growing, rice harvesting, sugar cane cutting, weeding, cassava harvesting, selling food, etc.), during weekends to earn some money while I was in school, and I had to show my parents that I could do it.

With dignity and pride I made my way from Chumpuang Suksa high school to Khon Kaen University, Khon Kaen. Since then I never asked my parent for financial support anymore. I was first interested in physics because I thought it was quite challenging for a girl to study physics among guys. Unfortunately physics was not quite popular for girls and my mother did not agree with it. So, I turned to biology and never looked back. During university years, I enjoyed helping high school kids to have fun with science. I was a mentor for Physic camp and Biology Olympic camp, and an academic coordinator for Biology camp which is an outreach program for high school kids in a remote area.

My mother kept saying that I was destined to live far away from the rice farms where I grew up. Amazingly, my life has gone so far since the day I left home for school. Fortunately, I won a scholarship to study abroad supported by the Development and

Promotion of Science and Technology Talents project of Thailand (DPST). My family and the villagers were so proud of me. I was excited to see another part of the world but so scared to be at a place where nobody spoke the same language. It was hard for me to imagine living outside Thailand because I never had a chance to converse with English-speaking tourists.

After graduating from Khon Kaen University in 1996, I was offered a position as a lecturer in the Biology Department. Here, I spent time teaching biology to freshmen and looking for a graduate school to pursue a M.S.-PhD. After waiting to be accepted by a university in the U.S. for almost a year, I was finally accepted to NCSU in 1997. Once I made a decision to come to the U.S, another chapter of my journey had begun. I recorded every memory in a journal, about how excited or nervous I was, how interesting everything was, and how things were different from home. I have experienced different cultures, languages, religions, politics, as well as opinions. If there is one thing that I have learned from my time in the U.S., everyone has an opinion. This was quite different from the village where I came from. All these experiences have helped me grow up quite a bit. I am so grateful and thankful for all opportunities I have had. Hopefully someday, a story of my journey will be told to people back home.

Besides experiencing life outside school, I have learned quite a lot about science and research that will definitely help my career. I definitely love science and I hope to be a great scientist someday. Upon completion of my studies, I am looking forward to return home, teach students at Khon Kaen University, as well as develop a research. I also hope to come back to the U.S. to do postdoctoral research once I get an offer.

After all these years, I have earned such a tremendous experience. I truly understand how important the education is. Therefore, I intend to help kids in my village and nearby

communities to have a better education. So far I have been supporting only my siblings. Hopefully, I can develop an outreach program for kids soon.

ACKNOWLEDGEMENTS

I wish to thank Dr. Dominique Robertson for her guidance as my advisor and mentor. I owe her a debt of gratitude for her patience and kindness to me as an international student for whom English is not a first language. She has taught me quite a lot of things while I have been here. She made sure I understood what we talked about and guided me. I also wish to thank the other members of my Graduate Advisory Committee, Dr. Linda Hanley-Bowdoin, Dr. Judith Thomas, and Dr. William F. Thompson, for their kindnesses, great advice, and their time.

There are a number of others I wish to thank. First, I am most indebted to Chip Peel for being one of the nicest friends I have known the U.S. He taught me how to speak correct English and how to perform some of research techniques. I would also like to thank Dr. Michael Turnage for his advice, guidance, and friendship. He was a great sounding board for ideas I had about our research. My colleague and classmate, Chad Jordan, is a wonderful friend, whom I enjoyed working with and talking to. I wish also to thank all the people who have offered help and support during my time here: Dr. Patricia Eagle, Mr. Kevin Cox, Dr. Pei-Lan Tsou, and Dr. Steve Nagar.

There are a number of people from outside my research whom I wish to acknowledge: Dr. Nina Allen and Dr. Eva Johannes and their lab for use of the dissecting microscope, the phytotron for growing plant specimens, Dr. Arthur Weissinger and his lab for use of the particle gun bombardment device, and Dr. Kent Burkey for use of a spectrophotometer. Finally, I wish to thank the members of the Botany Department for their support.

From a personal perspective there are many people to whom I offer my thanks and gratitude. My family and the people from my village and nearby communities always give me love, support, and encouragement. I also thank the members of the Thai Student Association and all other friends at Raleigh for all friendship and help. I also thank Dr. Miklos Kiss for his love, encouragement, and help. I also wish to give a special word of thanks to my dear friend Barbara Bergman. As my sponsor, she was my family and made me feel at home in Raleigh.

Finally, I wish to thank the DPST project and the Royal Thai Embassy for their financial support of my education.

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ABBREVIATIONS and SYMBOLS

35S	cauliflower mosaic virus 35S promoter
aa	amino acid
AL1	viral replication protein gene
AR1	coat protein gene
A	antisense
bp	base pair
BLAST	basic local alignment search tool
CaMV	cauliflower mosaic virus
CbLCV	cabbage leaf curl virus
CH42	chlorata42, *sulfur* homolog gene from Arabidopsis
ChsA	chalcone synthase
Col	columbia Arabidopsis strain
ddm1	decreased DNA methylation
dpi	day post inoculation
dsDNA	double stranded DNA
dsRNA	double stranded RNA
GFP	green fluorescent protein
G-VIGS	geminivirus-induced gene silencing
HDGS	homology dependent gene silencing
IR-PTGS	inverted repeat-PTGS
miRNA	micro RNA
mom	morpheus'molecule
nt	nucleotide
ORF	open reading frame
PCNA	proliferating cell nuclear antigen;
PCR	polymerase chain reaction
Poly A	polyadenylated
PTGS	post-transcriptional gene silencing
PVY	potato virus Y
PVX	potato virus X
Rb	retinoblastoma
RdRP	RNA dependent RNA polymerase
RNAi	RNA interference
RT-PCR	reverse transcriptase-polymerase chain reaction
rbcS	riburose-1,5-bisphosphate carboxylase oxygenase
S	sense
sde	silencing defective
sgs	suppressor of gene silencing
siRNA	small interference RNA
som	somniferous
S-PTGS	sense transgene induced PTGS
ssDNA	single stranded DNA
ssRNA	single stranded RNA
su	*sulfur* gene

T-DNA	transfer DNA from *A. tumafacians*
TGMV	tomato golden mosaic virus
TGS	transcriptional gene silencing
TRV	Tobacco rattle virus
VIGS	virus-induced gene silencing
WDV	wheat dwarf virus
w.p.i.	weeks post inoculation
wt	wild type

Chapter 1: Introduction

Geminiviruses

Genome structure, host range, and insect vectors

Geminiviruses are a group of small DNA viruses characterized by twin icosahedral particle morphology. Based on molecular characterization, they are classified into three genera with respect to their genome structure, host range, and insect vectors [1-3]. The Mastreviruses have members consisting of one single-stranded circular DNA, infect monocot plants, and are transmitted by leafhoppers. This genus is typified by maize streak virus (MSV) and wheat dwarf virus (WDV). Curtoviruses also have a monopartite genome and are transmitted by leafhoppers, but infect only dicotyledonous hosts. Members of this genus include tomato leaf curl virus (TLCV) and beet curly top virus (BCTV). The third genus, the Begomoviruses, have members consisting of two single-stranded circular DNA genomes. They infect a wide range of dicotyledonous plants and are transmitted by whiteflies. Examples of this genus include African cassava mosaic virus (ACMV), bean golden mosaic virus (BGMV), tomato golden mosaic virus (TGMV), and cabbage leaf curl virus (CbLCV) [1-4]. The Begomoviruses have been widely studied as models for replication and transcription [3]. Several geminiviruses cause major crop losses in many counties around the world.

Geminiviruses are now being used to study virus/host interactions, virus movement, and, recently, gene silencing. This dissertation thesis focuses primarily on gene silencing mediated by two members of the Begomoviruses: TGMV and CbLCV.

Begomoviruses: Tomato Golden Mosaic Virus (TGMV) and Cabbage Leaf Curl Virus (CbLCV)

TGMV and CbLCV infect a number of dicotyledonous plants and are transmitted by whiteflies. They both have a bipartite genome consisting of two circular, single-stranded DNAs designated as the A and B components as shown in Fig. 1. These two components are similar in size, about 2.6 kb. TGMV and CbLCV share the same genome organization, but they are dramatically different at the amino acid sequence level. Overall, CbLCV has 60% homology in amino acid sequences to TGMV for the AL1 gene or has an average of 69% homology for the products of three genes AL1, BL1 and BR1 [5]. TGMV infects many crops of the family Solanaceae such as petunia and tobacco [6], while CbLCV has a distinct host range and infects a variety of Brassicaceae including cabbage and Arabidopsis [7]. They both infect *Nicotiana benthamiana*.

The cloning of the TGMV and partial sequence of CbLCV genomes were first reported by Bisaro et al. [8] and Abouzid et al. [9], respectively. In TGMV, both A and B components share a ~200 base pair common region (CR). The common region is highly conserved between the two genomes but not conserved between viruses, and contains the viral origin of replication and transcriptional elements [10]. In contrast, the common regions of the A and B components of CbLCV are not identical and share only 80% sequence identity [7].

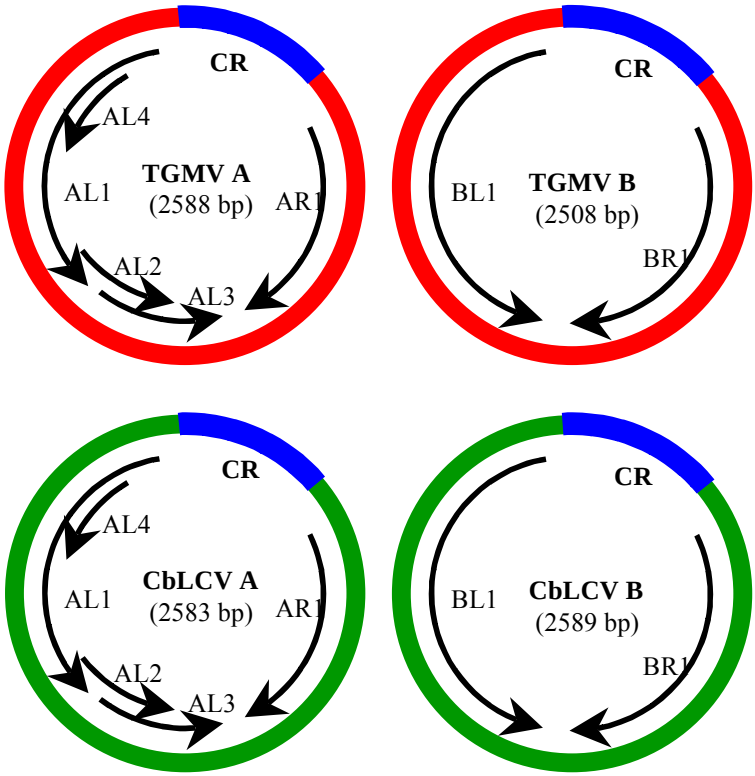

Figure 1. TGMV and CbLCV genome. TGMV and CbLCV both have two components, designated as A and B that are similar in size. Common region (CR) is indicated by blue boxes. The CR is conserved between the A and B genome, but varies between viruses. The A component encodes genes needed for replication, gene expression, and encapsidation. The B component contains two proteins needed for viral movement and symptom development.

Viral proteins and their functions

TGMV and CbLCV each have seven open reading frames (ORF). The A component encodes four leftward overlapping genes: AL1, AL2, AL3, AL4, and one rightward transcribed AR1 gene. AL1 is the only essential protein required for DNA replication in the presence of host proteins [6, 11]. It is a multifunctional protein and binds specifically to sequences in the common region that are conserved among A and B components [12, 13].

The AL3 protein is not required for TGMV replication but greatly enhances viral accumulation in infected plant cells. The disruption of the AL3 ORF results in a reduction of

single stranded DNA (ssDNA) and double-stranded dsDNA (dsDNA) accumulation, and there is a delay and attenuation of symptoms [14]. Evidence that both AL1 and AL3 are localized in plant nuclei of infected cells [15] suggests that AL3 might interact with AL1 in the replication process.

In vitro and in vivo mutagenesis of the *AL4* gene showed it was not essential for normal infection [6, 16]. The product of the *AL2* gene is required for virus infection. However, mutations in AL2 totally abolished systemic spread and symptom production without affecting viral replication [6, 14]. The AL2 protein itself transactivates transcription of the AR1 and BR1 genes of TGMV [17].

The coat protein, which is required for ssDNA packaging and transmission by insect vectors, is encoded by the AR1 ORF in the rightward direction from the common region. AR1 deletions attenuate symptoms of TGMV but viral infection is not prevented [18]. Therefore the AR1 is not essential for viral DNA replication or systemic spread. In both BGMV and TGMV, ssDNA accumulation was reduced upon the mutation of the AR1 gene [19, 20].

The BR1 and BL1 proteins encoded by the B genome are involved in symptom formation and systemic infection [21]. They both are involved in cell-to-cell spread that may occur though plasmodesmata and, in long distance transport, vascular tissues [20, 21].

Viral DNA replication and transcription

Geminiviruses replicate via double-stranded DNA intermediates in plant nuclei and induce host factors for their replication machinery [3, 22]. Once viral DNA is in a plant nucleus, a circular double-stranded DNA is formed using the original single strand (ss) viral DNA as a template for complementary strand synthesis. Rolling circle replication, a

mechanism common to bacterial ssDNA coliphage such as PhiX174 and M13, is used by geminiviruses to produce large amount of ssDNA. The viral strand DNA synthesis is initiated by a site-specific nick of the dsDNA template, while the complementary strand synthesis is generated by an unknown mechanism [1, 3, 22]. Rolling circle replication produces high viral DNA copy numbers per cell although it is not known how this level is regulated [23].

The mRNAs of geminiviruses are transcribed bidirectionally from multiple initiation sites in or near a common region (CR) and are polyadenylated. This results in multiple overlapping RNA species, and polycistronic RNAs are produced. For example, six RNAs are transcribed from TGMV A templates [3, 11].

Viral vectors and vector delivery

Geminiviruses have advantages as expression vectors for many reasons. First, they have small genomes that make them easily clonable. Secondly, vectors based on geminiviruses replicate to high copy numbers in infected cells, which can lead to high RNA expression. Thirdly, the coat protein, not needed for virus infection and movement, can be replaced with a foreign gene while maintaining the strong coat protein promoter which can drive expression of the new gene or RNA. Since geminiviruses replicate in plant nuclei but do not integrate into chromosomal DNA, this facilitates studies of DNA and chromatin [1, 2, 23, 24].

The A component of bipartite viruses encodes all the viral proteins required for replication, transcription, and encapsidation, while the B component contains proteins needed for viral movement and symptom development. The two components have to be co-infected for systemic spreading of the virus. The B genome cannot replicate in host cells without A

component proteins, while the A genome can autonomously replicate but cannot move without the B component proteins [2, 6].

Delivery of geminiviral vectors into plants is accomplished in many different ways. For example, mechanical transmission can occur using the sap of infected plants or vector DNA. Agroinfection, which involves T-DNA mediated transformation of the viral genome into plant cells, can also be employed. Finally, direct gene transfer technologies such as electroporation or particle gun bombardment has been used for transfection of protoplasts or intact plants [1, 2].

Virus induced gene silencing (VIGS)

Gene silencing: TGS and PTGS

Gene silencing is a homology-dependent process that causes the reduced expression of a gene. This phenomenon results in little or no phenotypic expression and very low mRNA levels of a gene or RNA sequence that was formerly expressed, or under non-silencing conditions would be expressed. It is found among species including plants (e.g., co-suppression), fungi (e.g., quelling), and animals (e.g., RNA interference) [25, 26]. In general, gene silencing is characterized into two classes: Transcriptional gene silencing (TGS) and post-transcription gene silencing (PTGS). TGS occurs at the DNA level and is characterized by changes in chromatin structure that reduce or prevent transcription. TGS could be the result of methylation or changes in chromatin associated proteins. There is evidence that the initiation of TGS may result from DNA pairing [27] or from RNA [28]. PTGS is a process that reduces cytoplasmic RNA levels and can be distinguished from TGS by nuclear run-on transcription assays of isolated nuclei combined with northern blot assays of cytoplasmic

RNA. If transcription occurs but transcripts fail to accumulate, PTGS is assumed while TGS has no transcription in the nucleus and no RNA accumulation in the cytosol [29-31].

PTGS is correlated with a lack of RNA accumulation in the cytoplasm. This phenomenon was first described in plants by a few research groups [32, 33]. Both groups were attempting to create petunias with darker purple flowers by over-expressing the gene coding for chalcone synthase (*chs*), an enzyme involved in anthocyanin pigment production. Surprisingly, the result was the opposite of what was expected. Instead of producing deeper purple flowers, many of the transgenic plants had white, pigment-free flowers, and others had flowers with white-purple patterns. They were surprised to find that, despite the strong promoter, the *chs* transgene was not expressed and the endogenous *chs* gene was also silenced in the white flower tissues. This phenomenon is now known as PTGS, in which silenced genes are still actively transcribed but the messenger RNAs are degraded before they can be translated into proteins. Since then, similar PTGS-like mechanisms are found in diverse organisms.

Gene silencing can be induced by many different sources including expression of a transgene, inverted repeats that produce dsRNA, or by transient expression from DNA and RNA viral vectors [26, 34]. Plants with multiple copies of the transgene are more likely than plants with a single copy to exhibit gene silencing [35]. However, the correlation has not been consistent for some systems. For example, plants with a single copy of transgene also exhibited gene silencing [36, 37]. Several studies have indicated that methylation of DNA is associated with gene silencing. For example, TGS correlates with methylation in the promoter, while PTGS correlates with methylation in the coding region [38, 39]. Gene silencing also occurs in organisms that lack DNA methylation, such as *Drosophila* [40].

Furthermore, PTGS and in some cases TGS are associated with accumulation of small RNAs (21-25 nucleotides) [41-43]. Therefore, there may actually be some underlying common mechanism between these two phenomena [43, 44].

Several methylation-deficient mutants have been identified in which TGS is inactive. These include *ddm1*, *som,* and *met1*. The DDM1/SOM locus encodes a protein belonging to the SWI2/SNF2 family that plays a role in chromatin remodeling [45, 46], while MET1 encodes a methyltransferase that is required for maintenance of methylation [47]. Another TGS mutant is *mom*. This locus was mapped to a gene required for maintenance of TGS, but there was no alteration in the methylation pattern of the transgene locus. MOM encodes a protein with no homology to any known proteins but has a region related to the SWI2/SNF2 family [48]. Morel et al. [49] reported that the *ddm1* and *met1* mutations not only affected TGS, but also PTGS. They concluded that DNA methylation does seem to play a major role in the TGS and PTGS silencing pathway, however, its function is far from clear.

The loss of gene expression can also occur if dsRNAs are present in cells. This experiment was first demonstrated in animal cells where dsRNA was injected into *C. elegan*. Subsequent degradation of a gene homologous to the dsRNA was observed [50]. In plants, dsRNA is thought to be produced by introduction sense/antisense RNA. Waterhouse and coworkers [51] demonstrated that there was increased silencing in plants expressing genes in both sense and antisense orientation compared with plants expressing either sense or antisense alone. This result suggested that dsRNA is formed and possibly triggers the degradation of RNA and all homologous endogenous mRNA.

The mechanism of how RNA degradation occurs is not well understood. Waterhouse et al. [51] suggested that dsRNA may serve as a template for RNA dependent RNA

polymerase (RdRP) to produce complementary RNA (cRNA) and this cRNA will hybridize with mRNA of transgene and endogenous gene, and RdRP will cleave all homologous mRNA. The discovery of RdRP in plants, and of mutants in RdRP that are defective in silencing, has supported this model. Despite the unclear nature of the genetic mechanism of dsRNA-induced gene silencing, Chuang and Meyerowitz [52] successfully used dsRNAs as a way to trigger gene silencing in Arabidopsis. By designing a plasmid construct carrying inverted repeats of a gene that can produce a hairpin dsRNA structure in plant cells, they successfully downregulated four genes that are needed in flower production and the phenotypic results were the same as the phenotypes created by antisense suppression or mutagenesis.

Virus induced gene silencing (VIGS) is another type of homology dependent RNA degradation phenomena. Plants infected with viruses carrying fragments with sequence homology to plant genes, cause RNA degradation of all homologous RNA. VIGS will be described in detail later in this chapter.

PTGS: Models and Mechanisms

Researchers have previously proposed three major models for post-transcriptional gene silencing: 1) Threshold model, 2) Aberrant RNA model, or 3) Ectopic pairing model [29, 53, 54]. Threshold models propose that gene silencing is a response to inappropriately high levels of expression of the targeted gene. This hypothesis is based upon the relationship between the increased number of gene copies and silencing. Aberrant RNA models propose that PTGS is activated by the presence of RNA that is somehow different from most other RNAs (aberrant RNAs) [54]. The nature of these aberrant RNAs is also a matter of debate, but they might include double-stranded sequences or antisense RNAs. This model postulates

that aberrant RNAs (either antisense or dsRNAs) are formed and recognized as duplex substrates for RNase degradation. Alternatively, dsRNA may inhibit translation if they anneal to mRNA in the cytoplasm. Non-sense mediated decay or degradation may then follow [55].

The third model suggests that PTGS is triggered by a physical interaction or pairing between homologous DNA sequences or by DNA-RNA interactions. This model is based upon evidence that PTGS occurs in transgenic plants containing multiple copies of a transgene. So far it is known that more than one mechanism is likely to be involved for many PTGS events. For example, an ectopic DNA interaction might lead to transcription of aberrant RNA species that elicit the silencing response, or a threshold level of aberrant RNA may in turn be required to trigger silencing [54].

Table 1. Components of the PTGS pathway.

Proteins	*Arabidopsis*	*C. elegan*	*N. crassa*
RdRP	SGS2/SDE1	EGO1	QDE1
Translation initiation factor	AGO1	RDE1	QDE1
RecQ DNA helicase			QDE3
RNA helicase	CAF/SDE3		
RNaseD	CAF	Mut7	
Chromatin remodeling/methylation	DDM1/MET1		
Dicer		DCR1	

Sources: Fagard et al. [56] and Voinnet [26].

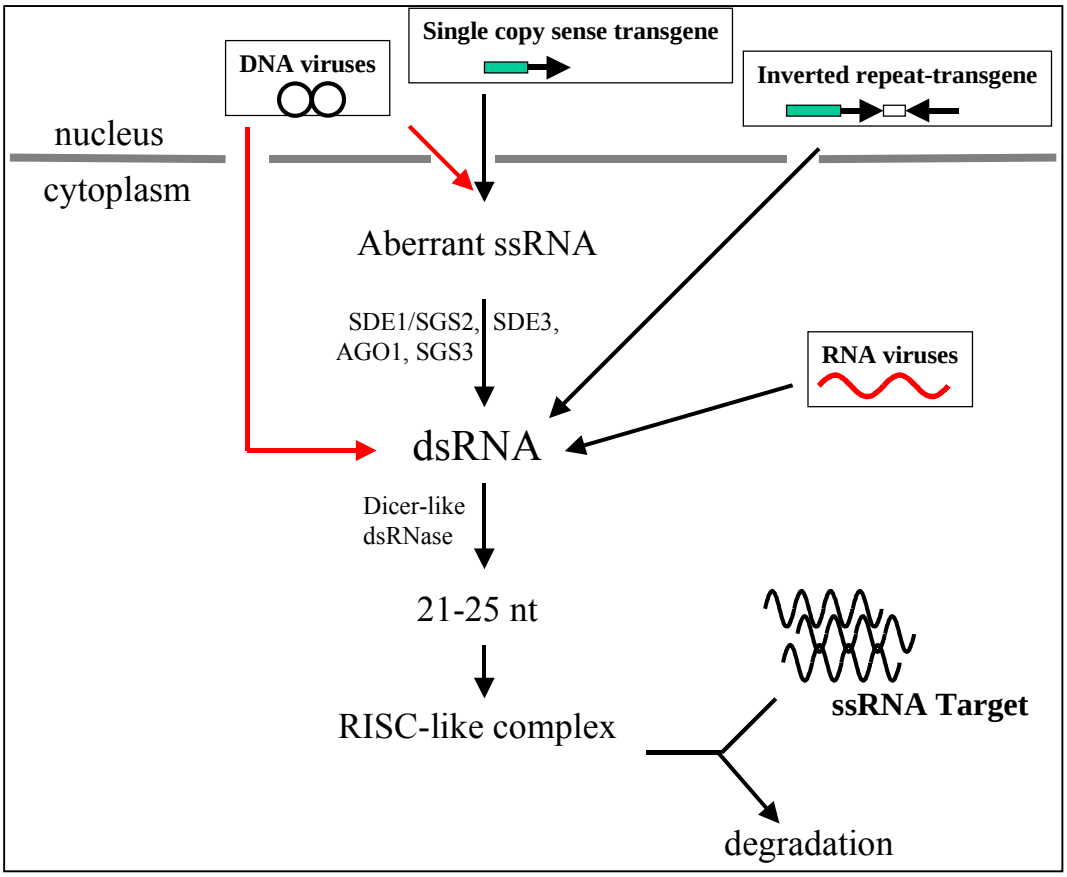

Figure 2. A model of PTGS in plants. Transgene loci arranged as inverted repeats or RNA viruses can directly produce dsRNAs. dsRNAs could also be synthesized by a cellular RdRP (SDE1/SGS2) companied with SDE3, SGS3 and AGO1 using aberrant ssRNAs as templates derived from either DNA viruses (e.g. geminiviruses) or highly transcribed single copy transgene loci. The dsRNAs from either these sources can be cut by RNases (e.g. DICER-like) generating small RNA 21-25 nucleotides of both polarities. These small RNAs are then incorporated into a RNA-induced silencing complex (RISC), multicomponent ribonuclease, and act as guides for sequence specific degradation of homologous target RNAs (Adapted from Matzke et al. and Voinnet et al. [31, 57].

The phenomenon of PTGS is functionally equivalent to RNAi in animals and quelling in fungi. Genetic and biochemical analysis of PTGS, RNAi, and quelling have demonstrated that these phenomena are derived from an ancestral mechanism [58]. Similar genes were identified among species, such as RdRP, translation initiation factor, and an RNase D-like protein (Table 1) [57, 58]. It is generally agreed that dsRNA is a potent trigger of gene silencing in a wide range of organisms. How dsRNAs are produced is still not completely

understood, but the finding that a gene encoding an RdRP required for PTGS [59, 60] suggests that this enzyme is involved in the dsRNA production process, supporting the hypothesis previously proposed by Waterhouse et al. The dsRNAs are probably recognized as a template and then are degraded into small 21-25 nt RNAs that are incorporated into an RNase complex. The RNase complex can then target homologous RNAs for degradation [26, 31, 57] (Fig. 2). Despite the presence of small dsRNAs in several species exhibiting PTGS, the relationship of these small dsRNA fragments to the biological functions of PTGS is unclear. How abundant are these RNA fragments and how are they generated? Are they silencing signals?

Gene silencing induced by RNA and DNA viruses

PTGS mediated by viruses can occur with DNA viruses, which replicate in the nucleus, and with RNA viruses, which replicate in the cytoplasm (Table 2). Infection of *N. benthamiana* by PVX, a single–stranded RNA potexvirus, led to virus replication and accumulation of viral RNA in the cytoplasm. Infection of recombinant PVX virus carrying the coding sequence of the phytoene desaturase (PDS) gene in either sense or antisense orientation led to PTGS of the endogenous PDS gene. In this case, PDS RNA and PVX-PDS both were degraded [61]. It was also reported that infection of transgenic *N. benthamiana* plants expressing a p35S-GFP transgene by a recombinant PVX virus carrying a coding sequence of the GFP gene in either sense or antisense orientation led to degradation of GFP transgene mRNA. In this case both, endogenous GFP and PVX-GFP RNA were efficiently and uniformly degraded [39, 61].

Silencing induced by a DNA virus was first reported by Kjemtrup et al. [62] and Atkinson et al. [63]. Tomato Golden Mosaic Virus (TGMV) was engineered to carry gene

fragments with homology to either transgene or an endogenous gene in plants. The genome of TGMV was modified and the coat protein (CP) was replaced with foreign DNA fragments from the firefly luciferase gene (luc) or an endogenous plant gene involved in chlorophyll biosynthesis (magnesium chelatase, *su*). *N. benthamiana* plant infected with TGMV::su exhibited leaves with yellow sectors, caused by a lack of chlorophyll. The loss of chlorophyll was observed in plants infected with TGMV carrying both 5' and 3' fragments or expressed in sense or antisense orientation. Transgenic plants expressing the luciferase gene showed little or no luminescence in systemically infected leaves after TGMV::luc inoculation. Transcripts corresponding to *su* or luc were greatly reduced in plants, while the tobacco elongation initiation factor 4A and histone transcripts remained unchanged, demonstrating that gene silencing had occurred [62]. Atkinson et al. [63] reported that transgenic petunia plants expressing a CaMV 35S promoter-driven chalcone synthase A (*ChsA)* gene from DNA replicon derived from tomato yellow dwarf virus (TYDV) produced flowers with white sectors. Steady stage levels of *ChsA* RNA from both the *ChsA* transgene and endogenous were downregulated in white flowers. This result suggested that *ChsA*, a key enzyme in anthocyanin biosynthesis, was silenced or inactivated.

Like PTGS, a TGS-like mechanism can be triggered by viruses. TGS mediated by DNA viruses has been reported in transgenic *Brassica napus* plants expressing a p35S-GUS transgene [64]. Plants inoculated with cauliflower mosaic virus (CaMV) reduced viral accumulation in systemically infected leaves resulting from the homology of the viral and transgene promoter region. In this case, however, PTGS of the 35S RNA from CaMV also was seen.

Table 2. Virus-induced gene silencing in plants.

	Group	Plant species	Target nuclear genes	References
I. RNA viruses				
TMV	Tobamovirus	*N. benthamiana*	Phytoene desaturase	Kumagai 1995
PVX	Potexvirus	*N. benthamiana*	Phytoene desaturase, GFP transgene	Ruiz 1998
			cellulose synthase	Burton 2000
TRV	Tobravirus	*N. benthamiana*	Phytoene desaturase,	Dalmay 2000
			Rubisco small subunit, LEAFY homologue, GFP transgene	Ratcliff 2001
		Arabidopsis	Phytoene desaturase, GFP transgene	
TEV	Potyvirus	*N. benthamiana*	TEV coat protein transgene	Lindbo 1993
PsbMV	Potyvirus	*Pisum sativum*	PSbMV replicase transgene	Jones 1998
PPV	Potyvirus	*N. benthamiana*	PPV replicase transgene	Guo 1999
II. DNA viruses				
*TYDV	Geminivirus	*Petunia hybrida*	Chalcone synthase transgene	Atkinson 1998
TGMV	Geminivirus	*N. benthamiana*	Magnesium chelatase, luciferase transgene,	Kjemtrup 1998
			GFP transgene, PCNA	Peele 2001
CbLCV	Geminivirus	Arabidopsis	Chlorata42, GFP transgene	Turnage 2002

Source: Voinnet [57] and Turnage et al. [5]
* VIGS using DNA replicons

The mechanism leading to the establishment of VIGS has been widely investigated. Our best understanding comes from RNA virus studies. PTGS can suppress the expression of transgene or endogenous genes, and also cause the degradation of the triggering viral RNA vector [65]. It is thought that VIGS occurs because of factors such as multiple copies of transgene, double-stranded RNA production, or ectopic pairing of DNA. It could also be that aberrant RNAs are produced as the result of modification to chromatin structure and/or DNA methylation [56, 66].

A striking aspect of gene silencing is the involvement of diffusible signals. In several silencing systems, grafting of a non-silenced scion onto a silenced stock caused the scion to become progressively silenced [67]. A similar feature is also observed in which a single leaf of GFP transgenic plants was challenged with constructs carrying GFP sequences via *Agrobacterium* infiltration or biolistic bombardment of the homologous DNA. Silencing spreads so that the whole plant exhibits the silencing phenotype, loss of green fluorescence [68, 69]. This result provides evidence that the silencing signal which probably includes RNA can be transmitted cell-to-cell, and perhaps the phloem for long distance transport [70].

Interestingly, VIGS against a transgene is somehow different from VIGS of an endogenous gene. VIGS of a GFP transgene has a virus-independent maintenance stage while VIGS of endogenous PDS could not be initiated and maintained without virus replication [61]. Additionally, Jones et al. [39] reported that methylation was associated with VIGS of a transgene of GFP when transgenic plants expressing GFP were infected with a PVX vector carrying the GFP coding region. In contrast, no methylation was detected with VIGS of an endogenous plant gene, ribulose-1, 5-bisphosphate carboxylase oxygenase (rbcS).

Contrast between RNA and DNA viruses

Even though gene silencing effects can be triggered by both RNA and DNA viruses, it is thought that mechanisms mediated by these two types of viruses are somehow different. Here, we are interested in RNA viruses (e.g., potexviruses and poteyviruses) and DNA viruses (e.g., geminiviruses). Several different features exist between these viruses. First, most RNA viruses replicate in the cytoplasm while DNA viruses replicate in plant nuclei. Secondly, the RNA viral genome replicates through an RNA intermediate and uses its own

polymerase such as RdRP. In contrast, geminiviruses (although not CaMV) replicate via dsDNA intermediates in plant nuclei and, probably, use host proteins for their replication machinery including DNA polymerase delta and PCNA [66, 71]. Owing to the different replication strategy of the DNA and RNA viruses, the silencing initiation and consequences of viral degradation might be different [66].

Anti-silencing

Many RNA viruses including potyviruses, cucumoviruses, and potexviruses encode proteins that can suppress gene silencing. Anti-silencers were discovered by experiments in which silenced transgenes in plants were reactivated after virus infection or after introduction of genes encoding candidate suppressor proteins. Voinnet et al. [72] showed that the spatial pattern and degree of suppression varied extensively between viruses. Several suppressors have been reported, including HC-Pro, 2b, p19, p25, and more. HC-Pro is a viral movement protein required for long-distance movement though the phloem and for maintenance of genome amplification and is encoded by tobacco etch virus (TEV). Transgenic plants expressing HC-Pro showed reversal of either transgene or endogenous gene silencing [73]. Small RNA species associated with PTGS were also absent in these transgenic plants, suggesting that HC-Pro targets a step probably upstream of small RNA production [74]. HC-Pro also interacted with a calmodulin-related protein, rgs-CaM, a host protein that itself functions as an RNA silencing suppressor [75]. This suggests that HC-Pro interacts with host proteins for silencing suppressor machinery.

The cucumber mosaic virus (CMV) 2b protein is also a movement protein located in a plant nucleus and functions in the long-distance movement of the virus. Infection of plants containing a silenced GFP reporter transgene with CMV resulted in silencing suppression,

but only in newly emerged tissues that developed after infection. It is thought that 2b may target a different step in the silencing pathway, either an initiation or a signaling step [76]. The 19 kDa (p19) and 25 kDa (p25) movement proteins of the PVX potexvirus and of tombusviruses also blocked silencing induced by a transgene in similar manner to the 2b protein, but not by virus infection. P19 and P25 possibly inhibit a step involved in the systemic signaling pathway [77, 78].

DNA viruses also have anti-silencing properties. Turnage, M. (unpublished results) showed that GFP silenced *N. benthamiana* plants inoculated with TGMV showed GFP fluorescence, but only in new growth 3 weeks post inoculation. The same result was observed in GFP silenced Arabidopsis plants that were inoculated with CbLCV. These results imply that both TGMV and CbLCV have an anti-silencer (not yet known) that targets some part of the silencing pathway.

PTGS and plant defense response

The association between PTGS and virus resistance comes from several experiments. First, transgenic plants expressing a viral coat protein transgene were not susceptible to virus infection anymore [79]. Transgenic plants expressing the coat protein of tobacco etch virus (TEV) developed symptoms of virus infection on the inoculated leaves but symptoms did not develop in systemic leaves. The systemic leaves became resistant to secondary infection with viruses related or closely related, and RNA virus level declined. It was proposed that a cytoplasmic RNA degradation mechanism was induced by virus infection [79]. A similar conclusion was drawn from a genetic analysis of transgenic tobacco plants expressing the RdRP of PVY [80]. Secondly, many silencing mutants of Arabidopsis were hypersusceptible to infection by some viruses (CMV) such as *sde1/sgs2*, *sde3, sgs3,* and *ago1* [59, 60].

Moreover, evidence that many viruses contain a silencing suppressor such as HC-Pro, 2b, p19, and p25 support the idea that PTGS is a major anti-virus mechanism.

Although it is becoming clear that PTGS serves as a natural defense mechanism against viruses, some researchers think that plants may use gene silencing as a more general mechanism to regulate the expression of endogenous genes [54, 72]. This hypothesis is supported by evidence that a genetic mutant, *ago1*, that interferes with PTGS also showed developmental abnormalities and was unfertile [81]. Recently, MicroRNAs (miRNAs), numerous small ~21-24 nucleotide RNAs, similar to small interfering RNAs (siRNAs), were described in many organisms including Arabidopsis, *Drosophila*, *C. elegans*, and human [82-84]. Llave et al. [82] showed that most or all of the miRNAs arises from structural precursor RNA, from intergenic regions (IGRs) and some from coding sequences and transposon elements. Additionally, it has been reported that a group of miRNAs were involved in the regulation of developmental timing of gene expression of the *C. elegans* [85]. miRNAs and siRNAs both were associated with the down regulation of gene expression, therefore they are likely to function in a similar manner [83, 84]. However, whether these small RNAs indeed serve a broader gene regulatory purpose in plants remains to be determined.

Thesis summary

Geminiviruses replicate in plant nuclei and induce host proteins for their own DNA replication processes. So far, only one essential host protein has been identified. PCNA, an auxiliary factor for DNA polymerase δ, was induced in TGMV infected mature leaves of *N. benthamiana* and in transgenic plants expressing AL1 protein [15]. An attempt to identify genes required for viral DNA replication was initially done (Eagle and Robertson,

unpublished data). Whether these genes are required for viral replication remain to be determined.

Chapter 2 describes experiments optimizing TGMV as a silencing vector and its use as a potential screen for genes associated with viral DNA replication. If a virus silences an essential gene needed for DNA replication, systemic infection will be attenuated or prevented. One candidate gene, clone 9, was tested for its potential role in viral DNA replication. Viral accumulation in systemic leaves of *Nicotiana benthamiana* was prevented in plants inoculated with TGMV carrying a small fragment of this gene.

In later chapters, experiments were done using Arabidopsis as a plant system and CbLCV as a silencing vector. Arabidopsis offers many advantages for this study, such as the small size and space requirement of the plants, its short generation time, its complete genome sequence, and the availability of numerous mutations. Here, an investigation on the mechanism of geminivirus induced gene silencing is described.

Chapter 3 compares gene silencing systems mediated by two viruses: TGMV and CbLCV. TGMV B has been successfully used as a vector to silence endogenous genes (from Chapter 2). The B component of CbLCV was tested for a potential silencing vector. We found that the CbLCV B vector was not as effective as the CbLCV A vector. This suggests that different versions of silencing vectors derived from Begomoviruses need to be tested for optimal silencing.

In Chapter 4, several Arabidopsis silencing mutants were tested for CbLCV mediated gene silencing. These mutants are defective in PTGS of transgene or endogenous genes. We are interested in identifying which genes are required for the geminivirus-mediated silencing pathway. Our results showed that several genes are required in the pathway. Interestingly,

some of these genes, which are not required for silencing induced by RNA viruses, are required for the geminivirus silencing pathway suggesting that RNA and DNA viruses interact differentially with the plant silencing machinery. We conclude that multiple silencing pathways exist.

Chapter 5 describes a preliminary experiment. Here, VIGS was used as a tool to suppress plant gene expression. Three candidate genes were tested for their potential roles in viral DNA replication: calmodulin-related protein (CaMRP), *A. thaliana* g1-related protein (AtG1RP), and proliferating cell nuclear antigen (PCNA). Arabidopsis seedlings were inoculated with a recombinant virus carrying a fusion of *CH42* gene and one cDNA fragment of those three genes. Silencing phenotypes varied among these genes, as well as DNA viral accumulation levels. Two candidate genes associated with the silencing pathway were also tested: SGS2 (SDE1) and SDE3. We found that only downregulation of the SGS2 gene by VIGS inhibited silencing.

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Chapter 2

A Silencing Assay for a Gene Associated with Geminivirus Replication

Nooduan Muangsan performed all the cloning and testing of clone 9 except PCR-based subtraction and RNA blot analysis.

Key Words: *Nicotiana benthamiana*, virus-induced gene silencing, AL1-upregulated genes, viral DNA replication

Summary

Geminiviruses replicate in mature cells by inducing host proteins for DNA replication. AL1 is the only virally encoded protein absolutely required for viral DNA replication and is sufficient for proliferating cell nuclear antigen (PCNA) induction in differentiated cells. To identify other host genes required for viral DNA replication, PCR-based subtractive hybridization was performed. RNA from mature leaves from the 35S-AL1 transgenic plants were subtracted against leaves of similarly grown wild type *Nicotiana benthamiana* plants. One of the clones, Clone 9 showed high homology to an unknown protein of Arabidopsis and significant similarity to a putative transcription factor, the goliath g1 protein *Drosophila melanogester.* The mRNA level of Clone 9 was increased in 35S-AL1 transgenic tissue and TGMV-infected tissue, but was very low or undetectable in mature leaf tissues of nontransgenic plants. To determine if Clone 9 was required for TGMV DNA replication, geminivirus mediated gene silencing was used to downregulate Clone 9 expression. Virus replication can be prevented if the virus silences a gene needed for its own replication. Short fragments of Clone 9 carried by TGMV dramatically reduced viral DNA accumulation in most plants. This suggests that Clone 9 is needed for TGMV infection, and may be needed for virus DNA replication or movement.

Introduction

Geminiviruses are single-stranded DNA viruses which replicate via double-stranded DNA intermediates in plant nuclei. A small number of viral proteins are encoded in the geminiviral genome, therefore they rely on host proteins for their own DNA replication processes [1, 2]. Nagar and colleagues [3] showed that the geminivirus tomato golden mosaic virus (TGMV) induced the accumulation of proliferating cell nuclear antigen (PCNA), an accessory protein of DNA polymerase δ, during DNA replication in differentiated plant cells. This finding motivated the investigation of other host factors that would be required for viral DNA replication in mature plant cells.

The geminivirus TGMV has a bipartite genome of two circular molecules designated the A and B components (TGMV A and TGMV B), each of which is ~2.5 kb in size. TGMV A is capable of replication in the absence of TGMV B while TGMV B relies on TGMV A proteins for its replication [2, 3]. The TGMV A component encodes five proteins: AL1, AL2, AL3, AL4, and AR1. AL1 and AL3 are needed for viral DNA replication whereas AR1 codes for coat protein and is required for insect transmission [4]. The *AL4* gene was not essential for normal infection [4, 5]. The product of the *AL2* gene is required for virus infection. Mutation of the AL2 totally abolished systemic spread and symptom production without affecting viral replication [5, 6]. The AL2 protein itself transactivates transcription of the AR1 and BR1 genes of TGMV [7]. The BR1 and BL1 proteins encoded by the B genome are involved in symptom formation and systemic infection [8]. They both are involved in cell-to-cell spread which may occur though plasmodesmata and, in long distance transport, vascular tissues [8, 9].

AL1 is the only essential protein for TGMV DNA replication. It is a 40-kDa, multifunctional protein containing several domains: a DNA binding domain, a DNA cleavage/ligation domain, and a protein oligomerization domain [2, 10]. The AL1 protein binds specifically to sequences in the common region that are conserved between TGMV A and TGMV B components [11]. Several studies have shown that TGMV infection and the AL1 protein interact with cell cycle regulatory proteins, causing plant cells to enter S phase. First, many TGMV infected cells and some cells from the 35S-AL1 transgenic plants contained large nuclei [3]. Chromatin condensation which is characteristic of early mitotic prophase occurred in a high proportion of TGMV infected cells ([12]; Nagar, S. unpublished). Secondly, transgenic plants expressing AL1 protein under the CaMV 35S promoter complemented TGMV infection with a mutation in AL1 when co-inoculated with the B component [13]. Additionally, this AL1 transgenic plant can induce PCNA accumulation in plant cells without the presence of other viral proteins [3]. Finally, TGMV AL1 and another related Rep A protein of wheat dwarf virus (WDV) can interact with a plant homologue of pRb (retinoblastoma related protein) [14, 15]. Mammalian viruses encode proteins that target pRb and stimulate quiescent cells to reenter the cell cycle [16]. Based on these findings, it is suggested that AL1 might use the same interaction machinery as mammalian oncoproteins to cause plant cells to reenter S phase.

In plants, DNA replication plays a central role in plant growth and development, but molecular components required for this process are not well identified. As already mentioned, viral replication depends on cellular DNA replication proteins, however, the molecular basis for linking viral DNA replication and the induction of cellular DNA

replication remains to be elucidated. Geminiviruses thus serve as a model system for understanding plant DNA replication machinery.

Because of the significant involvement of AL1 in TGMV infection and host induction, and because AL1 is the only viral protein required for viral DNA replication in the presence of host factors, we asked whether AL1 induces other host factors for viral DNA replication. We used gene silencing mediated by geminiviruses as a tool to create a specific gene "knock out" in plants. We then assayed for viral replication in the silenced plants. We show here that a short fragment of clone 9 dramatically reduced viral DNA accumulation levels in upper leaves of most plants, and minimal or undetectable symptoms were observed, while the silencing of *su*, which is not required for viral replication [17], had little effect on viral DNA accumulation.

Results

Identification of AL1-upregulated genes

AL1 itself can induce an accumulation of PCNA in transgenic plants expressing AL1 protein under the constitutive 35S promoter [3] suggesting that AL1 may also induce other host proteins for viral DNA replication. To identify genes upregulated by AL1, PCR-based subtractive hybridization was carried out to obtain differentially expressed genes between two populations, the 35S-AL1 transgenic and nontransgenic tissues (Eagle and Robertson, unpublished). The outline of PCR-based subtraction hybridization (as shown in Fig. 1) was performed according to the manufacturer's instructions using a PCR-select kit (BD Bioscience, USA). Several cDNA clones from the subtracted library were sequenced (Eagle, unpublished). Homology searches were performed using the BLAST programs against the

non-redundancy (nr) database [18]. A partial DNA sequence from each cDNA clone was used as a query for amino acid sequence similarity to sequences deposited at GenBank. Several cDNA clones were identified that could be upregulated in mature leaves of the 35S-AL1 transgenic plants (see Table 1).

My studies initially focused on genes that have homology to known proteins, and northern blot hybridization was performed to confirm whether these genes were upregulated in the 35S-AL1 transgenic tissue or their expressions were enriched in infected tissue. A few of the cDNA clones tested showed high levels of expression in the 35S-AL1 transgenic tissue and in TGMV-infected tissue (Muangsan and Eagle, data not shown). These included clone 9, clone 37, and clone 41 (see Table 1). Other clones were not upregulated and were therefore not studied further.

Clone 9 was particularly interesting for two reasons. First, it showed high homology to an unknown protein of *A. thaliana* containing similarity to a goliath regulatory protein from *D. melanogester*, which is a zinc-finger protein associated with mesoderm formation [19]. An examination of this clone revealed that the deduced amino acid sequences, encoded by a 961 bp partial sequence, shares 41% amino acid identity to an *Arabidopsis* protein (accession No. NP_5637171.1). The *Arabidopsis* ortholog of clone 9 is an expressed protein containing a zinc-finger motif (Fig. 2A) and shares amino acid sequence similarity to proteins from several organisms (see Table 2). Second, Northern blot analysis showed that clone 9 was expressed in meristematic tissue, not in mature leaf tissue, but was also upregulated in TGMV-infected tissue and in the 35S-AL1 transgenic tissue (Fig. 2B). Based on these findings we anticipated that clone 9, a putative transcription factor, might play a role in regulating gene expression during DNA replication or cell cycle-associated processes.

Optimizing a silencing system using TGMV B as a cloning vector for downregulation of the gene expression

Gene silencing was used to create the loss-of-function of clone 9. The silencing system based on TGMV has been previously developed by Kjemtrup et al. [17] and Peele et al. [20] where it was shown that plant genes can be silenced upon infection by the virus carrying sequences homologous to endogenous plant genes. TGMV A or TGMV B can both be used as silencing vectors. However, silencing of *su*, the sulfur gene which is required for chlorophyll production, was more extensive if a TGMV B vector was used with a combination of wild type TGMV A [20], suggesting that TGMV B is a more efficient silencing vector. It has also been reported that TGMV vectors have a size constraint for foreign DNA insertion [21] and large DNA fragments inserted into the virus can be deleted. If deletions occurred randomly, some viral molecules might retain enough of the insert to propagate silencing. If deletions producing functional viral molecules were rare, it would not be possible to clone larger fragments and expect silencing to be propagated. To address this problem, TGMV B was tested for a size limitation for propagation of a foreign DNA insert. The *su* gene was used as a visual maker of gene silencing because downregulation of this endogenous gene is known to produce a visible phenotype, yellow tissue because of a lack of chlorophyll [17]. 455 and 935 bp fragments of a *su* cDNA (pLVN44) were cloned into TGMV B at an *Xba*I site, 20 bp downstream from a stop codon of the BR1 gene and before the polyadenylation site (Fig. 3), and co-inoculated with wild type TGMV A into intact plants. We found that only one of ten plants from TGMV::su455 and TGMV::su935 exhibited extensive silencing at 2 weeks post inoculation (w.p.i) compared with plants infected with TGMV B carrying a 154 bp fragment of *su* (Fig. 4A). The rest of the plants

showed minimal silencing with several yellow spots in inoculated leaves and white or yellow areas along the veins of systemically infected leaves. Minimal or undetectable symptoms were observed in these plants.

DNA gel blot analysis of genomic DNA isolated from infected plants at 2 w.p.i demonstrated that a deletion occurred in the TGMV B::su455-bp construct, reducing the size to less than that of the TGMV B::su154-bp construct (Fig. 4B top panel), and a shorter *su* fragment was strictly maintained (Fig. 4B bottom panel). Viral DNA accumulation in the other infected plants was greatly reduced suggesting that movement of the virus with large inserts is compromised and deletions do not rapidly occur in this system. However, a few plants showed extensive silencing in new leaves after 6 w.p.i. (Fig. 5A), and the PCR analysis of viral DNA isolated from these leaves revealed that a deletion had occurred so that the *su* insert size had decreased below 150 bp (Fig. 5B). The conclusion drawn from these results is that there is a narrow size range for insertion of foreign DNA into the B component, and that 150 bp is close to the optimal size.

Silencing of clone 9 resulted in the reduction of viral accumulation in systemically infected leaves

According to the above results, a small fragment of a gene should be used to obtain efficient silencing from the TGMV B vector. To determine if virus replication could be prevented in plants silenced with a clone 9 gene fragment, a 106 bp fragment with 96 nt of clone 9 ORF was inserted into a TGMV B vector and co-inoculated with TGMV A into *N. benthamiana* plants. If the *clone 9* is needed for TGMV replication, plants infected with the viral construct carrying this gene fragment should have less viral DNA accumulation. Silencing of the *su* gene, which is not needed for viral replication, was used as a visual

marker for symptoms, silencing spread and viral DNA accumulation. Plants infected with a geminivirus carrying the clone 9 cDNA fragment exhibited a striking feature of severe symptoms, especially at 4 w.p.i. in inoculated leaves, but minimal or no symptoms in systemic leaves (Fig. 6a, 6b and 6c). Fewer symptoms were produced in inoculated leaves of plants infected with the virus carrying a *su* gene fragment (Fig. 6g and 6h). Analysis of viral DNA isolated from inoculated leaves and systematic leaves of these plants at 4 w.p.i showed that viral DNA accumulation was dramatically reduced in systemic plant cells (Fig. 7 lane 4, 6, 8, 10, 12), while it was not affected in *su* silencing plants (Fig. 7 lane 2).

Systemic silencing of su was delayed or inhibited in plants infected with TGMV A::su/TGMV B::Clone 9

Silencing mediated by RNA viruses, e.g. PVX, is divided into two stages: initiation and maintenance. Initiation of transgene silencing requires viral replication while the maintenance stage is viral replication independent [22]. However, silencing of endogenous genes, e.g. PDS, was not maintained in the absence of the virus, so that both initiation and maintenance of endogenous gene silencing required viral replication [22]. Silencing of the endogenous *su* gene mediated by geminiviruses, which are DNA viruses, behaves similarly in that viral DNA replication and movement is required for extensive silencing in systemically infected leaves [20]. In *N. benthamiana* plants infected with TGMV A::su without TGMV B, systemic silencing of *su* was not observed (Peele, C. unpublished data). At the same time no silencing occurred if the plants were infected with TGMV B::su alone. This implies that initiation of silencing first requires viral DNA replication, and secondly, a silencing signal, probably RNA, requires proteins encoded by the TGMV B component for

long distance movement to recipient cells in systemically infected cells where viral replication and amplification of the silencing signal has taken place [20].

We hypothesized that systemic silencing of *su* would be affected if the virus could not replicate in systemically infected leaves. Systemic silencing was observed in plants infected with TGMV A::su as a coat protein replacement but was less extensive compared to silencing achieved by TGMV B::su [20]. The *su* silencing was monitored in plants infected with TGMV A::su/wt TGMV B, or with TGMV A::su/TGMV B::Clone 9. At 4 w.p.i., plants infected with TGMV A::su/TGMV B::Clone 9 showed severe symptoms in inoculated leaves but minimal symptoms in systemic leaves, similar to previous results with TGMV A/TGMV B::Clone 9 (Fig. 6d, 6e and 6i). Systemic silencing of the *su* gene in plants infected with TGMV A::su/TGMV B::Clone 9 was inhibited or delayed in systemically infected leaves, even though the initiation of *su* silencing did occur, demonstrated by the presence of many yellow spots in inoculated leaves in all infected plants (Fig. 6d, 6e and 6f). In contrast, systemic silencing of *su* was not delayed or inhibited in plants infected with TGMV A::su/TGMV B::PCNA or with TGMV A::su/TGMV B (Fig. 6f left and 6i left, respectively).

PCNA was induced in differentiated plant cells upon TGMV infection [3], and silencing of PCNA has been shown to greatly reduce primary growth and the reduction of viral DNA accumulation in new growth [20]. Systemic leaves from plants infected with TGMV A/TGMV B::Clone 9 or TGMV A::su/TGMV B::Clone 9 were healthy with minimal or undetectable symptoms (Fig.7j3 and 7j4) while the leaves from plants infected with wild type virus and TGMV A/TGMV B::su were mosaic for chlorosis, and yellow as a lack of chlorophyll, respectively (Fig. 6j1 and 6j2).

Discussion

Geminiviruses have provided an excellent system for understanding plant DNA replication and cell cycle regulation because they rely on host proteins for their replication machinery. The studies done by Nagar et al. and Egelkrout et al. [3, 23], showing PCNA induction upon virus infection, and expression in AL1 transgenic plants raised a question about other host factors that might be induced and required for viral replication. It is shown here that several genes were upregulated in transgenic plants expressing the AL1 protein by using a PCR-based subtractive hybridization technique, and that silencing of at least one gene caused the reduction of viral accumulation in systematically infected leaves.

The 35S-AL1 transgenic plants (generated by Hanley-Bowdoin et al.[13]) were used because of the significant involvement of AL1 as a viral replication initiation protein, and because AL1 alone can induce PCNA accumulation in differentiated cells of transgenic plants [3, 23]. TGMV-infected leaf tissue is a poor choice for starting material because of the complication of virus and host interactions. Not only genes activated by AL1 are obtained, but also several other defense genes and genes that interact with other virally encoded proteins would likely to be induced.

Several genes showed differentially expression in the 35S-AL1 transgenic plants. Surprisingly, none of the genes obviously indicated whether they belong to DNA synthesis or to the cell cycle related proteins. Many genes had homology to the chlorophyll a/b binding protein family or the photosynthesis-related proteins and some belonged to proteins involved in stress response. A few of these proteins were upregulated in the 35S-AL1 transgenic tissue (data not shown) indicating that AL1 might also have a role in a stress response or

pathogenesis response even though the 35S-AL1 transgenic plants were phenotypically normal. In fact, the 35S-AL1 transgenic plants were difficult to maintain [13]. Toxicity of virally encoded proteins has been reported in transgenic plants expressing other TGMV A proteins, e.g. AL2 [24].

AL1 is the only viral protein essential for DNA replication in the presence of host factors. It has shown that AL1 binds maize pRB, known as a cell cycle regulator, through a novel domain [14, 25]. It is possible that the binding between AL1 and a plant Rb homologue results in releasing of E2F transcription factor, therefore E2F is free to activate the transcription of genes which might be involved in cell cycle or DNA synthesis machinery. It is also possible that AL1 can bind other transcription factors, as yet unknown, involved in processes other than DNA replication. Therefore further investigation is needed.

To further demonstrate that the AL1-upregulated genes somehow participate in geminivirus replication, the loss-of-function of gene expression using gene silencing mediated by a geminivirus was used. A nuclear-localized DNA virus carrying sequence complementary to chromosomal genes can silence all copies of the chromosomal genes [17]. In plants, gene silencing mediated by geminiviruses and RNA viruses is classified as post-transcriptional gene silencing (PTGS) in which homologous RNAs, either plant RNAs or virus RNAs, are degraded after transcription. It is thought that the degradation process requires a double-stranded RNA (dsRNA) template for plant endonuclease digestion, and 21-25 nt RNAs are produced as the result [26]. dsRNA is a potent activator for PTGS in *Caenorhabditis elegans* and several other organisms [27-29]. It is also known that 21-25 nt small RNAs are present in plants associated with PTGS [30]. These small RNA species, which are possibly derived from dsRNA are key components of gene silencing [26].

We hypothesized that if silencing signals are produced in a few cells of inoculated leaves and then spread throughout the plant, DNA virus replication would be prevented once the virus has moved into the plant cells in systemic leaves where a plant gene needed for viral replication is silenced.

We cloned a short fragment of clone 9 into a TGMV B plasmid and co-innoculated it with wt TGMV A or TGMV A::su into *N. benthamiana* plants. Viral DNA accumulation was greatly reduced in systemic leaves, accompanied with undetectable viral symptoms and systemic silencing of *su* was inhibited or delayed. These results suggest that clone 9 might have potential function in viral DNA replication. However, we cannot rule out that the reduction of viral accumulation in systemically infected leaves could be due to the inhibition of viral movement. For example, clone 9 might regulate plant gene expression involved in viral movement.

We think that the clone 9 is more likely to be involved in cell cycle regulation or DNA replication process for two reasons. First, Clone 9 is a putative transcription factor. Second, viral symptoms were observed in systemically infected leaves of plants silenced for clone 9 at 1-2 w.p.i., indicating that at least viruses can move in the plants. Silencing of the PCNA gene that reduced viral accumulation in systematic leaves accompanied by primary growth reduction has been demonstrated [20]. In contrast, normal phenotypes of new growth were observed in plants silenced in the clone 9. This implies that the clone 9 could be a potential target for resistance. In the absence of this gene, plants could be resistant to virus infection. In order to test this possibility, the level of clone 9 transcripts from systemic leaves and inoculated leaves needs to be elucidated. T-DNA knockout or hairpin silencing of clone 9 in *N. benthamiana* and then challenges with wt TGMV would be necessary.

To conclude, virus replication could be prevented if the virus silenced a plant gene needed for its own replication or movement. A full-length gene was not needed for downregulation of the endogenous gene using geminivirus mediated gene silencing. However, the full-length sequence of clone 9 remains to be isolated and the role of the protein needs to be further determined.

Material and Methods

PCR-based subtractive hybridization (Performed by Dr. Patricia Eagle)

Suppressive PCR, used to create a library of short fragments differentially expressed in the 35S-AL1 transgenic plants, was done according to the instructions of the manufacturer (BD Bioscience, USA). Briefly, polyA RNA was isolated from mature leaves of two populations, wild type *N. benthamiana* and the 35S-AL1 transgenic *N. benthamiana*. cDNA was separately synthesized using RT-PCR and subjected to a *Rsa*I restriction endonuclease digestion. The cDNA population from the 35S-AL1 transgenic plants (tester) was divided into two pools: tester 1 and tester 2, following addition of two different types of adapters while no adapter was added to wild type cDNA population (driver). First, an annealing reaction was carried out between tester 1 and excess driver, and tester 2 with excess driver, separately. Unhybridized cDNAs from each pool were then combined and a second hybridization was taken place. PCR was then performed at this stage using primers that recognize adapter sequences from tester 1 and tester 2. Only the fragments that have two different types of adapters were exponentially amplified. PCR products were subsequently ligated with vector pCR2.1 using T/A cloning Kit (Invitrogen), and transformed into *E. coli*.

Selected cDNA clones were then checked for insert size, sequenced, and characterized by homology search of known proteins using BLAST programs.

Plasmid DNA constructs

The TGMV A and TGMV B plasmids were constructed as previously described in detail by Kjemtrup et al. [17] and Peele et al. [20]. pTG1.3BXSR [31] was used as a vector for inserting foreign DNA into an *Xba*I site, 20 bp down stream of the BR1 ORF. Two different sized *su* fragments were cloned from pLVN44, which contains a *Nicotiana tabacum su* cDNA [32], into pTG1.3BXSR, a 455-bp *Sac*I/*Sac*I fragment and a 935-bp *Sac*I/*Acc*651 fragment. TGMV B::su154, TGMV B:PCNA122, and TGMV A::su have been described elsewhere [20]. TGMV B::Clone 9 was obtained by the insertion of a 106-bp *Ssp*I/*Eco*RV fragment into the *Xba*I site of pTG1.3BXSR. Before ligation reaction was performed, the insert and the vector fragments were both blunted via Klenow fill-in. Descriptions of all TGMV derived vectors are shown in Table 3.

Plant material and DNA bombardment

Five-week-old *N. benthamiana* seedlings in two-inch plastic pots were bombarded with a combination of 5 μg of each TGMV A and B DNA using the BIOLISTIC Particle Delivery System (Bio-Rad, Hercules, CA USA) as previously described [3]. In brief, 5 μg each of TGMV A and B DNA component vectors were added to 3 mg gold particles and agitated for three-five minutes. 50 μl of 2.5M CaCl2 and 20 μl of 0.1M spermidine were added, followed by three-five minutes agitation. Centrifugation was done for 10 second at 12,000 rpm. Then, DNA pellet was resuspended with 250 μl 200 proof ethanol, centrifuged briefly for 10 second, and resuspended with 65 μl of 200 proof ethanol. This gave 5 bombardments. The bombardment was done under a pressure of 1,100 PSI of He gas.

Southern blot and PCR analysis

Systemically infected leaf tissues or inoculated leaf tissues were harvested at 2 weeks or 4 weeks, or in one experiment 6 weeks after bombardment. Total DNA was isolated [33] and 5 μg from each plants was separated by electrophoresis, blotted, and probed with a Digoxigenin-labeled probe corresponding to the TGMV A AL1 (a 281-bp fragment of AL1 DNA) or a ^{32}P-dCTP labeling probe corresponding to a 786 bp of *su* cDNA. Digoxigenin-labeled probe was prepared using a Dig-High Prime kit (Boehringer Mannheim, IN, USA) followed by chemiluminescent detection according to the instruction's recommendation. The PCR products corresponding to 315-595 nt of the AL1 ORF were used as probes and were obtained using the following primers: AL1 upper primer 5'CGACAAAGACGGAGATAC TC 3' and AL1 lower primer 5' GTCTCATCTCGTCTGGCACG 3'. Cycling conditions were 30 cycles of 95°C for 1 minute, 50°C for 1 minute, and 72°C for 7 minutes. The DNA probes specific for su cDNA were generated by random prime labeling using ^{32}P- labeled dCTP and the Rediprime DNA labeling system (Amersham Life Science, Buckinghamshire, England). The PCR analysis of insert size in the B component was done as previously described [20] using primers: BR1 5' GTCGGGATATTCTCTCAAAGG 3' and BL1 5'TCTACTATTGGGCTAACAGG 3' in a 100-μl reaction with 10 ng template DNA. Cycling conditions were 30 cycles of 95°C for 1 minute, 54°C for 1 minute, and 72°C for 7 minutes. 10 μl of each PCR samples was separated through a 2% agarose gel and photographed.

Northern blot analysis

To determine if subtracted cDNA clones from the PCR-based subtractive hybridization were induced in either TGMV infected tissue or AL1 transgenic tissue, total

RNA was isolated from mature leaves of wild type, TGMV-infected, the 35S-AL1 transgenic plants, and young seedlings. RNA gel blot analysis was carried out following fractionation of the total RNA samples in 1.2% formaldehyde-agarose gels. Random primer P^{32}-labeled probes were performed using a specific 961 bp of clone 9 cDNA (RNA blot of clone 9 was performed by Dr. Patricia Eagle) or other cDNA clones.

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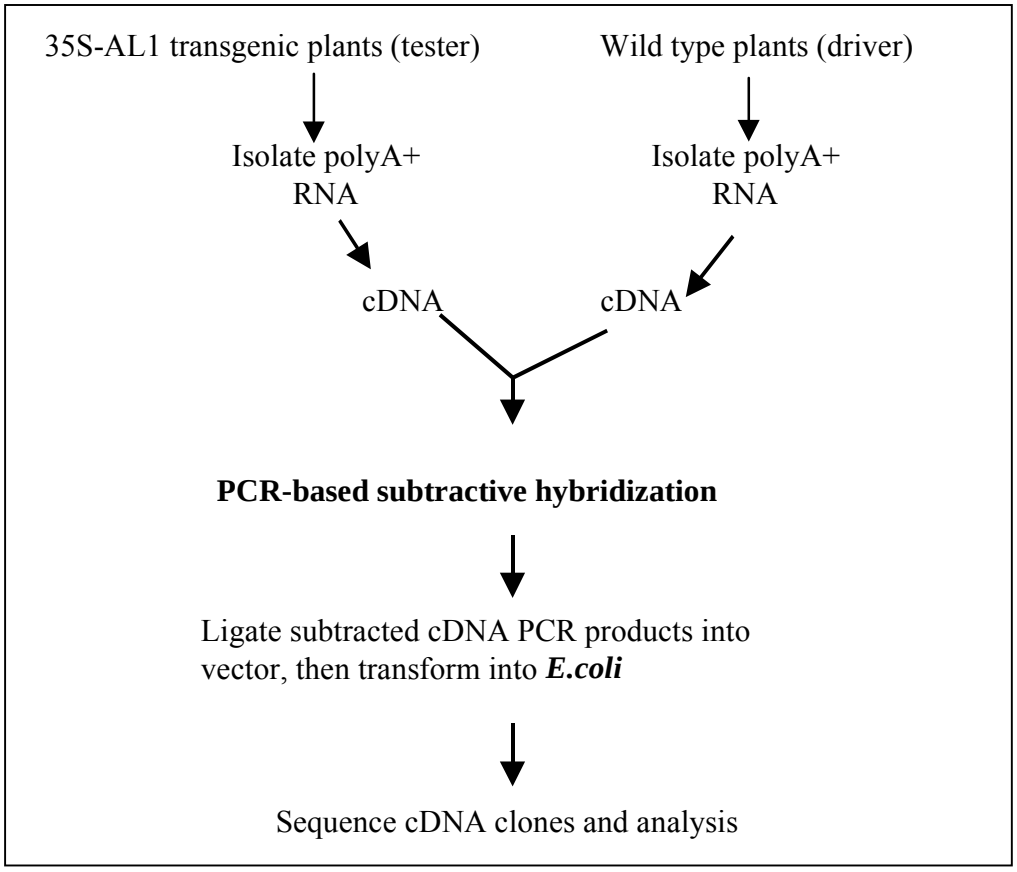


Figure 1. Summary of our method for identifying AL1-upregulated genes.
The PCR-based subtractive hybridization used cDNA from two mRNA sources as shown. The differentially expressed cDNAs (tester) were present in the 35S-AL1 transgenic cDNA but were absent (or present at lower levels) in wt plants. Subtracted cDNAs were subsequently amplified by PCR, facilitating the enrichment of less abundant cDNAs. Products from the PCR reaction were inserted into a pCR2.1 vector plasmid and then transformed into *E. coli*. DNA sequencing of the cDNA clones and nucleic homology searches using the BLAST program at the National Center for Biotechnology Information (NCBI) website were then performed according to Altschul et al. (1990).

Table 1. Putative homologues of some cDNA clones upregulated in the 35S-AL1 transgenic plants.

Clones	Homolog (protein)	Score/E value/ Percent amino acid identity	GenBank Accession No.[a]
[b]Clone 9	Unknown protein (*A. thaliana*)	122/6e-24/41%	NP563717.1
[c]Clone 13	Unknown protein (*A. thaliana*)	43.1/5e-12/46%	NP174979
Clone 13.6	Expressed protein (*A. thaliana*)	70.1/4e-09/53%	NP566194
Clone 14.7	PSII 10kD Phosphoprotein (*N. tabacum)*	122/1e-27/94%	NP054529
Clone 15.8	Chlorophyll a/b binding protein 36-(LCHII) (*N. tabacum*)	244/9e-65/99%	P27494
Clone 16.22	Chlorophyll a/b binding protein 7 (LHCII) (*N. tabacum*)	160/2e-55/97%	P27491
Clone 17.29	Phosphoribulokinase (*P. sativum*)	140/3e-33/94%	T06463
Clone 18.3	NADH dehydrogenase subunit 5 (*Arctoscopus haponias*)	29.3/9.6/41%	NP148761
Clone 20.5	Glycine-rich protein (N. tabacum)	62.4/2e-09/40%	P23137
Clone 25.1	Calmodulin-related protein (*A. thaliana)*	71.6/9e-18/45%	NP198593
Clone 26.4	Thioredoxin (*A. thaliana*)	114/7e-39/76%	NP564566
Clone 27.6	Endosomal protein-like (*A. thaliana*)	142/5e-34/76%	BAB10022
Clone 28.7	Poly A binding protein (*N. tabacum*)	98.2/1e-20/83%	AAF66824
Clone 29.11	Proline-rich protein (*A. thaliana*)	82.4/9e-16/66%	T01345
[b]Clone 37	Chlorophyll a/b binding protein (*A. thaliana)*	37.4/0.035/45%	AAD27878
[b]Clone 41	Chloroplast drought-induced stress protein (*A. thaliana*)	125/2e-28/49%	NP177735

[a] similar sequences as identified by BLASTX searches using partial sequences of cDNA clones as query sequences translated in all reading frames against a protein sequence from NCBI database. Source: http://www.ncbi.nlm.nih.gov/BLASTX

[b] Upregulated genes in the 35S-AL1 transgenic tissue and TGMV-infected tissue

[c] No change in gene expression pattern

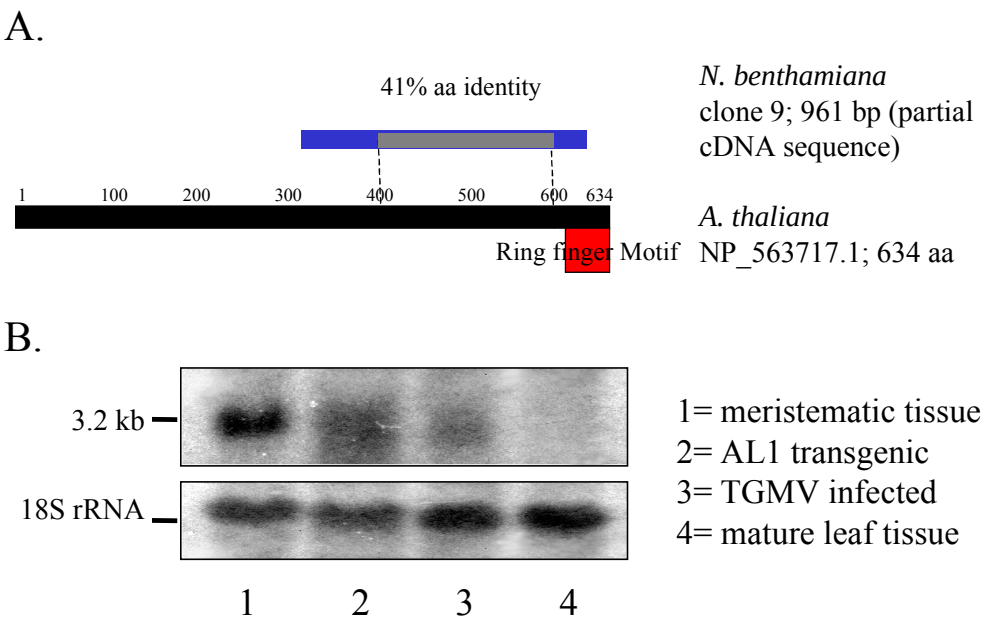


Figure 2. **Characterization of clone 9.**
A. Schematic alignment of predicted amino acid sequence from a partial cDNA clone, a 961-bp clone 9 fragment to *Arabidopsis* (accession No. NP_653171.1). A gray box indicates the region of amino acid identity whereas blue boxes indicate the non-homology region.
B. Northern blot analysis of clone 9 (performed by Dr. Patricia Eagle). Total RNA was separated on a formaldehyde-agarose gel, blotted, and probed with a ^{32}P-labeled probe corresponding to a 961-bp fragment of the clone 9 cDNA. The clone 9 transcript which is approximately 3.2 kb in size was present in meristematic tissue (lane 1) but not in mature leaf tissue (lane 4). It was also upregulated in the 35S-AL1 transgenic tissue (lane 2) and TGMV-infected tissue (lane 3).

Table 2. Unigene cluster of the *Arabidopsis thaliana* unknown protein (At1g04790; F13M7.22)*, putative homologue of clone 9.

Organisms	Protein accession #/amino acid sequence	Description	Percent identity of amino acid sequence	Length of aligned region
A. thaliana	NP_563717.1/ 634 aa	Expressed protein	100 %	633 aa
H. sapiens	NP_060904.1/ 276 aa	Goliath protein; likely ortholog of mouse g1-related zinc finger protein	40%	77 aa
M. musculus	NP_067515.1/ 419 aa	G1-related zinc finger protein	40%	77 aa
C. elegans	NP_497129.1/ 473 aa	Zinc finger protein	37%	77 aa
D. melanogaster	NP_523864.1/ 248 aa	goliath	29%	126 aa

* Source http://www.ncbi.nlm.nih.gov/UniGene

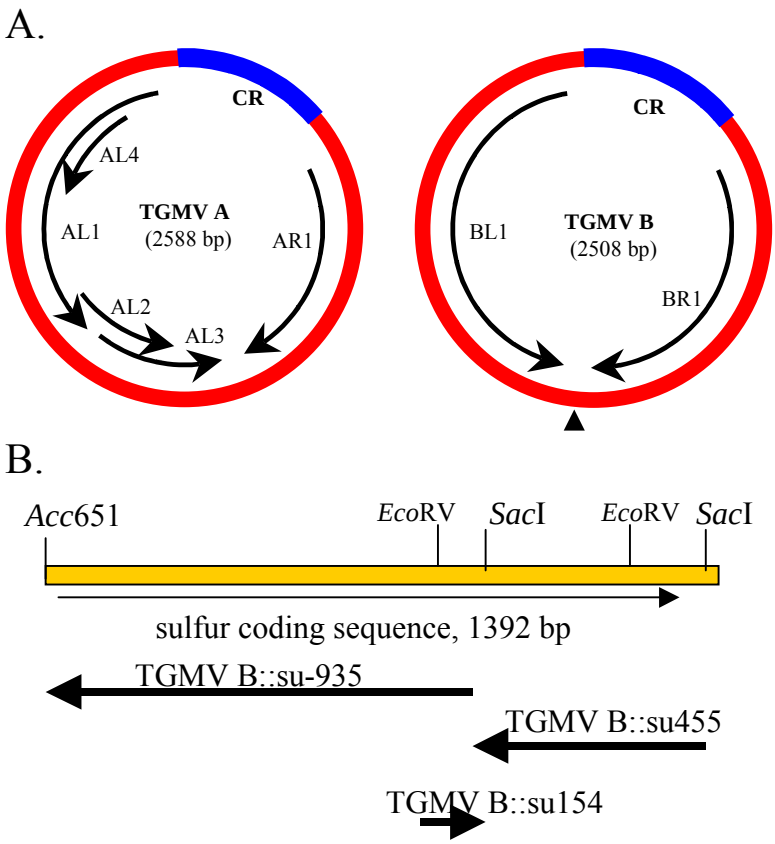
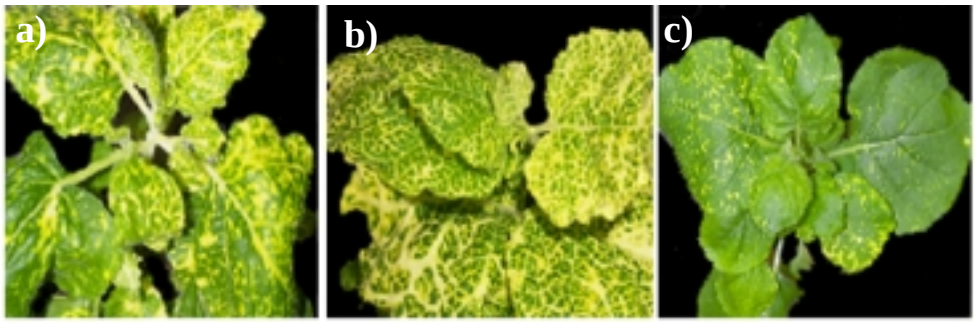


Figure 3. **Wild type TGMV genome and *su* cDNA fragment used as a visual maker for gene silencing.**
A. The wild type TGMV A and B genome, ~2.5 kb in size. Each component shares a common region (CR) that includes the origin of replication. The A component encodes the AL1, AL2, and AL3 genes needed for replication and gene expression, AL4 has no function, and AR1 coat protein needed for insect transmission. The AR1 ORF can be replaced with foreign DNA up to 0.8 kb. The B component encodes two genes, BL1 and BR1, required for movement and symptom development. Foreign DNA was inserted into the B component vector at 20 bp downstream of the BR1 stop codon, before a polyadenylation site (arrow).
B. The full length *su* cDNA 1392 bp. Three su cDNA fragments, 935-bp *Acc*651/*Sac*I in antisense orientation, 455-bp *Sac*I/*Sac*I in antisense orientation, and 154-bp *Eco*RV/*Sac*I in sense orientation, were used to make TGMV B::su935, TGMV B::su455, and TGMV B::su154, respectively.

A.



B.

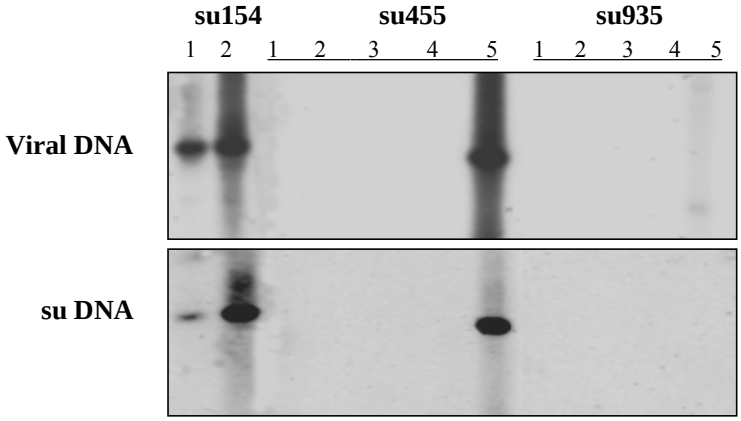


Figure 4. **Analysis for the upper size limitation in TGMV B-derived vectors**.
A. Silencing phenotypes of plants infected with TGMV B::su154, TGMV B::su455 and TGMV B::su935 at 2 w.p.i. All five plants infected with TGMV B::su154 and one plant out of five plants infected with TGMV B::su455 showed extensive silencing; loss of chlorophyll, in stems, veins, and systemic leaves with minimal viral symptoms (a, b). Nine plants infected with either TGMV B::su455 or TGMV B::935 showed less extensive silencing with numerous yellow spots in systemic leaves or a few yellow spots in some plants (c). Viral symptoms were also greatly attenuated in these plants.
B. Southern blot analysis of DNA isolated from systemic infections of plants inoculated with different vectors. Linearized DNA cut with a restriction enzyme, *Bam*HI, was separated by electrophoresis, blotted, and probed with a 281 bp TGMV AL1 DNA fragment. DNA isolated from two plants infected with TGMV B::su154 (lane 1 and 2), five plants from TGMV B::su455 (lane 3-7), and five plants from TGMV B::su935 (lane 8-12). Only plants with extensive silencing of *su* had high viral DNA accumulation (lane 1, 2, and 7). Bottom panel, same blot as above but probed with a ^{32}P-labeled 786 bp fragment from *su* cDNA.

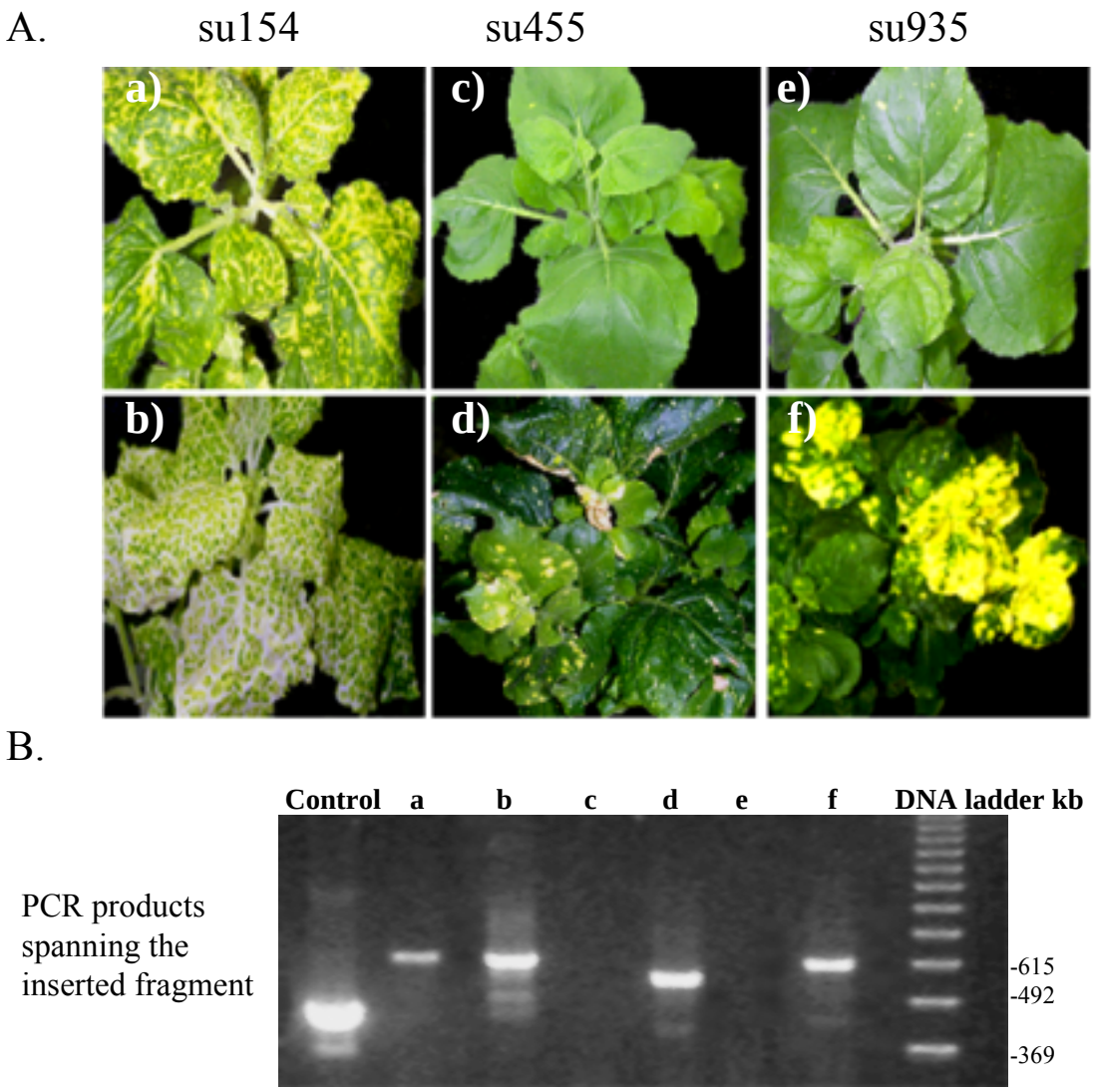


Figure 5. **PCR analysis of silencing tissues at 2 and 6 w.p.i.**
A. Silencing phenotypes of some plants infected with TGMV B::su154 (a, b), TGMV B::su455 (c, d), and TGMV B::su935 (e, f). A few plants infected with either TGMV B::su455 or TGMV B::su935 showed extensive silencing of *su* in new growth at 6 w.p.i. (d, f), while a few yellow spots were observed in systemic leaves at 2 w.p.i (c, e).
B. PCR products spanning the inserted fragment of plants from above panel. The 154 bp insert was stable (a and b) whereas the 455-bp and 935-bp inserts showed deletions, with less than 154 bp of insert remaining (c, d, e, and f). Control shows the PCR product from wt B component plasmid with no insert. a and b used total DNA from systemically infected leaves and show that propagation of the 154-bp insert was stable, even though deleted forms were present (B).

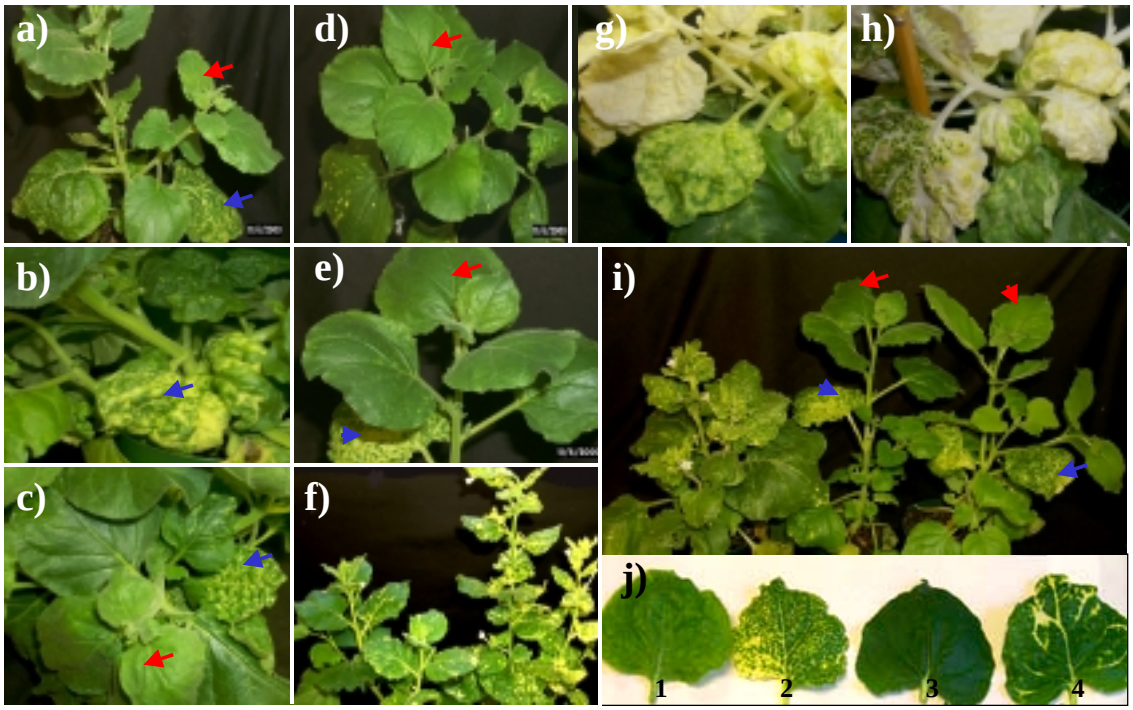


Figure 6. Silencing phenotypes of clone 9.
Plants infected with TGMVA/TGMV B::Clone 9 (a, b, c, i right) showed severe symptoms in inoculated leaves including leaf curling, and chlorosis (blue arrows) while minimal symptoms were observed in systemically infected leaves (red arrows). This experiment was repeated two times independently with five plants in each experiment. The same results were obtained from all experiments. Plants infected with TGMV A::su/TGMV B::Clone 9 (d, e, f, i middle) also showed similar phenotypes. The initiation of *su* silencing of these plants was observed in inoculated leaves in all five plants (several yellow spots) but only one out of five plants showed systemic silencing of *su* in new growth at 6 w.p.i. (f, left). Silencing of *su* in plants infected with TGMVA/TGMV B::su154 was typically extensive with minimal symptoms in systemic leaves (g, h), while less extensive *su* silencing was observed from plants inoculated with TGMV A::su/TGMV B (f, right). Plants inoculated with TGMVA::su/TGMV B::PCNA showed *su* silencing in systemic leaves with the reduced stem elongation (i, left). The uppermost systemic leaves of plants that were infected with either TGMVA/TGMVB::Clone 9 (j3) or TGMVA::su/TGMV B::Clone 9 (j4) were symptom-free, while the leaves from wt TGMV infection or TGMV B::su154 showed chlorosis (j1) or curling with numerous yellow spots (j2), respectively. The photographs were taken at 4 w.p.i, except f and j were taken at 6 w.p.i.

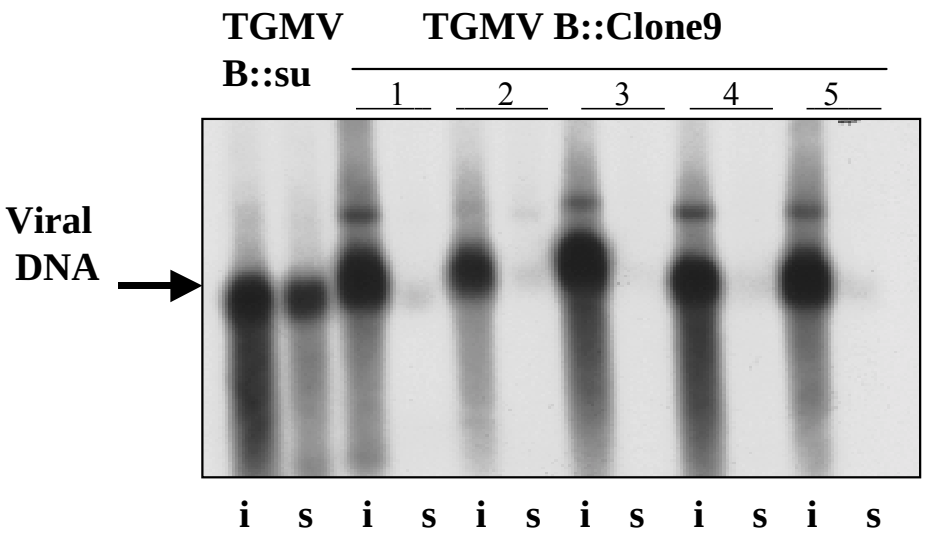


Figure 7. Southern blot analysis of viral DNA accumulation in infected plants. Total DNA was extracted from inoculated leave (i) and systemically infected leaves (s) at 4 w.p.i. DNA samples (5 µg per lane) were analyzed by gel blotting, using a Dig-labeled probe corresponding to TGMV AL1 DNA. Viral accumulation in plants infected with TGMV A/TGMV B::Clone 9 (lane 4, 6, 8, 10, 12) was dramatically reduced in systemically infected leaves. Viral accumulation in systemic leaves of plants infected with TGMV A/TGMV B::su was a little reduced (lane 2). The experiment was repeated twice and the same result was obtained.

Table 3. Describes the TGMV A and TGMV B plasmids used for the experiments here in. CP, Chip Peel; NM, Nooduan Muangsan

Name	Description
pCPTGMV A::su	786 bp *Acc*651/*Eco*RV fragment, corresponding to nt 0-786 of su cDNA, antisense orientation
pCPTGMV B::su154	154 bp *Sac*I/*Eco*RV fragment, corresponding to nt 785-939 of the su cDNA, sense orientation
pNMTGMV B::su455	455 bp *Sac*I/*Sac*I fragment, corresponding to nt 939-1394 of the su cDNA, antisense orientation
pNMTGMV B::su935	935 bp *Sac*I/*Acc*651 fragment, corresponding to nt 0-935 of the su cDNA, antisense orientation, 18 nt 5'untranslated region and 917 nt ORF
pNMTGMV B::Clone 9	106 bp *Ssp*I/*Eco*RV fragment, corresponding to 96 bp 5' cDNA of the Clone 9 961 bp fragment.
pCPTGMV B::PCNA	122 bp fragment of PCNA cDNA

Chapter 3

Geminivirus-based vectors for gene silencing in Arabidopsis

Michael A. Turnage, Nooduan Muangsan, Charles G. Peele and Dominique Robertson

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Nooduan Muangsan performed the cloning of pNMCbLCV B and pNMCbLCV B::CH42, and testing them for silencing effect.

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TECHNICAL ADVANCE

Geminivirus-based vectors for gene silencing in *Arabidopsis*

Michael A. Turnage, Nooduan Muangsan, Charles G. Peele[†] and Dominique Robertson[*]
Department of Botany, North Carolina State University, Raleigh, NC 27695, USA

Received 8 November 2001; accepted 18 November 2001.
[*]For correspondence (fax +1 919 515 3436; e-mail niki_robertson@ncsu.edu).
[†]Present address: Biolex, 480 Hillsboro Street, Suite 100, Pittsboro, NC 27312, USA.

Summary

Gene silencing, or RNA interference, is a powerful tool for elucidating gene function in *Caenorhabditis elegans* and *Drosophila melanogaster*. The vast genetic, developmental and sequence information available for *Arabidopsis thaliana* makes this an attractive organism in which to develop reliable gene-silencing tools for the plant world. We have developed a system based on the bipartite geminivirus cabbage leaf curl virus (CbLCV) that allows silencing of endogenous genes singly or in combinations in *Arabidopsis*. Two vectors were tested: a gene-replacement vector derived from the A component; and an insertion vector derived from the B component. Extensive silencing was produced in new growth from the A component vectors, while only minimal silencing and symptoms were seen in the B component vector. Two endogenous genes were silenced simultaneously from the A component vector and silencing of the genes was maintained throughout new growth. Because the CbLCV vectors are DNA vectors they can be inoculated directly from plasmid DNA. Introduction of these vectors into intact plants bypasses transformation and extends the kinds of silencing studies that can be carried out in *Arabidopsis*.

Keywords: geminivirus, gene silencing, RNAi, *Arabidopsis*, cabbage leaf curl virus.

Introduction

With the completion of the sequencing of the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), it has become clear that new tools and approaches are needed to determine the function of the many presumed genes for which no function has been assigned. We previously demonstrated that plant genes can be silenced using the geminivirus tomato golden mosaic virus (TGMV) in the permissive host plant *Nicotiana benthamiana* (Kjemtrup *et al.*, 1998; Peele *et al.*, 2001). Silencing produced a phenocopy of a mutation of the gene; that is, the silenced tissue appeared to have lost the function of the silenced gene. Silencing of genes that would be lethal in null mutants was demonstrated using this virus (Peele *et al.*, 2001). This paper describes the development of similar geminivirus based silencing vectors functional in *Arabidopsis*.

There are two primary reasons for developing this system in *Arabidopsis*: the wealth of genetic and sequence information available for *Arabidopsis* which allows easy verification of clones and phenotypes; and the fact that this virus is distantly related to TGMV. Bipartite geminiviruses in the genus Begomovirus infect a variety of vegetable crops and legumes. Geminivirus vectors, with their highly conserved genetic structure (Table 1), and wide range of host plants (Bradeen *et al.*, 1997; Ingham *et al.*, 1995; Paximadis *et al.*, 1999; Timmermans *et al.*, 1994), could provide a powerful methodology for studying gene function in a wide range of economically important plant species.

Several strategies have been employed to achieve gene-silencing in *Arabidopsis*. Double-stranded RNA (Smith *et al.*, 2000), and vectors derived from the RNA viruses Potato virus X (PVX) (Dalmay *et al.*, 2000a) and Tobacco rattle virus (TRV) (Ratcliff *et al.*, 2001) cause homology-based silencing reactions in this plant; however, these approaches have significant limitations in utility for functional genomics. Current technology for efficient silencing with double-stranded RNA requires non-trivial cloning to

Table 1. Amino acid similarity of selected new world (NW) and old world (OW) begomoviruses

Virus	Average[a]	AL1/AC1[a]	BL1/BC1[a]	BR1/BV1[a]	Source[b]
Cabbage leaf curl virus	**100 (100)**	**100 (100)**	**100 (100)**	**100 (100)**	**NW**
Bean calico mosaic virus	76 (86)	84 (90)	75 (87)	70 (82)	NW
Squash leaf curl virus	74 (84)	81 (88)	75 (86)	67 (79)	NW
Sida golden mosaic virus	73 (84)	63 (76)	82 (90)	73 (86)	NW
Cucurbit leaf crumple geminivirus	73 (84)	79 (88)	74 (86)	66 (79)	NW
Bean golden mosaic virus	72 (84)	63 (75)	82 (91)	70 (86)	NW
Dicliptera yellow mottle virus	72 (82)	63 (74)	82 (89)	71 (82)	NW
Bean dwarf mosaic virus	72 (82)	63 (75)	82 (89)	71 (83)	NW
Tomato mottle virus	71 (82)	63 (76)	80 (89)	70 (82)	NW
Tomato leaf curl virus	71 (81)	65 (75)	81 (89)	66 (78)	NW
Tomato leaf crumple virus	71 (83)	65 (78)	80 (89)	69 (83)	NW
Chino del tomato virus	71 (83)	64 (77)	81 (89)	69 (82)	NW
Potato yellow mosaic virus	70 (80)	63 (75)	82 (88)	64 (77)	NW
Taino tomato mottle virus	70 (82)	62 (76)	80 (90)	69 (80)	NW
Abutilon mosaic virus	69 (81)	59 (73)	81 (89)	68 (81)	NW
Tomato golden mosaic virus	**69 (81)**	**60 (74)**	**78 (88)**	**68 (80)**	**NW**
Tomato rugose mosaic virus	68 (80)	60 (74)	76 (86)	67 (79)	NW
Pepper huasteco virus	67 (80)	52 (68)	82 (91)	68 (81)	NW
Havana tomato virus	66 (78)	62 (75)	70 (79)	67 (79)	NW
Cassava latent virus	44 (60)	53 (68)	44 (61)	35 (52)	OW
Indian cassava mosaic virus	42 (60)	51 (68)	43 (58)	33 (55)	OW
Vigna mungo yellow mosaic virus	42 (61)	52 (68)	44 (63)	30 (52)	OW
South African cassava mosaic virus	41 (58)	51 (67)	42 (59)	29 (49)	OW
Mungbean yellow mosaic virus	41 (59)	51 (67)	43 (61)	28 (48)	OW
Watermelon chlorotic stunt virus	41 (59)	51 (68)	43 (58)	30 (52)	OW
West African cassava mosaic virus	40 (57)	51 (67)	41 (56)	29 (48)	OW
Indian mungbean yellow mosaic virus	40 (60)	51 (67)	44 (62)	28 (50)	OW
Tomato yellow leaf curl virus	39 (59)	53 (68)	39 (59)	24 (50)	OW

[a]Values given indicate percentage of identity with the amino acid sequence of the corresponding *CbLCV* gene; percentage of similarity is given in parentheses. Average is the average of the percentage identity (similarity) values determined for the products of the three genes *AL1*, *BL1* and *BR1*. [b]NW, new world isolates; OW, old world isolates.

produce the dsRNA template, as well as the time commitment to produce transgenic *Arabidopsis*. Use of RNA virus vectors is limited by the transient and limited silencing produced when endogenous plant genes (rather than integrated transgenes) are the target of silencing (Ratcliff *et al.*, 2001; Ruiz *et al.*, 1998). It has been proposed that the silencing signal which is transmitted throughout a plant is perpetuated by transgenes, but not by endogenous genes (Fagard and Vaucheret, 2000a; Fagard and Vaucheret, 2000b; Morel *et al.*, 2000). This difference in signal transmission has been linked to differences in the RNA-directed methylation of the silenced gene (Jones *et al.*, 1999; Pelissier and Wassenegger, 2000; Pelissier *et al.*, 1999) and may account for the difficulty in silencing endogenous genes. We demonstrate here that, unlike silencing induced by other viruses in *Arabidopsis*, silencing induced by Cabbage leaf curl virus (CbLCV) is effective against both transgenes and endogenous genes.

CbLCV is a bipartite geminivirus belonging to the Begomovirus genus. Several features of CbLCV made it a likely candidate for a silencing vector in *Arabidopsis*. First, it was known that CbLCV is infectious in *Arabidopsis* (Hill *et al.*, 1998); second, the genetic map is similar to that of TGMV, a virus with which we have considerable experience. Although the genetic map was similar, they have diverged significantly at the sequence level (Table 1), thus CbLCV serves as an indicator of the general applicability of this type of silencing to other geminivirus/plant combinations.

Results and discussion

Construction of infectious plasmid constructs of CbLCV A and CbLCV B

Geminiviruses are small, circular DNA viruses that can be inoculated into plants using *Agrobacterium* or microprojectile bombardment of plasmid DNA (Elmer *et al.*, 1988; Kjemtrup *et al.*, 1998; Sung and Coutts, 1995; Timmermans *et al.*, 1994). To construct plasmid vectors for CbLCV, partially duplicated viral genomes containing two direct repeats of the origin of viral replication were generated for both CbLCV components (Figure 1). Introduction of these plasmids into plant cells allowed production of unit length

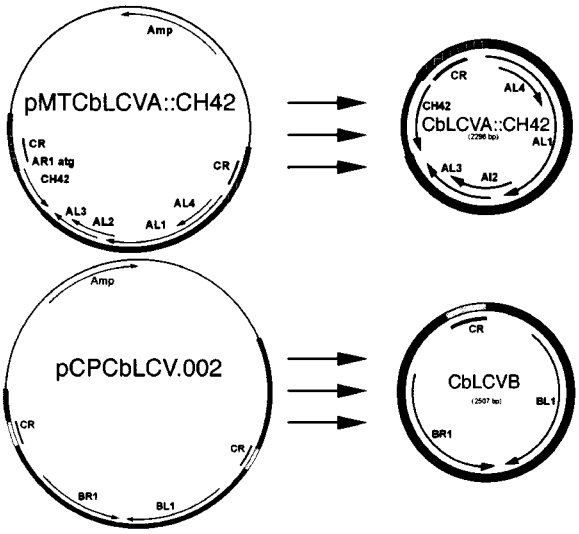

Figure 1. Plasmid DNA introduced into plants (left) and replicating viral DNA vector (right) for the A and B components of the CbLCV silencing system.
Upper plasmid and vector is the *AR1*-deleted A component containing a *CH42* fragment transcribed by the viral *AR1* promoter. The lower plasmid and vector is the wild-type CbLCV B component which is co-bombarded with the A component. Locations of viral genes *AL1, AL2, AL3, AL4, BR1* and *BL1* are indicated. CR denotes the common region which contains the viral origin of replication. Thin solid lines indicate plasmid sequences carrying an ampicillin-resistance gene (Amp).

geminivirus DNA that replicates and subsequently causes a viral infection.

Plasmids containing partial direct repeats of the CbLCV A and B components were co-bombarded into *Arabidopsis* seedlings to test for infection. Severe symptoms (stunted growth and necrosis) were produced approximately 2–3 weeks post-bombardment (data not shown). To attenuate symptoms, and to determine if the coat protein was needed for viral infection, a CbLCV A component vector containing an *AR1* gene deletion was constructed and co-bombarded with the wild-type B component. The mutant virus produced attenuated symptoms (Figure 2a,b). DNA gel blots demonstrated that it was infectious and could be used as a vector (data not shown). Because some geminivirus/host combinations require the coat protein for infectivity of the virus (Pooma *et al.*, 1996), it was important to establish the infectivity of the *AR1* mutant. The *AR1* gene encodes the coat protein, which is required for transmission by insect vectors (Sung and Coutts, 1995; Timmermans *et al.*, 1994). Therefore the CbLCV A component vectors are not insect-transmissible.

Construction of silencing vectors

The ability of the *AR1*-deleted vectors to propagate silencing was first tested in transgenic *Arabidopsis* carrying the *mGFP5* gene (Haseloff *et al.*, 1997), which expresses the green fluorescent protein under control of the Cauliflower mosaic virus (CaMV) 35S promoter. Transgenic lines were used which gave visible GFP expression throughout development. A fragment of the *GFP* coding sequence was cloned into the *AR1* deletion site of CbLCV A, and this construct co-bombarded with the CbLCV B component into *Arabidopsis* plants. Approximately 2–4 weeks after infection of the plants viral symptoms developed in new growth of the infected plants, and the green fluorescent signal was no longer seen (data not shown). RNA gel-blot analysis of RNA from silenced leaves demonstrated a dramatic reduction in the level of mRNA for the *GFP* transgene, as expected for silenced tissue. Levels of *GFP* transcript were not reduced in plants infected with the *AR1* mutant (Figure 3).

Highly expressed transgenes are often more susceptible to homology-dependent silencing than are endogenous plant genes (Fagard and Vaucheret, 2000a; Fagard and Vaucheret, 2000b; Jones *et al.*, 1999; Kjemtrup *et al.*, 1998; Morel *et al.*, 2000; Pelissier and Wassenegger, 2000; Pelissier *et al.*, 1999; Ruiz *et al.*, 1998). In order to determine the applicability of silencing from CbLCV vectors to endogenous genes, fragments of the *Chlorata 42* gene (*CH42*) (Koncz *et al.*, 1990), encoding a component of the magnesium chelatase complex required for chlorophyll production, and of the *phytoene desaturase* (*PDS*) gene (Scolnik and Bartley, 1993), which is necessary for carotenoid pigment production, were cloned into the *AR1*-deleted CbLCV A vector, singly and in combination. Silencing of the endogenous *CH42* gene was expected to prevent synthesis of chlorophyll; in *Nicotiana* species silencing of this gene phenocopies the mutant sulfur phenotype, resulting in yellow tissue (Kjemtrup *et al.*, 1998). Silencing of the *PDS* gene in *Nicotiana* or in *Arabidopsis* results in loss of carotenoids and photobleaching, seen as near-white tissue (Dalmay *et al.*, 2000b; Ruiz *et al.*, 1998). Infection of *Arabidopsis* with virus containing the *CH42* gene fragment caused viral symptoms and a yellow 'sulfur' phenotype in new growth 3–4 weeks after infection (Figure 2e,f). Infection with virus containing the *PDS* gene fragment resulted in viral symptoms in the new growth, but the bleached white silencing phenotype was initially variable in these plants – some showed nearly complete bleaching in the infected tissue, while others showed little sign of *PDS* silencing (Figure 2g,h). However, in all plants infected with CbLCV containing *PDS*, bleaching of infected tissue spread to include all the symptomatic tissue by approximately 6 weeks post-infection.

To test the utility of this system for silencing multiple genes, an *AR1*-deleted CbLCV A vector was constructed that contained fragments of two endogenous genes, *CH42* and *PDS*. Plants infected with this virus were of two types: one group of plants showed *CH42* silencing in new growth

Figure 2. Endogenous gene silencing from the CbLCV A component vector.
Arabidopsis plants co-bombarded at the four- to six-leaf stage with CbLCV A vectors and wt CbLCV B were photographed approximately 4 weeks post-bombardment.
(a,b) Plants infected with empty vector (*AR1*-deleted CbLCV A and wild-type CbLCV B) showed no silencing.
(c,d) Plants infected with CbLCV A containing a fragment with homology to two endogenous genes, *CH42* and *PDS*, showed extensive silencing (white tissue) in new growth.
(e,f) Plants infected with CbLCV A::CH42 showed extensive *CH42* silencing (yellow tissue) in new growth.
(g,h) Plants infected with CbLCV A::PDS showed delayed silencing of PDS. The PDS-silencing phenotype was not extensive at this point, but continued to spread so that it was as extensive as *CH42* silencing 6 weeks post-inoculation.
Plants in (a) and (h) show yellow leaf margins due to bombardment damage.

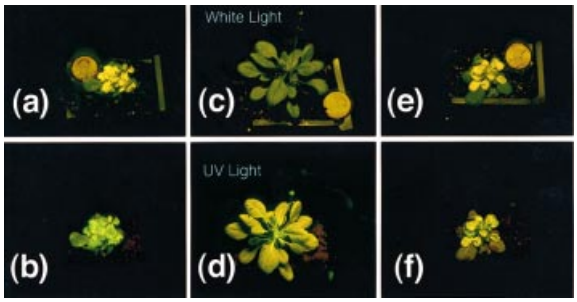

Figure 4. Silencing of multiple genes from a single viral vector.
GFP transgenic *Arabidopsis* plants were photographed with white light (a,c,e) or ultra-violet illumination (b,d,f).
(a,b) Plant infected with CbLCV A::CH42, showing silencing of *CH42* but not *GFP*.
(c,d) Uninfected control showing GFP expression.
(e,f) Plant infected with CbLCV A::CH42GFP shows silencing of both *CH42* and *GFP*.
A penny was photographed in the white light panels to indicate relative sizes of the plants. Infected plants also contained the wt CbLCV B component.

after 4 weeks. The silencing phenotype changed over the next 2 weeks, from yellow to white (Figure 2c,d). The second group of plants displayed white tissue only, and appeared to lack carotenoids. The two responses suggest either that generation of silencing signals for the two genes occurred independently, or that initiation of silencing of the *PDS* gene was variable. Although the delayed onset of the *PDS*-silenced phenotype is consistent with variable initiation of silencing for this gene (Figure 2g,h), it

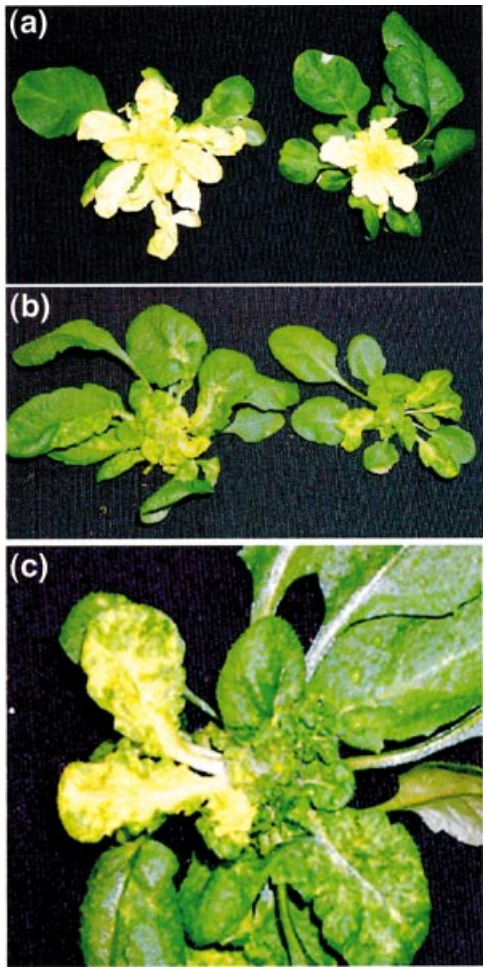

Figure 5. Silencing was not extensive when CbLCV B component vectors were used to carry homologous DNA.
Arabidopsis infected with CbLCV A component vectors (a) or B component vectors (b,c) carrying CH42 gene fragments 27 d.p.i. Plants in (b) and (c) showed variable and weak silencing of CH42, but strong symptoms.

is also possible that post-translational events caused differential loss of carotenoids in the infected plants.

Silencing multiple genes

Silencing of multiple genes with a single viral construct would allow silencing of a gene of unknown function to be linked to silencing of a gene with a visible and predictable phenocopy. Silencing multiple genes would also allow identification of genes of possible redundant function. If multiple genes in a pathway were silenced, enhanced phenotypes might be achieved compared to silencing a single gene in the pathway, and epistatic interactions could be determined. Although the results for *AR1*-deleted CbLCV vectors carrying both *PDS* and *CH42* sequences strongly suggested silencing of two genes, an additional

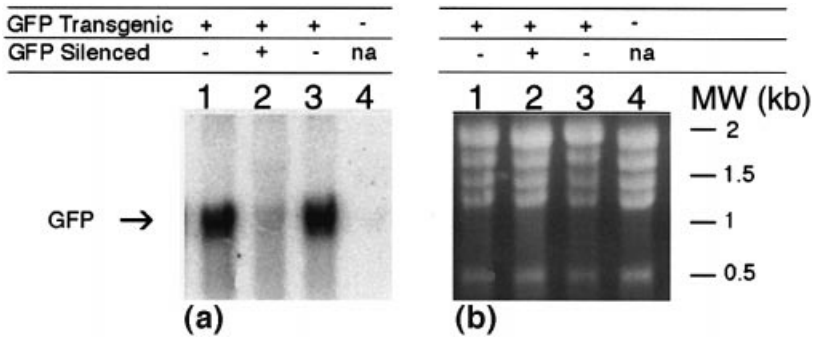

Figure 3. Silencing of *GFP* in transgenic *Arabidopsis* requires homologous sequence in the CbLCV vector.
RNA was isolated from *GFP* transgenic plants that were uninfected, lane 1; infected with CbLCV A::GFP and silenced for *GFP*, lane 2; infected with empty vector (*AR1* deletion), lane 3; and from non-transgenic *Arabidopsis*, lane 4. All infected plants also contained the CbLCV B component.
(a) RNA transferred to nitrocellulose and hybridized to ^{32}P-labeled *GFP* sequence. Arrow indicates position of the *GFP* transcript.
(b) Ethidium stained gel for loading reference.

set of genes with easily distinguishable phenotypes was tested.

A second *AR1*-deleted CbLCV A vector was constructed that carried fragments of the *GFP* transgene and the *CH42* endogenous gene, and was used to infect *Arabidopsis* carrying a 35S::*mGFP5* transgene (control plant, Figure 4c,d). Approximately 4 weeks post-bombardment, new growth of the *Arabidopsis* plants showed symptoms and loss of both GFP fluorescence and chlorophyll, indicating that both the *GFP* transgene and the *CH42* endogenous genes were silenced (Figure 4e,f). *GFP* transgenic plants bombarded with virus vector containing only the *CH42* fragment produced yellow tissue in new growth, but were still strongly fluorescent for GFP when viewed with UV illumination (Figure 4a,b), demonstrating the sequence specificity of the silencing response.

Silencing from a CbLCV B component vector is not extensive

Previous results for TGMV suggested that the B component might serve as a better vector for silencing initiation and spread (Peele *et al.*, 2001). To determine if a similar vector constructed from the B component of CbLCV would be effective in silencing, a 144 bp fragment of the *CH42* gene was inserted downstream of the *BR1* gene and before the putative polyadenylation site. Co-bombardment of this vector with the *AR1*-deleted CbLCV vector produced plants that showed symptoms, but weak initiation and spread of silencing (Figure 5c,d). While in the TGMV/*N. benthamiana* virus host combination the *AR1*-deleted A component vector produces limited spread of endogenous gene silencing, while the B component vector shows extensive spread. The opposite response was found for CbLCV: the *AR1*-deleted A component vector showed extensive spread (Figure 5a), while silencing from the B component vector was limited and patchy (Figure 5b,c). These results suggest that each geminivirus/host combination must be optimized for producing effective silencing vectors.

General utility of the system

As with other silencing systems, certain cautions are in order in evaluating the phenotype caused by virus-induced gene silencing. First, symptoms caused by viral infection, although reduced compared to a non-modified virus, are still noticeable and could interfere with interpretation of the silencing phenotype. Plants infected at early stages of development (four- to six-leaf stage) showed extensive silencing in rosette leaves, but also showed stunted growth and often did not produce inflorescences, flowers or seed. Infecting mature plants (newly formed rosettes) produced less severe symptoms and silencing (for *CH42*) which extended into the inflorescence stems and siliques (data not shown). Silencing was not transmitted through the seed to subsequent generations.

One of the goals of our research is to develop a silencing system that will easily transfer to economically important crop species, allowing the use of virus-induced gene silencing to determine the function of genes in these plants. The begomoviruses have a similar gene organization within species and infect a wide range of crop plants. We transferred silencing technology from TGMV vectors to the distantly related CbLCV (Table 1). Many of the new world isolates of Begomoviruses, covering a wide host range, have amino acid sequences no more divergent from CbLCV than is TGMV (Table 1), suggesting that these viruses will be amenable to development as silencing vectors. Although the sequences of the old world isolates of Begomoviruses are more divergent, their similar genetic structure should allow these viruses to be developed as silencing vectors, applying the information we have gained from CbLCV and TGMV.

We recently described the silencing of a DNA replication associated gene, *proliferating cell nuclear antigen* (*PCNA*), using TGMV vectors (Peele *et al.*, 2001). The ability of a geminivirus to silence a meristematic gene was surprising because TGMV does not penetrate the meristem; however, silencing generated by the TGMV vector extended throughout meristematic tissues, as demonstrated by

immunolocalization of PCNA. If CbLCV vectors such as TGMV can be used to initiate silencing of meristematic genes, this system will be a powerful tool for studying processes such as cell-cycle control and differentiation.

Experimental procedures

Bombardments

Arabidopsis plants (ecotype Columbia) were co-bombarded with equal mixtures of A and B component vectors, as previously described for *Nicotiana benthamiana* (Kjemtrup *et al.*, 1998), using a Bio-Rad PDS 1000 He Particle gun. Briefly, 5 μg each of A and B component plasmids were added to 3 mg gold particles followed by 50 μl 2.5 M $CaCl_2$ and 20 μl 0.1 M spermidine. Microcarrier holders and surfaces of the bombarding apparatus were disinfected with 5% benzalkonium chloride before and after use, and between bombardments with different constructs. For experiments with seedlings, the plate or pot containing the seedlings was bombarded twice, with the pot or plate rotated 90° between bombardments

In some experiments, *Arabidopsis* seedlings were grown aseptically on agar-based media. *Arabidopsis* seeds were sterilized by soaking for 7 min in 50% bleach, rinsed with three changes of sterile water, and resuspended in 0.1% type M agar (Sigma: http://www.sigmaaldrich.com). Individual seeds were transferred to the surface of a Petri plate containing MS media, 2% sucrose and 1% agar using a 200 μl pipetteman. Twelve to 20 seeds were transferred to each plate in a 5 cm diameter area. Seedlings were allowed to grow with 8 h light at 22°C, 16 h dark at 20°C. When the seedlings reached the four- to six-leaf stage (≈6 weeks), plants were infected with the CbLCV constructs by bombardment. Three to five days post-bombardment the seedlings were individually transplanted to 5 cm pots containing Promix potting soil, and the plants were allowed to continue growing under the same light and temperature regime. Using these conditions, approximately half the plants were infected by virus.

An alternative method for bombarding seedlings was used in several experiments, in which seeds were sown in a 7.5 cm pot (approximately 15–20 seeds per pot). The seedlings were allowed to grow under 8 h light at 22°C, 16 h dark at 20°C. When the seedlings reached the four- to six-leaf stage (10–12-leaf stage in one experiment), the plants were infected with the CbLCV constructs. Ten to 15 days post-bombardment, the seedlings were individually transplanted to 2′ pots and the plants allowed grow under the same light and temperature regime. Using these conditions, approximately a third to half the plants were infected by virus.

To infect mature plants, plants were individually grown in 3′ pots with 8 h light at 22°C, 16 h dark at 20°C until the rosette covered the surface of the pot. Each pot was bombarded using our standard procedure (see above), and plants were allowed to grow under the same light and temperature conditions. This procedure resulted in 100% infection and silencing, seen in inflorescences and siliques.

A component vector construction

Plasmids containing the A (GenBank accession number U65529) and B (GenBank accession number U65530) components of CbLCV were the generous gift of Ernest Hiebert and James Strandberg, University of Florida at Gainesville.

The CbLCV A genome partial duplication was made in two cloning steps. First, a 1.6 kB *Eco*RI/*Hin*dII fragment of CbLCV A was isolated and ligated into pBluescript SK+ II that had been digested with *Eco*RI and *Hin*dII. The resulting plasmid was subsequently digested with *Kpn*I and *Hin*cII, the ends blunted with Klenow polymerase, and the plasmid recircularized. This destroyed the *Hin*cII, *Xho*I and *Acc*65I sites in the pBluescript polylinker. This plasmid, pCPCbLCVA.001, contained the common region and the *AL1* coding region of CbLCV A.

In the second cloning step, a 1.8 kB *Eco*RI/*Xmn*I fragment of CbLCV A was isolated and ligated into pCPCbLCVA.001, digested with *Eco*RI and *Sma*I. This plasmid, pCPCbLCVA.003, contained the entire CbLCV A genome plus a duplicated common region. Deletion of the *AR1* gene and introduction of a polylinker was accomplished by replacing the *AR1* sequences with two PCR generated fragments. pCPCbLCVA.003 was digested with *Bam*HI and *Bgl*II, removing one copy of the duplicated common region, the coding region of *AR1*, and the 5′ terminus of the *AL3* coding sequence. The larger fragment, composed of pBluescript with a single common region and *AL1*, was isolated. A 133 bp DNA fragment was produced by PCR amplification using CbLCV-3 (5′-GGA CGG ATC CTA TGT AAC TC-3′) and CbLCV-4 (5′-GCT AGC GGT ACC CTC GAG TCT AGA GGA TCC AGA TCT GAG CTC GCT AGC CTA ATC CTG TGT ATG CG-3′) followed by *Bam*HI digestion. This 133 bp fragment, containing the 3′ terminus of the *AL3* coding sequence and several introduced restriction sites, was ligated into the *Bam*HI/*Bgl*II digested pCPCbLCV.003, restoring the open reading frame of *AL3* to create pCbLCVA.005. A 435 bp DNA fragment, containing a copy of the viral common region and several introduced restriction sites, was produced by PCR using primers CbLCV-1 (5′-ACT AGT GGA TCC CCC G-3′) and CbLCV-2 (5′-GCT AGC GAG CTC AGA TCT GGA TCT AGA CTC GAG GGT ACC GCT AGC CGC TTA GGC ATT TCG-3′). This 453 bp fragment was digested with *Bam*HI and ligated into *Bam*HI-digested pCPCbLCVA.005 to produce pCPCbLCVA.007. pCPCbLCVA.007 contains the partially duplicated CbLCV A genome (with two common regions), but has had the coding region of the *AR1* gene replaced with a polylinker to facilitate insertion of foreign DNA fragments. The *AR1* promoter and the translational start (ATG) for the *AR1* gene are present upstream of the polylinker.

A-component plasmids used for silencing were constructed by introducing fragments of the gene to be silenced into the polycloning site of the *AR1*-deleted A genome vector (pCPCbLCV.007). For silencing of the *Chlorata 42* gene, a 360 nucleotide *Xba*I and *Bgl*II fragment of the *CH42* gene was generated using pPCV002GC as template. Primers CH42-1R (5′-ACT GTT AGA TCT TTA GTT GAT CTG-3′) and CH42-1 L (5′-AAT CCC TTC TCT AGA AAC CGT ATT CCA ACC-3′) were used to introduce the flanking restriction sites used for cloning into pCPCbLCV.007 to produce plasmid pMTCbLCVA::CH42.

For *GFP* silencing, a 388 nucleotide fragment from pBINmGFP5ER was amplified by PCR using primers GFP-1011 L (5′-GTT GGC TTT GGT ACC GTT CTT TTG CTT GTC GGC-3′) and GFP-1011R (5′-TTT CTG TCA GTC TAG AGG GTG AAG GTG ATG C-3′) to introduce restriction sites for *Acc*65I and *Xba*I. These sites were used in pCPCbLCV.007 to produce plasmid pMTCbLCVA:: GFP, and in pMTCbLCVA::CH42 to produce pMTCbLCVA:: CH42GFP.

For silencing of *PDS*, a 370 nucleotide fragment was produced via reverse transcription of 3 μg total *Arabidopsis* RNA followed by PCR using the Gene Amp RNA PCR kit (http://www.appliedbiosystems.com). Primers PDS-1 (5′-CTG AGG TAC CGC ACT TTC ATC TGG AGG TTG-3′) and PDS-2 (5′-ATC CTC TAG ACC AGT CCC CAT CTT CAT CC-3′) containing sites for *Acc*65I and

*Xba*I were used to introduce the fragment into plasmid pCPCbLCV.007 to produce pMTCbLCVA::PDS, and into pMTCbLCVA::CH42 to produce pMTCbLCVA::CH42PDS.

Construction of CbLCV B component vectors

Partial duplication of the CbLCV B component was made in two steps. First, a 1.4 kB *Eco*RI/*Eco*RV fragment of CbLCV B was isolated which contained the common region of the virus and part of the *BR1* coding region. This fragment was ligated into *Eco*RI- and *Sma*I-digested pBluescript SK+ II, to produce pCPCbLCVB.001. In the second step, a 2.5 kb *Eco*RI fragment consisting of the entire CbLCV B genome was isolated from the original clone provided by Dr Heibert. This fragment was ligated into *Eco*RI linearized plasmid pCPCbLCVB.001, producing pCPCbLCVB.002. This plasmid contains a partial duplication of the B component of the CbLCV genome, with duplications of the common region and part of the *BR1* gene.

The B component vector pNMCbLCVB was made by ligation of a palindromic 10 bp oligonucleotide AAGGTACCTT, containing an embedded *Acc*651 site, into pCPCbLCVB.003 at the *Hin*cII site. The oligonucleotide reconstructed the *BR1* stop codon while allowing foreign DNA to be inserted downstream of *BR1* and before the putative polyadenylation site. pNMCbLCVB::CH42 was made by was digestion of pNMCbLCVB with *Acc*651, addition of nucleotides using Klenow polymerase and ligation with a 144 bp *CH42* fragment. The *CH42* fragment was obtained using *Eco*RV and *Bam*HI digestion of pPCV002GC, rendered blunt via Klenow DNA polymerase. The presence of *CH42* sequence was confirmed both by PCR amplification and sequencing.

Photography

CbLCV-infected plants were photographed by using a Kodak digital camera, DC290. Images were processed using Adobe PHOTOSHOP.

Vector availability

The vectors described in this paper will be made freely available to other investigators for research use. It should be noted that the CbLCV is a pathogen on certain crop plants, and its importation into some regions may be discouraged or prohibited. We have removed the coat protein from the A component vector. This would prevent transmission by whiteflies, which is the normal means of transmission of this group of viruses. It is possible that a recombination between this vector and a wild geminivirus could result in an insect-transmissible CbLCV with a coat protein from another geminivirus. It is expected that users of this system will observe the necessary containment measures to prevent such recombination from occurring.

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Chapter 4

Geminivirus-induced gene silencing uses a unique genetic pathway

Muangsan, Nooduan; Beclin, Christophe; Turnage, Michael; Vaucheret, Herve, and Robertson, Dominique.

Nooduan Muangsan performed all the experiments except generating transgenic lines

Key Words: cabbage leaf curl virus, gene silencing, Arabidopsis, mechanism

Abstract

Virus-induced gene silencing (VIGS) is a sequence-specific RNA degradation process in higher plants that can occur if there is homology between plant genes and sequences carried by the viral vector. This study sought to identify genetic components of silencing mediated by the DNA geminivirus, Cabbage leaf curl virus (CbLCV). Several mutant lines of Arabidopsis known to be defective in transgene and endogenous gene silencing were tested for geminivirus-induced gene silencing (G-VIGS) of an endogenous gene, CH42. SGS1, SGS2, SGS3 and AGO1 are known to be required for silencing induced by high level transcription of a single-copy sense transgene (S-PTGS) [1-3]. In contrast, SGS2, SGS3 or AGO1 (as tested) are not required for silencing mediated by inverted repeats (IR-PTGS) [4]. We found that only SGS2 and SGS3 are required for G-VIGS mediated by CbLCV vectors, independent of the orientation of transcripts produced from the viral promoter. The onset and spread of silencing was greatly reduced in the *sgs2* and *sgs3* mutants, suggesting that SGS2 and SGS3 play a major role in the induction and maintenance of G-VIGS. SGS2 [2] is known to encode one member of a family of RNA dependent RNA polymerases (RdRP) in Arabidopsis. However, we found that expression of a tomato RdRP could not replace the SGS2 function. Finally, G-VIGS was unaffected in mutants affecting transcriptional gene silencing (TGS), *som* and *mom*, supporting the idea that G-VIGS is PTGS-specific. Taken together, these results suggest that silencing mediated by geminiviruses has requirements that are distinct from previously described silencing pathways in plants.

Introduction

Homologous-dependent gene silencing (HDGS) is a genetic mechanism found in diverse organisms that controls RNA levels either by transcriptional gene silencing (TGS), or by post-transcriptional gene silencing (PTGS) [5, 6]. Several studies have demonstrated that TGS and PTGS are mechanistically related. First, both processes are affected by DNA methylation and changes in chromatin structure [7-9]. Second, dsRNA encoded by either transgenes or viruses can induce TGS or PTGS, depending on whether the dsRNA has sequence homology with the promoter or the open reading frame (ORF) sequence [9-11]. Finally, PTGS and in some cases TGS, are associated with the accumulation of small RNAs (21-25 nucleotides) [8, 12, 13]. Therefore, there may be a common underlying mechanism between these two phenomena [8, 14].

Virus-induced gene silencing (VIGS) is similar to PTGS in that RNAs are specifically degraded if there is a similar sequence between viruses and plant genes [15, 16]. The degradation includes both plant and viral RNAs [9, 17, 18]. As a result, a sequence specific silencing signal is produced that is able to propagate from cell to cell and at long distances [17, 19-21]. It should be noted that, in plants, the long distance silencing signal has only been observed with transgenes. Twenty-one to twenty-five nucleotide RNAs, corresponding to the sense and antisense strands of the target RNAs, and methylation of coding sequence are both associated with VIGS [9, 12, 18, 22, 23]. In later stages, VIGS leads to the elimination of RNA viruses which may occur by the same mechanism as recovery [17, 24, 25]. In fact, PTGS is thought to represent a natural mechanism for plant protection against viruses and transposons [2-3, 26-28].

PTGS exists among various organisms, e.g., quelling in fungi [29] and RNA interference (RNAi) in *Caenorhabditis elegans* and *Drosophila* [30, 31]. Recently, gene silencing has become one of the most valuable tools for reverse genetic studies because it gives researchers a rapid way to determine the loss-of-function phenotype for a gene [32, 33].

Several genes associated with PTGS have been identified in a number of systems. These include Arabidopsis *sde2, sde3, sde4, sgs1, sgs2/sde1, sgs3* and *ago1* genes [1-3, 34-35]. Similar genes were also found in *Neurospora crassa* e.g. *qde* [29], and *C. elegans* e.g. *rde*, *mut* and *ego-1* [28, 31, 36] indicating that PTGS, quelling, and RNAi are evolutionally and mechanistically related. In Arabidopsis, some PTGS-deficient mutants such as the *sgs* and *sde* genes are morphologically normal, while the *ago1* mutant shows severe developmental abnormalities including sterility. It is now clear that a PTGS-related mechanism serves as a general mechanism for regulating plant development, and naturally occurring microRNAs have been found in plants [37].

Furthermore, TGS-deficient mutants have been identified including *ddm1*/*som, mom* and *met1* [38-40]. Both *ddm1/som* and *mom* mutant genes encode chromatin-associated proteins belonging to the SWI2/SNF2 family but only ddm1/som shows decreased methylation [38-40]. MET1 encodes a methyltransferase that is required for maintenance of methylation [23]. Morel et al. [7] reported that the *ddm1* and *met1* mutations did not only affect TGS, but also PTGS. They concluded that DNA methylation does seem to play a major role in the TGS and PTGS silencing pathway; however, its function is far from clear.

DNA viruses have been used to trigger a PTGS-like mechanism [16, 41, 42]. However, the silencing mechanism associated with geminiviruses is not well understood. RNA viruses replicate in the cytoplasm and are themselves targets of PTGS. In contrast,

geminiviruses replicate in nuclei and their genomes are not susceptible to PTGS. Silencing fragments encoded by geminiviruses are transcribed using RNA polymerase II and are polyadenylated. It is not known whether DNA genomes are targets of silencing although anti-silencing proteins from geminiviruses have been reported.

We were interested in identifying genetic components required for gene silencing mediated by a DNA geminivirus, CbLCV. The genetic requirements for geminivirus-induced gene silencing (G-VIGS) were investigated by testing several Arabidopsis mutants defective in either PTGS or TGS using CbLCV-derived vectors carrying a CH42 fragment. The mutants tested included *sgs1*, *sgs2*, *sgs3* [1, 2], *ago1* [3], *som* [43], and *mom* [40]. G-VIGS was inhibited in only two of the mutants. Our results genetically distinguish geminivirus mediated silencing from both S-PTGS and IR-PTGS.

Results

Inhibition of G-VIGS in sgs2 and sgs3 mutants, but not in sgs1 and ago1 mutants

Geminiviruses may induce silencing when mRNA produced from their genome has homology to RNA from another locus or by the production of dsRNA following readthrough of the viral 3'sequences opposite each other in the circular geminivirus genome [42]. We tested mutants known to be required for transgene silencing in Arabidopsis for their responses to G-VIGS of an endogenous plant gene. Previous work identified four classes of Arabidopsis mutants deficient in PTGS after ethyl methanesulfonate mutagenesis of a silenced 35S-GUS line, L1 [1, 3]. The *sgs1*, *sgs2*, *sgs3*, and *ago1* mutants each released PTGS of a highly transcribed sense transgene, the GUS transgene, and a nitrate reductase (*Nia*) transgene and its homologous host gene. Release was correlated with a decrease of

transgene methylation, indicating that all four genes regulate both RNA degradation and DNA methylation [1-3]. The mutants we tested were single-locus homozygous lines.

To determine if the *SGS1, SGS2, SGS3,* and *AGO1* genes were required for G-VIGS, we inoculated four-week-old seedlings of *sgs1-1, sgs2-1, sgs3-1, ago1-27* mutants and a silencing competent control, the L1 parental line containing a silenced GUS transgene, with a combination of CbLCV A carrying a fragment of the *chlorata42* (*CH42*) endogenous gene [42, 44] in place of the coat protein coding sequence, and wild type CbLCV B. *CH42* encodes a subunit of magnesium chelatase that is required for chlorophyll biosynthesis [42], thus the loss of gene expression can be easily seen as yellow regions on the leaves. These areas lack chlorophyll and are a visible marker for silencing.

We first tested a 360 bp fragment of *CH42* in antisense and sense orientations. Figure 1 shows silencing phenotypes at 25 days post inoculation (dpi). CbLCV::CH42-infected L1, *sgs1-1, ago1-27* plants showed extensive silencing (most or all systemically infected leaves were yellow), whereas infected *sgs2-1* and *sgs3-1* plants had less extensive silencing as seen with light green tissue in new growth. Additionally, infected *sgs2-1* and *sgs3-1* had little new growth and severe symptoms than other infected lines, demonstrating that these mutants were susceptible for CbLCV::CH42 infection. Similar phenotypes were obtained with CbLCV carrying the CH42 fragment in either antisense or sense orientations. The analysis of total chlorophyll content from systemically infected leaves also demonstrated that the loss of chlorophyll was more extensive in the infected L1, *sgs1-1* and *ago1-27* plants than in the infected *sgs2-1* and *sgs3-1* mutants (Fig. 2).

The extent of *CH42* silencing in the course of plant development in the infected mutants was closely observed. The percentage of infected plants showing silencing over

three experimental time periods (15, 20 and 25 dpi) increased during development for each genotype. In Figure 3A, two important features are noted. First, there is a delay of silencing initiation in the CbLCV::CH42-infected *sgs2-1* and *sgs3-1* plants. Second, the percentage of infected *sgs1-1* and *ago1-27* plants showing a delay was much lower; a smaller percentage showed silencing at 15 dpi (60% and 38%, respectively) compared to the L1 (83%). SGS1 or AGO1 mutants might be impaired for the establishment of G-VIGS. Similar results were found with CbLCV carrying the CH42 fragment in either antisense or sense orientations (Fig. 3A and 3B).

Previous work (Muangsan, Turnage and Robertson, unpublished) demonstrated that silencing phenotypes could vary from plant to plant in the same background line, depending on the age of seedlings or geminating conditions (on sterile medium or in soil) used for bombardment. For example, CbLCV::CH42-infected younger seedlings had more extensive silencing and severe symptoms than infected older seedlings. However, we found that similar results were obtained when four-week-old seedlings germinated on medium or six-week-old seedlings germinated in soil were used. In one experiment where six-week-old seedlings of *sgs2-1* mutant were used, only light green tissues were observed in new growth of the *sgs2-1* mutants that were inoculated with CbLCV::CH42, for both the antisense and sense constructs (Fig. 4). The persistence of some chlorophyll in all tissues was still consistent with earlier experiments showing that silencing was not totally abolished in the *sgs2-1*, but occurred with very low efficiency.

Taken together, our results show that G-VIGS was greatly reduced in the *sgs2* and *sgs3* mutants, but was not significantly different in the *sgs1* or *ago1* mutants compared to the L1 line that was actively silencing GUS by PTGS. There were no significant differences in

initiation or spread of *CH42* silencing between antisense and sense transcripts in these four mutants.

Enhanced silencing in Arabidopsis transgenic plants over-expressing SGS2

We showed above that G-VIGS of *CH42* was inhibited but not abolished in the *sgs2-1* mutant suggesting that G-VIGS was activated (at very low levels) in the absence of the SGS2 gene coding for one member of RdRP family in Arabidopsis. One of the possible roles of SGS2, as proposed by Matzke et al. and Sijen et al. [45, 65], was that it might be involved in the amplification of silencing. We anticipated that more silencing would be obtained if plants had more SGS2. Transgenic plants expressing SGS2 under the control of the 35S promoter (wt:L1:35S-SGS2) were tested, along with the *sgs2-1* mutant and line L1 using the same CbLCV::CH42 vector constructs described earlier. The 35S-SGS2 construct is functional. The L1 and *sgs2-1* transgenic lines were germinated on MS plates while the wt:L1:35S-SGS2 (heterozygous) were germinated on MS plates containing hygromycin (30 µg/ml), bombarded and then transferred to soil three days later. Most CbLCV::CH42-infected L1 and wt:L1:35S-SGS2 plants exhibited extensive silencing, while infected *sgs2-1* plants had weak silencing (Fig. 5).

In fact, CbLCV::CH42 mediated silencing in the infected wt:L1:35S-SGS2 plants was more extensive than in the infected L1 plants. The chlorophyll content of CbLCV::CH42-infected wt:L1:35S-SGS2 plants were approximately half of the amount in infected L1 plants, independent of *CH42* insert orientation (Fig. 6). We also found that silencing of *CH42* was initiated earlier (11 dpi) in the infected wt:L1: 35S-SGS2 plants (data not shown). Moreover, CbLCV::CH42-infected *sgs2-1* plants had little new growth and died prematurely. In contrast, the infected wt:L1:35S-SGS2 plants had attenuated symptoms and

lived longer than the infected L1 plants during the experimental period (47 dpi) (data not shown).

Some of the CbLCV::CH42-infected wt:L1:35S-SGS2 plants (7 out of 24 plants) from both the antisense and sense constructs showed a reappearance of green tissue in new growth at 20-25 dpi (Fig. 7A), suggesting that *CH42* silencing was inhibited. Newly initiated green leaves were more symptomatic than yellow leaves in individual reverting plants. Southern blot analysis of total DNA isolated from green or yellow tissues of these reverting plants demonstrated that the inhibition of silencing in green tissue was not due to the loss of viral DNA or inserted *CH42* fragments. Viral DNA of similar size was detected in both green and yellow tissues (Fig. 7B). At 47 dpi, only three of seven reverting plants were viable and each of these were inoculated with CbLCV::CH42 (sense orientation) (Fig. 7C). Because wt:L1:35S-SGS2 plants contained both 35S-GUS and 35S-SGS2 transgenes and originally produced inactive GUS due to PTGS, we asked if PTGS of GUS was inhibited with CbLCV::CH42 infection. Northern blot analysis of total RNA isolated from the CbLCV::CH42-infected wt:L1:35S-SGS2, reverting plants and *CH42* silencing plants, revealed the presence of *GUS* mRNA in the reverting plants but not in the *CH42* silencing plants (Fig. 7D). Thus it indicated that the PTGS of *GUS* was also inhibited. We reasoned that *SGS2* was probably cosuppressed in the reverting plants, possibly due to homology between the 35S CaMV promoter of both GUS and SGS2 transgenes or highly transcribed *SGS2* transgene.

Tomato RdRP had no effect on G-VIGS

The tomato RdRP (LeRdRP) [46] has similarity (37 % amino acid identity) to SGS2 in Arabidopsis [2] using BLAST 2 Sequences program [47], but the similarity extends

throughout the protein sequence, including a putative RNA recognition motif (RRM) in the N-terminus. The tomato RdRP was also found to be similar to EGO1 in *C. elegans* (27% identity) [36], and to QDE-1 in *N. crassa* (24% identity) [29], suggesting that it is likely to have a crucial role in the silencing mechanism. To investigate the role of this enzyme in G-VIGS, we hypothesized that if the tomato RdRP was required for G-VIGS, it would complement the *sgs2* mutant. The *sgs2-1* line and the same line complemented 35S-SGS2 or 35S-LeRdRP transgene were germinated on MS plates containing no antibiotics, hygromycin (30 µg/ml), or kanamycin (50 µg/ml), respectively, for four weeks until they had 6-10 leaves. They were then bombarded with both antisense and sense forms of CbLCV::CH42 as described earlier, and transferred to soil three days later. The 35S-SGS2 transgene restored *CH42* silencing, but the 35S-LeRdRP did not (Fig. 8). Similar phenotypes, light green or green tissues instead of yellow tissues and relatively high chlorophyll content, were observed in the *sgs2-1* mutant and the sgs2-1::35S-LeRdRP transformants. Three different lines of sgs2-1::35S-LeRdRP, all heterozygous for the 35S-LeRdRP transgene, were tested with similar results.

SOM and MOM were not required for G-VIGS

SOM/DDM1 and MOM are associated with the maintenance of TGS and their mutants can reactivate silenced genes [38, 40, 43]. The SOM/DDM1 locus encodes a protein belonging to the SWI2/SNF2 family that plays a role in chromatin remodeling, while MOM encodes a protein with no overall homology to any known protein, but has a region related to the SWI2/SNF2 family. While TGS release was correlated with decreased level of DNA methylation in *ddm1/som* mutants, no alteration in the methylation pattern of a 35S-*HPT* transgene locus in *mom* mutants was observed.

When *som8* and *mom* mutants were inoculated with CbLCV::CH42, *CH42* silencing was as extensive as the wt parental line A control and was maintained for at least 6 weeks (Fig. 9). However, in a few ddm1/som8 plants (less than five percent), CbLCV::CH42 DNA accumulated but there was no visible loss of chlorophyll. DNA gel blot hybridization showed that the CbLCV vector still carried the *CH42* insert and high levels of virus were present (Sanchez and Robertson, unpublished). These results demonstrated that SOM/DDM1 and MOM are not required for G-VIGS. Because som/*ddm1* causes other mutant phenotypes [43], we conclude that in a low proportion of plants a second locus was responsible for the lack of silencing in these plants. In contrast, out of 60 bombarded plants, there was a 100% correlation between silencing and presence of the virus in *mom* mutants.

Discussion

CbLCV is a member of the begomovirus group of geminiviruses which replicate in plant nuclei via dsDNA intermediates [48]. Its transcription process is similar to other geminiviruses that rely on host genes such as RNA polymerase II, and transcription occurs bi-directionally from a common region in both circular genomes [49-51]. In contrast, most RNA viruses replicate in the cytoplasm using a virally encoded RNA polymerase [52]. Vectors derived from geminiviruses can activate silencing of both transgene and endogenous genes of *Nicotiana benthamiana* and Arabidopsis [16, 42] if they carry homologous sequences. Unlike RNA viruses, geminiviruses cannot be eliminated from the plants by silencing, perhaps because circular DNA is not known to be degraded by the host.

Geminivirus-derived vectors trigger silencing in a sequence-specific manner resulting in mRNA degradation, known as PTGS [16, 41]. Because the geminivirus genes are

transcribed in plant nuclei, it is not known whether RNA degradation occurs in the nucleus or the cytoplasm. In the CbLCV vector used here, the AR1 ORF was replaced by a *CH42* fragment, such that *CH42* in sense or antisense orientation was transcribed from the AR1 promoter and polyadenylated by the host [42]. It has been proposed that silencing mediated by geminiviruses occurs either by the high activity of the AR1 promoter or by dsRNA produced by readthrough of the 3' region of transcripts from the rightward and leftward promoters [50, 53]. Readthrough transcripts have been detected from the TGMV A component carrying a silencing fragment (Peele and Robertson, unpublished).

We demonstrate here that G-VIGS is mediated by some of the known genetic components of plant gene silencing. We tested several silencing defective mutants based on the hypothesis that G-VIGS would be inhibited if a host gene required for silencing was mutated. Here we show that, regardless of orientation of transcripts derived from the viral promoter, *SGS2 (SDE1)* and *SGS3* were essential for G-VIGS of an endogenous gene, while *SGS1*, *AGO1*, *SOM* and *MOM* had minimal impact on silencing. This distinguishes G-VIGS from S-PTGS, IR-PTGS, and RNA-VIGS (Table 1).

The finding that G-VIGS was affected in a similar manner regardless of the orientation of silencing fragment suggests that antisense and sense transcripts from the virus initiate silencing using a similar mechanism. This result is similar to previous results showing that sense and antisense mRNAs were effectively targeted for PTGS-induced RNA degradation [54-56]. Considering these results, it is likely that dsRNA produced by the virus acts as an inducing signal for silencing.

Previous experiments (Muangsan and Robertson, unpublished) showed that *CH42* antisense transcripts derived from the viral promoter gave more extensive silencing than

CH42 sense transcripts at the initiation stage but not at the maintenance stage when wt Arabidopsis plants were used. This result suggested that silencing might be initially transcript-specific. However, this difference was not significant when the transgenic line L1 was used. L1 was chosen as the parental line for a selection that identified *sgs1, sgs2, sgs3* and *ago1,* and silences GUS by PTGS [1, 3]. It is possible that the difference in initiation of silencing in wild type plants was not seen in L1 due to previously activated silencing machinery.

Several models have been proposed to describe PTGS mechanisms [6, 14, 53, 57]. The basic process involves dsRNA production and degradation of this molecule by Dicer, a member of the RNaseIII family of nucleases in *Drosophlila* [58, 59]. Dicer is thought to produce small interference RNA (siRNA) [12, 60, 61]. It is still not understood how dsRNA is produced or what role siRNA has in the silencing process.

RdRP was shown to be required for PTGS ([2, 29, 36, 62] and this work). Silencing of the *CH42* endogenous gene mediated by CbLCV vectors was greatly reduced in *sgs2-1* mutant, whereas it was enhanced in transgenic plants containing 35S-SGS2. The requirement of an RdRP for G-VIGS suggests that dsRNA mediated silencing of an endogenous gene requires RdRP for amplification of the silencing signal or that dsRNA may need an RdRP to interact or target homologous RNAs. So far, the templates for RdRP and the requirements for sequence specific RNA production are not known [63]. siRNA might serve as a primer for RdRP to produce dsRNAs. Lipardi et al. [64] and Sijen et al. [65] reported that RdRPs do require a primer in vitro and in vivo, respectively. However, evidence that RdRP might be primer-independent for dsRNA production has also been presented [46]. Additionally, it is possible that RdRPs might have a distinct role in each specific organism, or on the specific

targets. The finding that tomato RdRP was not required for G-VIGS supports the ideas that RdRP is species-specific and/or different RdRPs might have different actions. Whether or not tomato RdRP is required for silencing in tomato has not yet been determined.

We also show that G-VIGS was inhibited, but not abolished, in *sgs2-1* and *sgs3-1* mutants, implying that silencing could occur in the absence of SGS2 or SGS3. The weak silencing may be due to the presence of a small amount of dsRNA formed by readthrough transcripts from the viral genome, bypassing the requirement for RdRP. SGS2 and SGS3 were dispensable for IR-PTGS due to the large amount of hairpin RNA produced by transcription of inverted repeats [4].

The SGS1, AGO1, SOM and MOM genes were dispensable for silencing mediated by CbLCV. However, the induction of the silencing was affected in both the *sgs1* and *ago1* mutants. *Sgs1* and *ago1* mutants showed developmental abnormalities including slow growth that could affect initiation and spreading of silencing. Moreover, it is known that CbLCV vectors replicate in plant nuclei and do not integrate into plant chromosomes, therefore chromatin configuration, which is affected by mutations of *mom* and *som,* may not affect G-VIGS.

Considering these results and others shown in Table 1, the findings that SGS2 (SDE1) and SGS3 but not SGS1 and AGO1 are required for G-VIGS indicate that G-VIGS uses a unique genetic pathway. The genetic requirements of G-VIGS are not equivalent to S-PTGS, IR-PTGS or RNA-VIGS but they all share some PTGS components. Here we propose a model for G-VIGS. G-VIGS occurs as result of highly transcribed RNAs (antisense or sense transcripts), coupled with dsRNA produced from readthrough transcription of the viral genes. The Arabidopsis specific RdRP protein encoded by the SGS2 (SDE1) gene may use such

RNAs as templates to synthesize dsRNA, which is then subjected to RNase digestion. SGS3 is also needed for initiation and maintenance of G-VIGS and may facilitate the RdRP activity of SGS2 (SDE1) by supporting reverse transcription of RNAs. Concurrently, a small amount dsRNA formed by readthrough transcripts, which bypass SGS2 and SGS3 actions, are directly processed into the degradation step. Alternatively, dsRNA produced from readthrough transcription could be a potent activator that is absolutely required for G-VIGS. In this case, a small amount of these molecules give weak silencing but silencing signals can be amplified in the presence of SGS2 and SGS3 leading to extensive silencing. In *Drosophila*, injection of a few molecules of dsRNA per cell resulted in extensive silencing that persisted to a next generation, suggesting that silencing signals are amplified, possibly due to cellular RdRPs [58]. Modifications of the CbLCV vector to prevent bidirectional transcription may help to resolve some of these questions.

Materials and Methods

Plant materials

The *sgs1-1, sgs2-1, sgs3-1, ago1-27* mutations were recovered after ethylmethane sulfonate (EMS) mutagenesis followed by a screen based on the reactivation of the PTGS-silenced sense 35S-GUS transgene at L1 locus [1, 3]. Line L1 carrying a single copy 35S-GUS transgene was silenced of GUS transgene. All mutants are homozygous for both L1 and the mutations. The *sgs2-1*::35S-SGS2 and sgs2-1::35S-LeRdRP are the *sgs2-1* mutants that were transformed with either 35S-SGS2 or 35S-LeRdRP from tomato, respectively. The generation of transgenic lines has been described in the literature [1, 3]. Seeds of *som8* and *mom* mutants were kindly provided by Dr. Paszkowski (Friedrich Miescher Institute, Switzerland). Briefly, the *som* mutants were obtained by the EMS mutagenesis of seeds with a stably silenced locus containing multiple copies of the hygromycin phosphotransferase gene (*hpt*). The *mom* mutants were recovered after random insertion of *Agrobacterium* transferred DNA (T-DNA) of the Arabidopsis transgenic line containing a 35S-*HPT* transgene locus that is hypermethylated and transcriptionally silenced (line A). The generation of *som* and *mom* lines has been described [40, 43].

Plasmid constructs

pMTCLCV A::CH42 is a CbLCV A-derived vector carrying a 360-bp fragment of *CH42* in antisense orientation in place of the AR1 ORF. This vector was described in detail elsewhere [42]. pNMCbLCV A::CH42 (sense) carries the same portion of the *CH42* gene in sense orientation relative to the AR1 promoter. The presence of the insert in the correct orientation was confirmed by sequencing. pCPCbLCV B.002 is a pUC-based vector containing 1.5 copies of CbLCV B. pNMCbLCV::luc was made by the ligation of a 623-bp

*Acc*651 fragment of luciferase cDNA (pCPTGMV::luc) into pCPCbLCVA.007 at an *Acc*651 site. pCPCbLCVA.007 contains 1.5 copies of CbLCV A with an AR1 deletion [42].

Plant Growth and Bombardment

Arabidopsis seeds were germinated either on a flat or on Murashiqe and Skoog (MS) medium containing no antibiotic solution, hygromycin (30 μg/ml), or kanamycin (50 μg/ml) for four weeks (6 to 10-leaf stage). The seedlings germinated on the flat were transferred to a 3'x3' pot with three-four seedlings per pot, the bombardment was then performed one-two weeks later. The seedlings germinated on the MS plates (15-20 seedlings/plate) were directly bombarded with plasmid DNA, and subsequently transferred to a pot of soil three days later. A combination of CbLCV A-derived vectors and a wild type CbLCV B vector was used to bombard the seedlings. The DNA bombardment was performed as described by Turnage et al. [42]. Plants were grown at 22/20°C under conditions of an 8 hr light/16 hr dark photoperiod (refer as a short day), 70% humidity and 120 μEm-2 lighting throughout the experiments.

Imaging

Photographs were taken at 25, unless noted, using a Kodak Digital camera. Photographs were transferred to a computer and manipulating using Adobe Photoshop v. 5.5.

DNA Extraction and Southern blot analysis

Systemically infected leaves from infected plants at 25 dpi were harvested for DNA isolation as described [66]. Five μg of each DNA sample were digested overnight with a restriction enzyme, *Acc*651 (fivefold excess), to linearlize viral DNA. The digested DNA was separated on a 1% agarose gel and transferred to a Hybond-N membrane and UV cross-linked [67]. The ^{32}P–labeled DNA probes corresponding to a 600-bp fragment of the CbLCV

AL1 cDNA was performed by using the Rediprime II random prime system (Amersham Biosciences, England, UK) according to the manufacturer's instructions. The AL1 probe was obtained by PCR using the upper primer (5'AGAGAGGAACATTCAGACGG 3') and lower primer (5'AGCACGATTGAGGGTATGCC 3'). A 797 bp cDNA fragment of the 25S ribosomal RNA from Arabidopsis (position 529-1325 nucleotide, GenBank accession No. X52320) was used as a probe to confirm equal loading of the gels. Two specific primers were used: 25S1 (5'GGGCTTTTGATACGCTTGTG3') and 25S2 (5'TAAGCGCCATCCATTTTCGG 3').

RNA isolation and Northern blot analysis

Systemically infected leaves of CbLCV::CH42-infected wt:L1:35S-SGS2 (silencing and reverting plants) were collected at 47 dpi and isolated for total RNA using phenol/chloroform followed by lithium chloride precipitation [68]. RNA samples (15 μg) were separated on a 1.2% (w/v) formaldehyde-agarose gel, transferred to the Hybond-N membranes, and probed with GUS or 25S specific probes [67]. DNA fragments were labeled by random priming incorporation of ^{32}P-dCTP (Amersham Biosciences, England, UK). A 584-bp *Acc*651/*Eco*RV fragment of pGHNC11 [69] corresponding to *GUS* cDNA was used as a probe.

Chlorophyll Extraction and Measurement

Systemically infected leaves relatively top of each plant were weighed and frozen at –80°C. Extraction buffer, N, N'-Dimethylformamide (DMF), was added to frozen tissues in a microcentrifuge tube and placed at 4°C in darkness for at least 48 hours. One ml of extract was diluted three times with 2.0 ml fresh DMF. The absorption spectrum at 720 nm of the extracted liquid in a 3-mm cuvette was measured with a Shimadzu, UV-visible recording

spectrophotometer UV-265FW (USDA LAB, North Carolina State University). Total chlorophyll content was calculated in mg per gram of fresh weight tissue as described [70].

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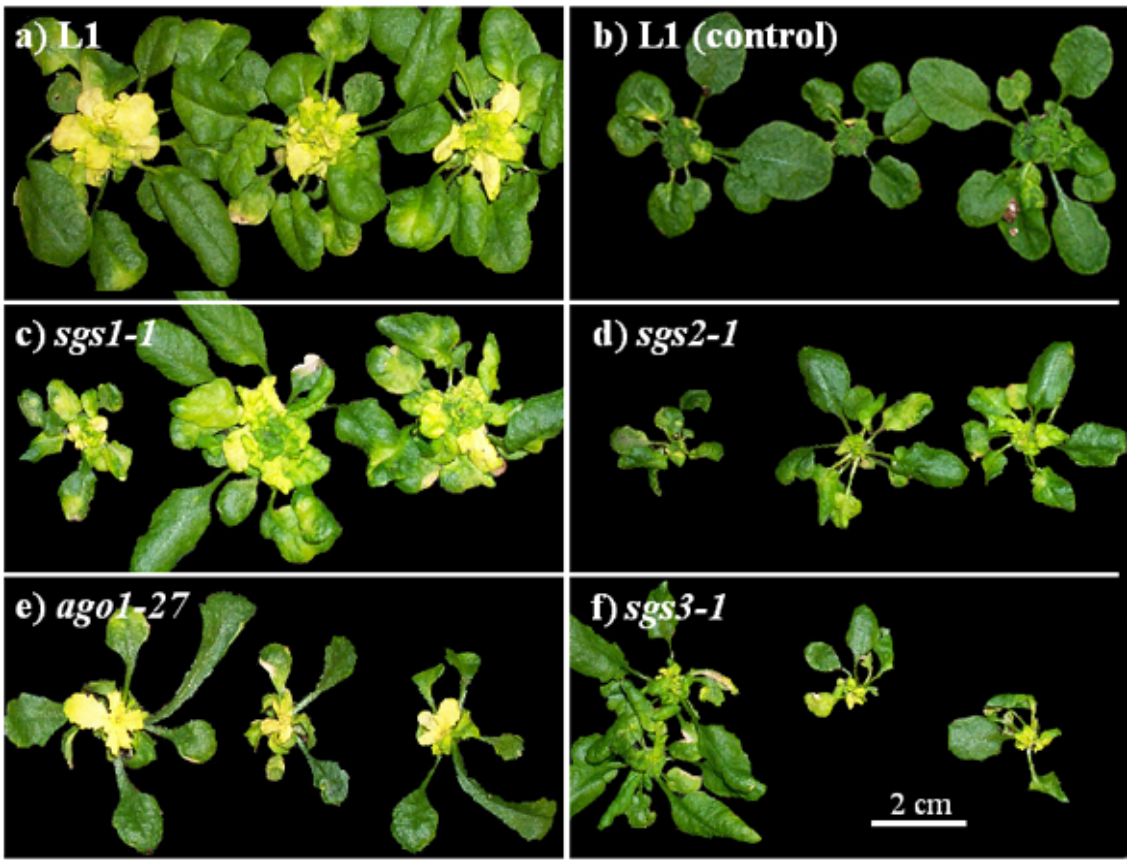


Figure 1. VIGS of a *CH42* endogenous gene in infected L1 plants and infected PTGS-deficient mutants.

Six to ten-leaf stage seedlings of PTGS-deficient mutants (*sgs1-1*, *sgs2-1*, *sgs3-1*, and *ago1-27*) and L1 plants were inoculated with CbLCV::CH42 (antisense orientation) whereas the L1 (control) plants were inoculated with CbLCV carrying a non-homologous gene fragment (luciferase). Photographs show plants 25 days post inoculation (dpi) in short-day growth condition. The CbLCV::CH42 infected L1, *sgs1-1, and ago1-27* plants (a, c, e) had yellow leaves in new growth, characteristic of the *CH42* silencing, in contrast to infected L1 (control), *sgs2-1*, and *sgs3-1* plants (b, d, f) which had green or light green leaves. The scale bar = 2 cm.

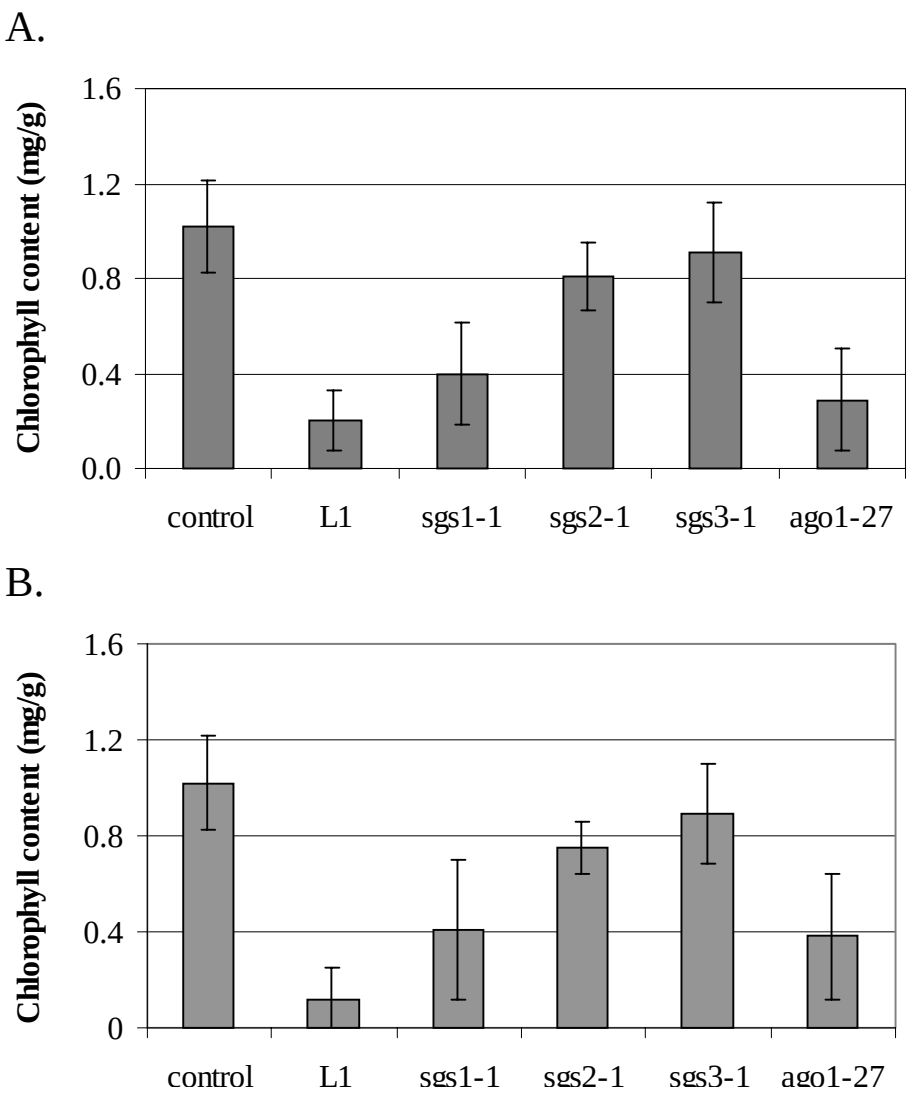
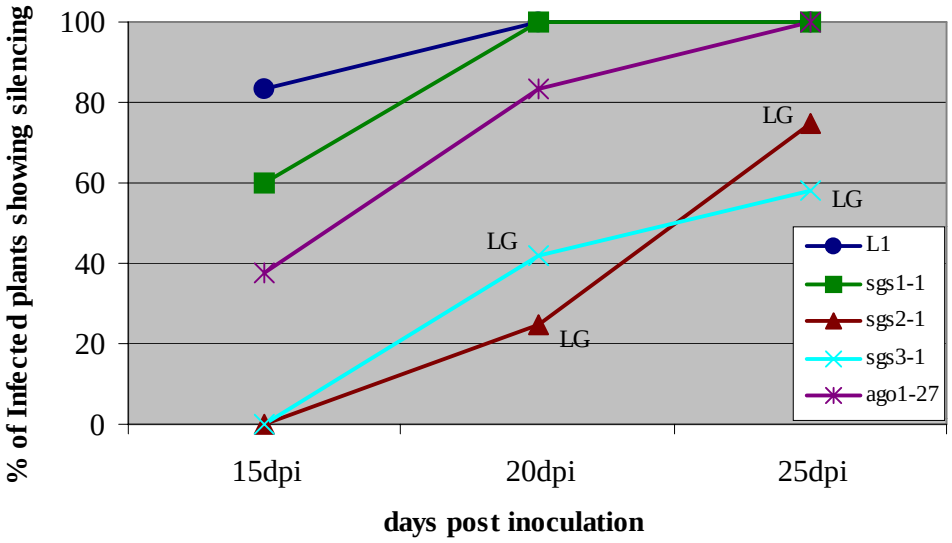


Figure 2. Chlorophyll contents of PTGS-deficient mutants.
Total chlorophyll content was determined from systemically infected leaves at 25 dpi of L1, *sgs1-1*, *sgs2-1*, *sgs3-1*, and *ago1-27* plants inoculated with CbLCV::CH42 carrying a CH42 fragment in antisense (A) or sense orientation (B) compared with control (L1 inoculated with CbLCV::luc). Each data point represents a minimum of five plants. Error bars indicate one standard deviation from the mean.

A. CbLCV::CH42 (antisense orientation)



B. CbLCV::CH42 (sense orientation)

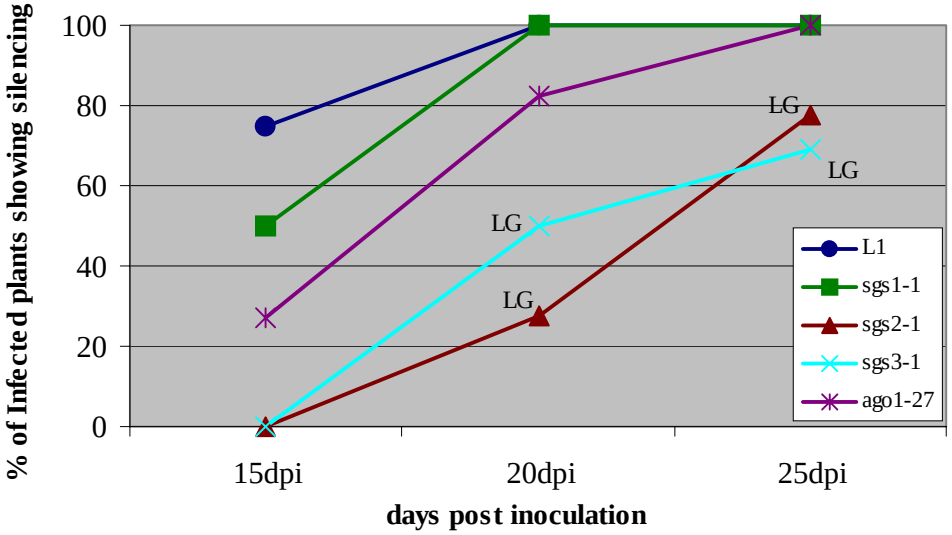


Figure 3. Percentage of infected plants showing silencing during plant development. Four-week-old seedlings (6 to 10-leaf stage) were inoculated with CbLCV carrying a *CH42* fragment in either antisense or sense orientations relative to the AR1 promoter. Infected plants exhibiting yellow or light green leaves, characteristic of *CH42* silencing, in new growth were scored at 15, 20, and 25 dpi. LG, light green, indicates that CH42 silencing was less extensive.

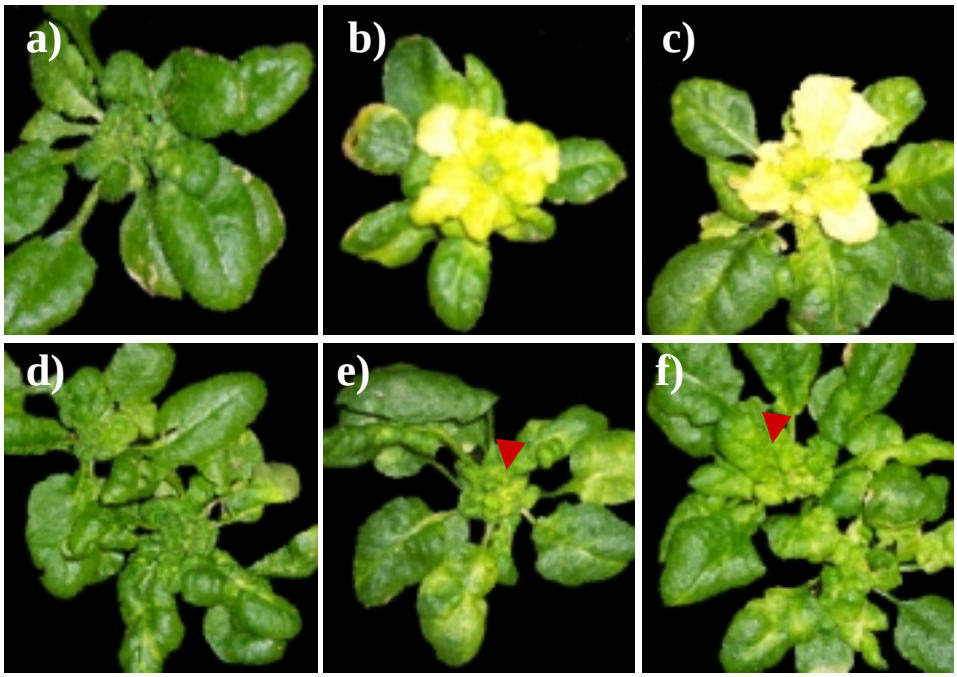


Figure 4. VIGS of *CH42* when six-week-old seedlings were inoculated with CbLCV-derived vectors.
Ten to twelve-leaf stage seedling of *sgs2-1* mutants and wt Arabidopsis Col. plants were used for the bombardments. Wt plants infected with CbLCV carrying CH42 in either antisense or sense orientation had yellow leaves in new growth (b, c). In contrast, *sgs2-1* mutant infected with CbLCV::CH42 had light green tissues regardless of insert orientation in new growth (e, f) (red arrows). Wild type and *sgs2-1* plants infected with CbLCV empty vector had green tissue in new growth (a, d). Photographs were taken at 24 dpi.

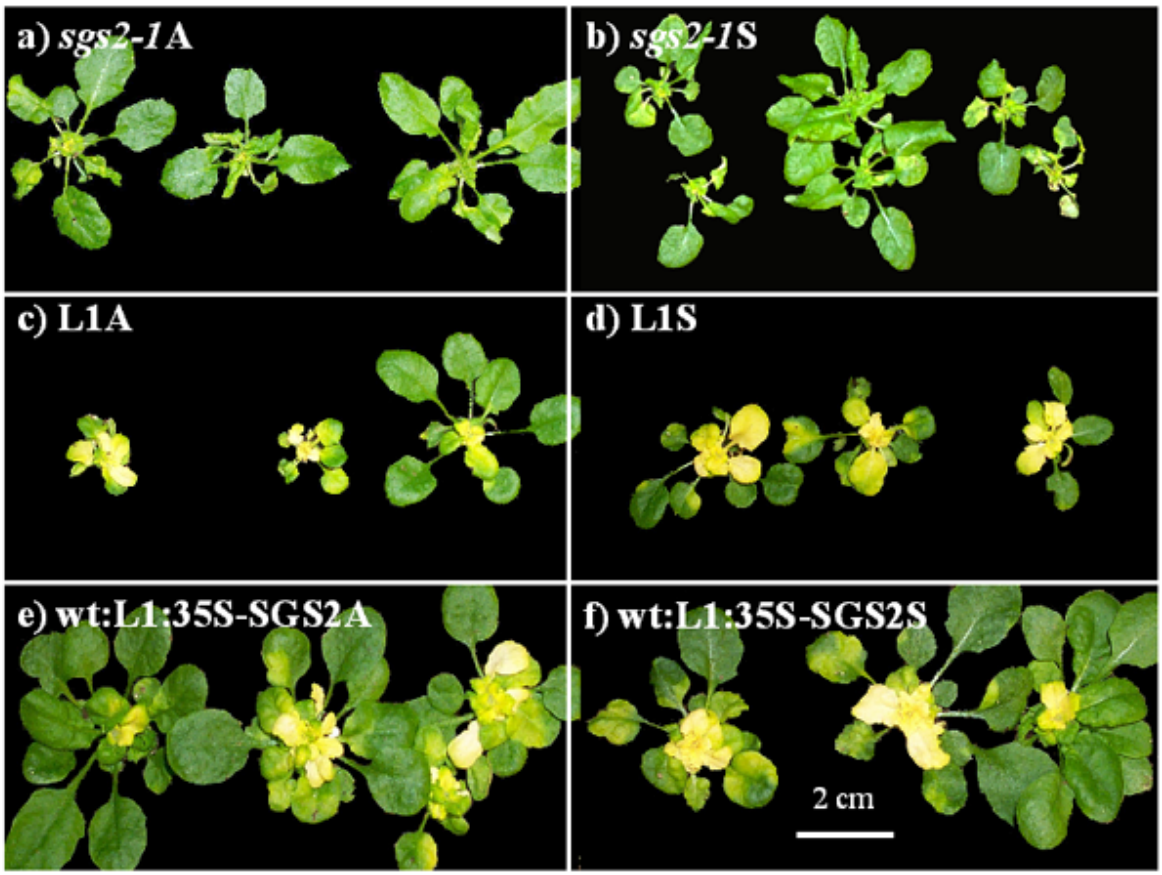


Figure 5. Silencing phenotypes in CbLCV::CH42 infected L1 and wt:L1:35S-SGS2 plants.
L1 and wt:L1:35S-SGS2 seedlings (six to ten-leaf stage) were inoculated with CbLCV carrying a CH42 fragment in either the antisense (a, c, e) or sense (b, d, f) orientation and photographed at 25 dpi. Silencing was inhibited in sgs2-1 plants, whereas L1 and wt:L1:35S-SGS2 plants exhibited yellow leaves, characteristic of *CH42* silencing, in new growth.

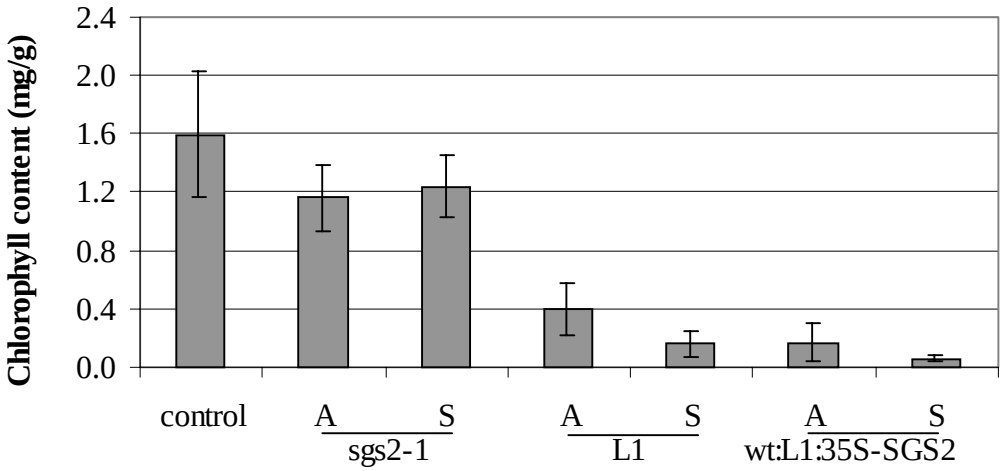


Figure 6. Chlorophyll contents in infected L1 and wt:L1:35S-SGS2 plants.
CbLCV::CH42 infected wt:L1:35S-SGS2 plants had a chlorophyll content lower (approximately twofold) than the infected L1 plants. Control was the L1 line inoculated with CbLCV::luc. Each data point represents a minimum of five plants. Error bars indicate one standard deviation from the mean. A, antisense; S, sense.

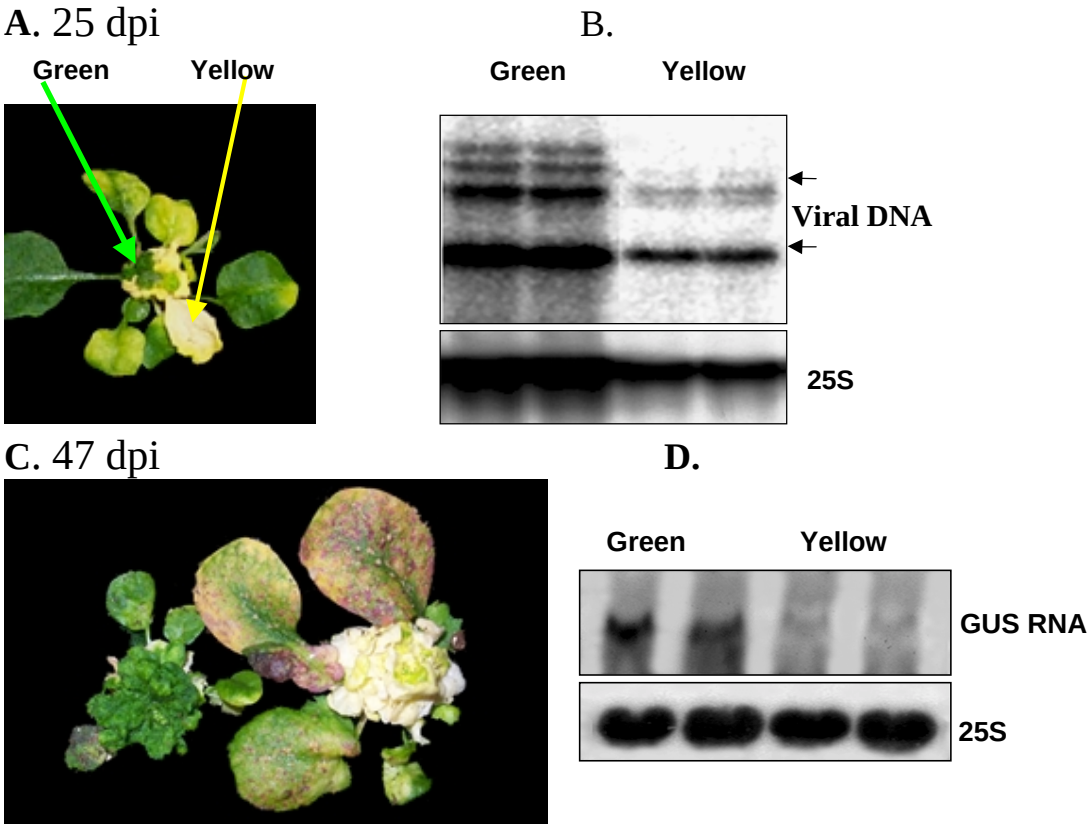


Figure 7. Silencing was unstable in some CbLCV::CH42 infected wt:L1:35S-SGS2 plants.
A. Green tissues reappeared in new growth of some wt:L1:35S-SGS2 silent plants at 25 dpi.
B. Total DNA was extracted from green tissues or yellow tissues of a pool of three plants and then, 5 µg per sample were digested with *Acc*651, loaded in duplicate on a 1% agarose gel, and transferred to Hybond-N membrane. The blot was probed with CbLCV AL1 or 25S probes.
C. At 47 dpi, some reverting plants continued to produce green leaves in new growth (left), whereas some reverting plants had extensive loss of chlorophyll (right).
D. Total RNA was isolated from green and yellow plants at 47 dpi (C). RNA samples, 15 µg per sample, were loaded in duplicate on a formaldehyde gel, and probed with GUS or 25S probes.

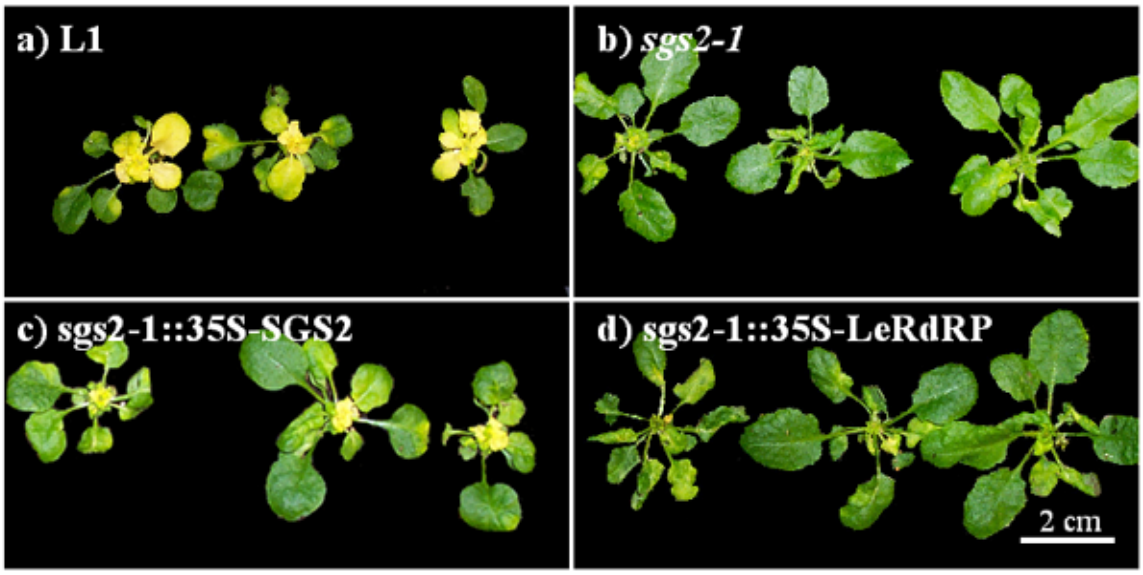


Figure 8. Tomato RdRP did not complement the *sgs2-1* mutation.
All plants were inoculated with CbLCV::CH42 (antisense orientation) and photographed at 25 dpi. *CH42* silencing (yellow tissues) was observed with infected L1 and *sgs2-1*::35S-SGS2 (the *sgs2-1* mutants transformed with 35S-SGS2). Only light green tissue or a few very small yellow leaves were observed in *sgs2-1* mutants and in *sgs2-1*::35S-LeRdRP (the *sgs2-1* mutant transformed with 35S-LeRdRP from tomato). Similar silencing phenotypes were seen with CbLCV::CH42 (sense orientation) infected plants.

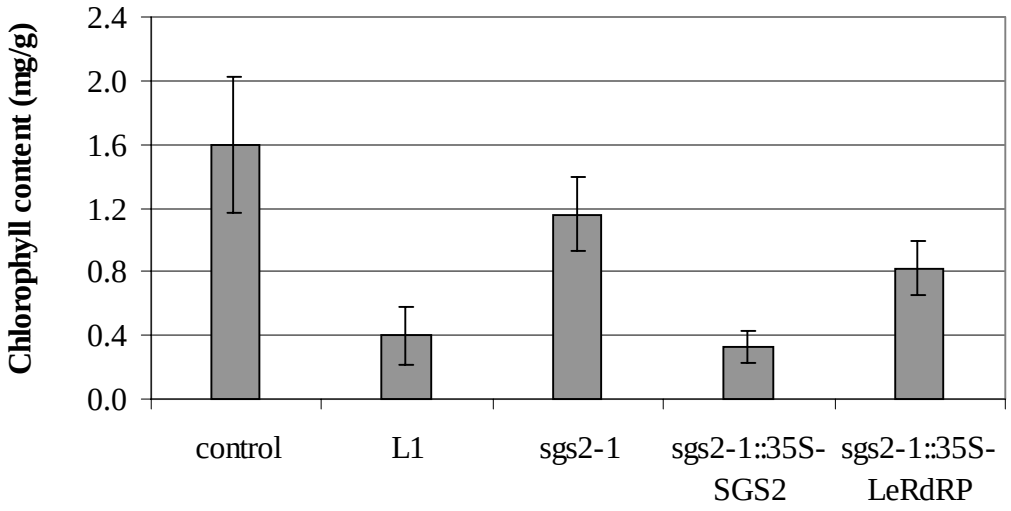


Figure 9. Chlorophyll content in CbLCV::CH42 infected plants.
Total chlorophyll content was determined from systemically infected leaves of L1, *sgs2-1*, *sgs2*-1::35S-SGS2, and *sgs2-1*::35S-LeRdRP plants were inoculated with CbLCV::CH42 (antisense orientation) at 25 dpi. Control was the L1 plants inoculated with CbLCV::luc. Each data point represents a minimum of five plants. Error bars indicate one standard deviation from the mean.

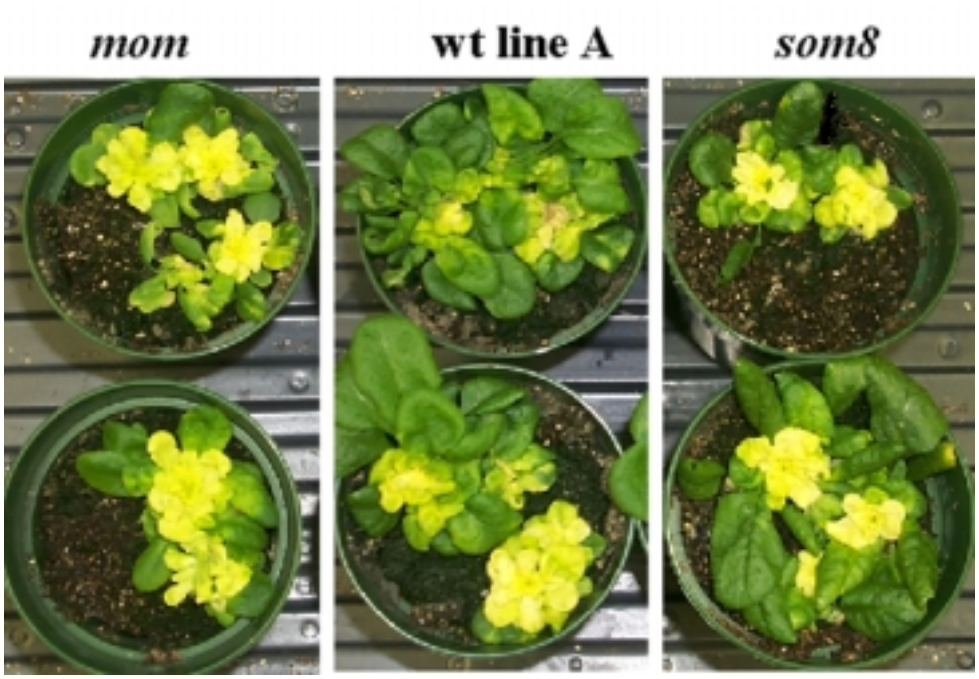


Figure 10. G-VIGS of *CH42* was unaffected in *som* and *mom* mutants.
Ten to twelve-leaf stage seedlings of *som* and *mom* mutants were inoculated with CbLCV::CH42 and photographed at 40 dpi.

Table 1. List of PTGS components required for endogenous gene silencing in plants.

Proteins	Description	S-PTGS	IR-PTGS	RNA virus (TRV)	DNA virus (CbLCV)
SGS1	unknown	✓	n/a	n/a	x
SGS2/SDE1	RdRP	✓	x	x	✓
SGS3	Coiled-coil protein	✓	x	n/a	✓
AGO1	Paz/Piwi domain	✓	x	n/a	x
SOM/DDM1	Chromatin remodeling/methylation	✓ (low frequency)	n/a	n/a	x
21-25 nt	Small nucleotide RNA	✓	✓	✓	✓
References		[1-3, 35]	[4]	[34, 62, 71]	This work; Turnage and Robertson, unpublished.

✓, Required or associated with; x, not required; n/a, not applicable

Chapter 5

Virus-induced gene silencing (VIGS) and Applications

Introduction

Previously, I tested clone 9 for its potential role in TGMV DNA replication (Chapter 2). Virus-induced gene silencing (VIGS) from TGMV-derived vectors was used to downregulate the clone 9 expression. Viral DNA accumulation was dramatically reduced in systemically infected leaves when *Nicotiana benthamiana* plants were inoculated with TGMV carrying a small fragment of clone 9 cDNA. Using the same method, silencing of proliferating cell nuclear antigen (PCNA), an auxiliary factor for DNA polymerase delta, reduced viral DNA accumulation [1]. These experiments have suggested that the target genes might play a role in viral DNA replication, and it would be useful to similarly test other *N. benthamiana* genes (see Chapter 2, Table 1) to determine if they are required for viral DNA replication. However, a complete sequence of those genes would be necessary; thus, *N. benthamiana* is not a desirable system to do such experiments now because of the lack of genomic sequence information for this plant. We therefore decided to use Arabidopsis as a plant system because of its complete genome sequence and because it is a host for the CbLCV geminivirus.

Three Arabidopsis candidate genes, homologs of AL1-upregulated genes from *N. benthamiana*, were tested to determine if they were required for viral DNA replication. These include *PCNA*, an *Arabidopsis thaliana* G1 related protein (*AtG1RP*), and a putative calmodulin-related protein (*CaMRP*). We also extended our study using VIGS to test two other candidate genes for their potential roles in the silencing machinery: SGS2 and SDE3, coding for an RNA dependent RNA polymerase (RdRP) and RNA helicase-like protein, respectively [2, 3]. The experiments to be described below were designed to develop CbLCV-derived vectors as a tool for functional genomic studies.

Results and Discussion

Silencing of a CH42 endogenous gene using CbLCV-derived vectors was orientation dependent.

A silencing system based on CbLCV-derived vectors was previously established [4] in which transgenes and endogenous genes were silenced if there was a similar sequence between plants and viruses. Because CbLCV A, despite the lack of the coat protein gene (*AR1*), was a better silencing vector than CbLCV B (Chapter 3), we decided to use CbLCV A as a cloning vector for this study. Before we cloned a gene fragment of interest into the CbLCV A vector, the orientation of a known insert was tested. *Chlorata 42* (*CH42*), coding for an enzyme required for chlorophyll production, was used as a visual marker for assessing silencing in this experiment. A description of plasmids used is listed in Table 1. A combination of CbLCV A-derived vectors and CbLCV B-derived vectors were used for DNA bombardment.

CbLCV A::CH42 (antisense)/wt CbLCV B-infected plants (A::CH42A/wt B) showed early signs of silencing, yellow tissue, and slightly more extensive silencing at 19 dpi (9 out of 11 plants) than did CbLCVA::CH42 (sense)/wt CbLCV B (A::CH42S/wt B)-infected plants (5 out of 9 plants) (Fig. 1). In addition, there was extensive silencing from plants inoculated with a combination of CbLCV A::CH42 (antisense) and CbLCV B::CH42 (antisense) (A::CH42A/B::CH42A) compared to plants inoculated with CbLCV A::CH42 (sense)/CbLCV B::CH42 (sense) (A::CH42S/B::CH42S) (Fig. 1). Interestingly, symptoms were attenuated in the A::CH42A/wt B infected plants compared to the others. However, silencing phenotypes of plants inoculated with either A::CH42A/wt B or A::CH42S/wtB were similar, at 25 dpi (data not shown). A::CH42A/B::CH42A infected plants maintained

their silencing extensiveness throughout the experiment, and that was not true with A::CH42S/B::CH42S infected plants. Silencing of sulfur, *su*, was orientation-independent when *N. benthamiana* plants were inoculated with TGMV-derived vectors [5]. Similar conclusions were drawn when *N. benthamiana* plants were inoculated with PVX vectors carrying PDS cDNA inserts in either the antisense or sense orientation [6]. In our study, however, the silencing of *CH42* was initially orientation-dependent and the silencing was more extensive when antisense transcripts were produced from the viral promoter.

Description of five endogenous genes from Arabidopsis thaliana

Five cDNA fragments corresponding to *PCNA*, *AtG1RP, CaMRP, SGS2* and *SDE3* genes from *A. thaliana* were generated by either polymerase chain reaction (PCR) of genomic DNA or Reverse Transcriptase-PCR (RT-PCR) of poly A+ RNA. Their positions and accession numbers are shown in Table 1. Although mRNAs encoding these genes ranged between 500 bp and 5.0 kb long, cDNA fragments of 154 bp to 800 bp are long enough to silence genes in the VIGS system [1]. Peele et al. [1] also have shown that a 92 bp fragment of *su* was sufficient enough to trigger the loss of gene expression based on TGMV. However, vectors based on geminiviruses have a size limitation for foreign DNA insertion (see Chapter 2). The fragment of foreign DNA insertion can be up to 0.8 kb in the AR1 replacement vector (A component) without affecting viral replication and movement [5]. A larger fragment is deleted, or inhibits viral movement [1]. The size of a cDNA fragment that is to be subjected to functional analysis in the VIGS system must therefore be given careful consideration.

Each of the cDNA fragments (~400 bp) was derived from the central portion of their genes. PCNA is highly conserved among species and required for DNA replication and repair

[7-9]. Previously, it has shown that systemic infection was reduced in plants silencing PCNA [1]. I tested a 412 bp Arabidopsis *PCNA* cDNA fragment corresponding to *PCNA1* on chromosome I; it shares 86% identity with *PCNA2* (chromosome II, accession No. AY089110) at the nucleotide level. A 398 bp *CaMRP* cDNA fragment that shares 84% identity to calmodulin-related protein 2 (touch-induced, *TCH2*) was also tested. The gene product, CaMRP, belongs to the calmodulin (CaM) family of proteins and contains four highly conserved Ca (2+)-binding EF-hands. Expression of the calmodulin-related *TCH* genes of *Arabidopsis* was rapidly up-regulated in plants after a variety of stimuli, including touch and wind [10, 11]. The other three cDNAs; 398 bp, 434 bp, and 437 bp of *AtG1RP*, *SGS2*, and *SDE3*, respectively, are homologous to a single gene and would not be expected to silence their family members.

A CbLCVA::CH42 vector [4] was selected as a cloning vector because plants infected with this vector developed a yellowish phenotype, loss of chlorophyll production, that can be a useful, visible maker in the analysis of viral replication and movement, or silencing phenotype. To maintain an optimal size for the virus, each of the five cDNA fragments (~400 bp) were inserted into the CbLCV::CH42 vector, downstream of the AR1 promoter and before a 360 bp *CH42* fragment, giving rise to CbLCV::PCNA, CbLCV::AtG1RP, CbLCV::CaMRP, CbLCV::SGS2, and CbLCV::SDE3. Based on the results obtained for *CH42* (above), all cDNA fragments were in the antisense orientation relative to the AR1 promoter.

VIGS of PCNA, CaMRP, and AtG1RP endogenous genes

Geminiviruses replicate in plant nuclei and induce host proteins for their replication machinery. PCNA accumulated at higher levels in mature plant cells infected with TGMV

than uninfected cells [12, 13]. Several host genes upregulated by TGMV were previously isolated from *N. benthamiana* but their functions remain to be identified (Eagle and Robertson, unpublished data). Many gene products have high similarity in amino acid sequence to Arabidopsis proteins; therefore, we asked if these homologs would be required for CbLCV replication. VIGS was used as tool based on the hypothesis that a virus cannot replicate if a gene needed for its own replication is silenced. We tested three Arabidopsis homologs including PCNA, AtG1RP (similar to clone 9 of *N. benthamiana*), and calmodulin-related protein (CaMRP) (similar to clone 25.1 of *N. benthamiana*).

To investigate the effect of three genes on viral DNA replication, wild-type Arabidopsis four-weeks-old seedlings were inoculated with a combination of a CbLCV A-derived vector and wt CbLCV B, and were grown under the short day photoperiod condition (8 hr light/16 hr dark). Plants infected with each of these constructs: CbLCV::PCNA, CbLCV:: CaMRP, and CbLCV::AtG1RP, developed a yellowish phenotype, a characteristic of *CH42* silencing, in new growth (Fig. 2). Although infected plants showed silencing, they were variegated and could be sorted into three categories: strong, moderate, and weak. Strong silencing (most or all systemically infected leaves were yellow) was 50%, 88%, and 80%, in CbLCV::PCNA, CbLCV::CaMPR, and CbLCV::AtG1RP plants, respectively (Fig. 3A). The remaining showed either moderate (a few systemically infected leaves were yellow) or weak silencing (most or all systemically infected leaves were light green or green). Chlorophyll analysis showed that the CbLCV::PCNA plants had a chlorophyll content slightly higher (twofold) than the others (Fig. 3B). These results suggested that silencing was slightly less efficient in the CbLCV::PCNA plants.

The CbLCV::PCNA, CbLCV::CaMPR, and CbLCV::AtG1RP plants had similar level of symptoms which were moderate compared to the control (wt plants infected with CbLCV::luc) (Fig. 4A). Symptoms were attenuated in some of the CbLCV::PCNA plants at 47 dpi (data not shown). Southern blot analysis of genomic DNA isolated from systemically infected leaves showed that viral DNA accumulation was greatly reduced only in the CbLCV::PCNA plants compared to other plants (Fig. 4B). Therefore, PCNA may be required for viral DNA replication. Silencing *CaMRP* or *AtG1RP* genes did not show the reduction of viral DNA accumulation, suggesting that these homologs of AL1-upregulated genes are not necessarily involved in viral DNA replication.

VIGS of SGS2 and SDE3 endogenous genes

Previously, we clearly showed that SGS2 was required for geminivirus-induced gene silencing (Chapter 4). Silencing of *CH42* was inhibited in a *sgs2* mutant that was inoculated with CbLCV::CH42. SDE3 appeared not to be required for silencing pathway (Appendix A). Because there is a difference of genetic background between the *sgs2* and *sde3* mutants in which *sde3* mutants contain a PVX amplicon [14, 15], we cannot rule out the possibility that a viral protein from the PVX amplicon in *sde3* mutants could replace the SDE3 function. SDE1 (identical to SGS2) and SDE3 (as tested) were not required for VIGS by some RNA viruses [2, 3], thus virally encoded proteins possibly replaced the SDE1 (SGS2) and SDE3 functions.

To determine if SDE3 is required for VIGS in the PVX-free condition, we targeted Arabidopsis *SDE3*, along with *SGS2,* using the same silencing method described above. Here we found that *CH42* silencing was more extensive in the CbLCV::SDE3 plants than in the CbLCV::SGS2 plants (Fig. 5A). While the CbLCV::SGS2-infected plants had 75% weak

silencing, the CbLCV::SDE3 plants had only 13% weak silencing. Most of the remaining population of CbLCV::SDE3 were either strong or moderate silencing (Fig. 5B). The silencing levels were further characterized by chlorophyll measurement. The CbLCV::SDE3 plants had a chlorophyll content threefold lower than that from the CbLCV::SGS2 plants. Similar chlorophyll contents were obtained from the CbLCV::SGS2 plants and the control plants where Arabidopsis plants were inoculated with a CbLCV A carrying a non-homologous fragment from the luciferase gene, and wt CbLCV B (Fig. 5C). Southern blot analysis of total genomic DNA revealed that the weak silencing occurred in the CbLCV::SGS2 plants was not due to the loss of viral DNA or inserted fragment (Fig. 5D). Taken altogether, these results indicated that SDE3 was not necessarily required for silencing mediated by CbLCV. It is possible that SDE3 might be required to enhance the silencing effect.

Conclusions

We showed here that VIGS could be used as a rapid and powerful tool to downregulate the expression of endogenous genes. At least two groups of genes were tested using this system: viral DNA replication-associated genes and silencing machinery-associated genes. As expected, viral DNA accumulation was reduced when PCNA was silenced. Viral DNA accumulation was not reduced in plants bombarded with *CaMRP* or *AtG1RP* constructs, suggesting that both genes are probably not needed for viral replication. In addition, we also showed that the VIGS could be used to test genes associated with silencing. Silencing was inhibited only if a gene required for the silencing pathway was

silenced. According to our results, SGS2 was required for the silencing pathway but not SDE3. Overall, the results were consistent to our earlier hypotheses.

Materials and methods

Plasmids and DNA constructs

Five CbLCVA-derived vectors were similarly constructed: pNMCbLCV::CaMRP, pNMCbLCV::PCNA, pNMCbLCV::AtG1RP, pNMCBLCV::SGS2, and pNMCbLCV::SDE3. 412 bp and 398 bp cDNA fragments corresponding to *PCNA* and *AtG1RP* cDNA, were obtained by RT-PCR using poly A mRNA (GeneAmp®PCR, Applied Biosystems, CA, USA). Specific primers are: *PCNA*-specific primers (upper primer, 5'TCACTCTAGACTATGGATTCGAGTCACGTTGC 3' and lower primer, 5' TAGCGGTACCAATGTCACCGGCAGTAGAAAC 3'), and *AtG1RP*-specific primers (upper primer, 5'GGATCTAGACGCCTCCTTGAAGATGTTGTTG 3' and lower primer, 5'TCTGGGGTACCATCTAGTTCAAGGCCACCAAG 3'). 400 bp, 434 bp, and 437 bp cDNA fragments corresponding to *CaMRP*, *SGS2*, and *SDE3* cDNAs were amplified by PCR from genomic DNA of Arabidopsis ecotype Colombia. Specific primers were used: *CaMRP*-specific primers (upper primer 5' CGTTTCTAGAAACTGCTTAGGATCAATGG 3' and lower primer 5' CAACGGTACCATCACCATCAGAATCAAC 3'), *SGS2*-specific primers (upper primer 5' GCTTCTAGAGGCGGAAGGTGTAAAGCGTGGTC 3' and lower primer 5' TAGCGGTACCTGTTGGGAGGGATGAGTTTC 3'), and *SDE3*-specific primers (upper primer 5' GCTTCTAGAGGCGGAAGGTGTAAAGCGTGGTC 3' and lower primer 5' ACGGATTGGTACCTTCCCTCTCATCGCATC 3'). All primers were designed to carry the restriction enzymes, *Xba*I and *Acc*651, at 5' end of upper primers and lower primers,

respectively. The PCR products were digested with the restriction endonuclease *Xba*I/A*cc*651, purified, and inserted into pMTCbLCVA::CH42 at *Xba*I/*Acc*651 sites, behind the AR1 promoter before the *CH42* fragment. These fragments were in the antisense orientation to the AR1 promoter. A description of plasmid vectors used in this study was listed in Table 1.

RT-PCR and PCR conditions

The RT-PCR was performed following the instruction recommendation (GeneAmp® PCR, Applied Biosystems, CA, USA) using 100 ng of poly A RNA templates. The PCR amplification was done in a 100-µl reaction with 10 ng DNA templates. Cycling conditions were 30 cycles of 95°C for 10 minute, 54°C for 1 minute, and 72°C for 7 minutes.

Plant growth and bombardment, Chlorophyll extraction and measurement, DNA isolation and Southern blot analysis were described in Chapter 4.

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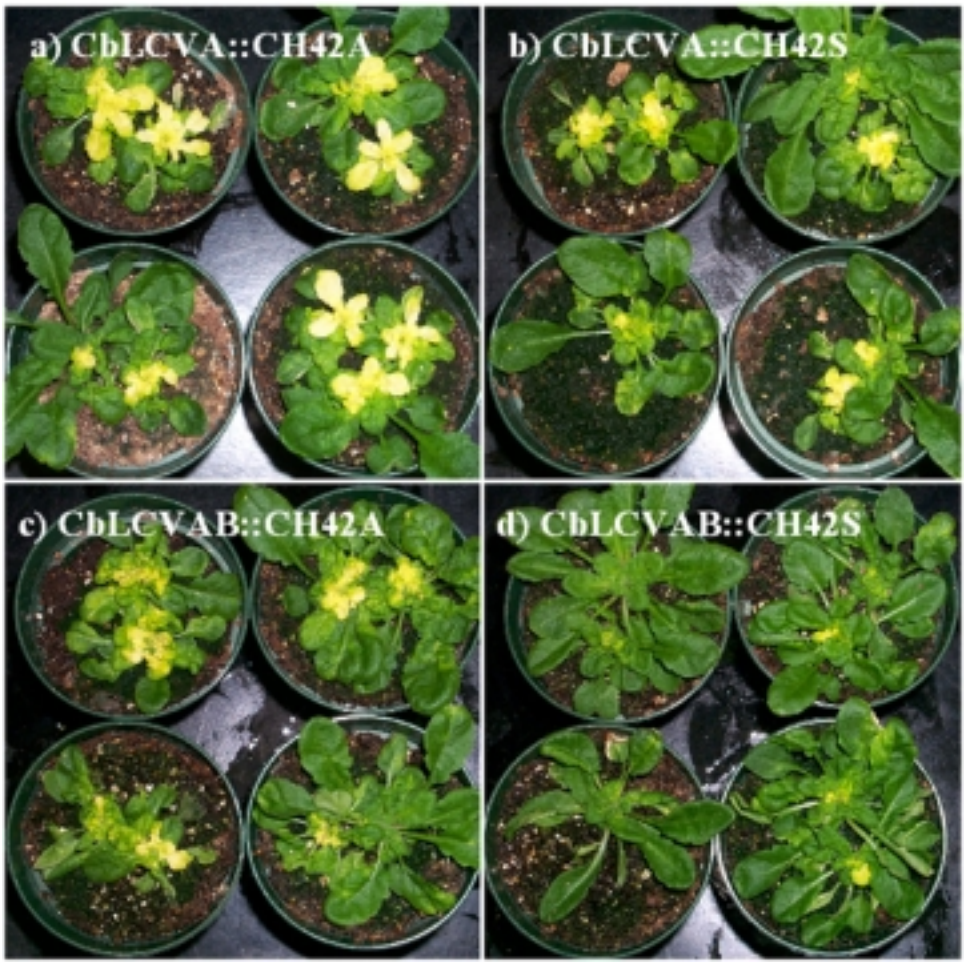

Figure 1. **VIGS of *CH42* was orientation dependent.**
Wild type Arabidopsis plants 6-weeks-old were inoculated with a combination of CbLCV A- and CbLCV B-derived vectors, and photographed at 19 dpi. CbLCV::CH42 (antisense orientation) infected plants (a, c) showed more extensive silencing than CbLCV::CH42 (sense orientation) infected plants (b, d). A and S indicate antisense and sense orientations, respectively.

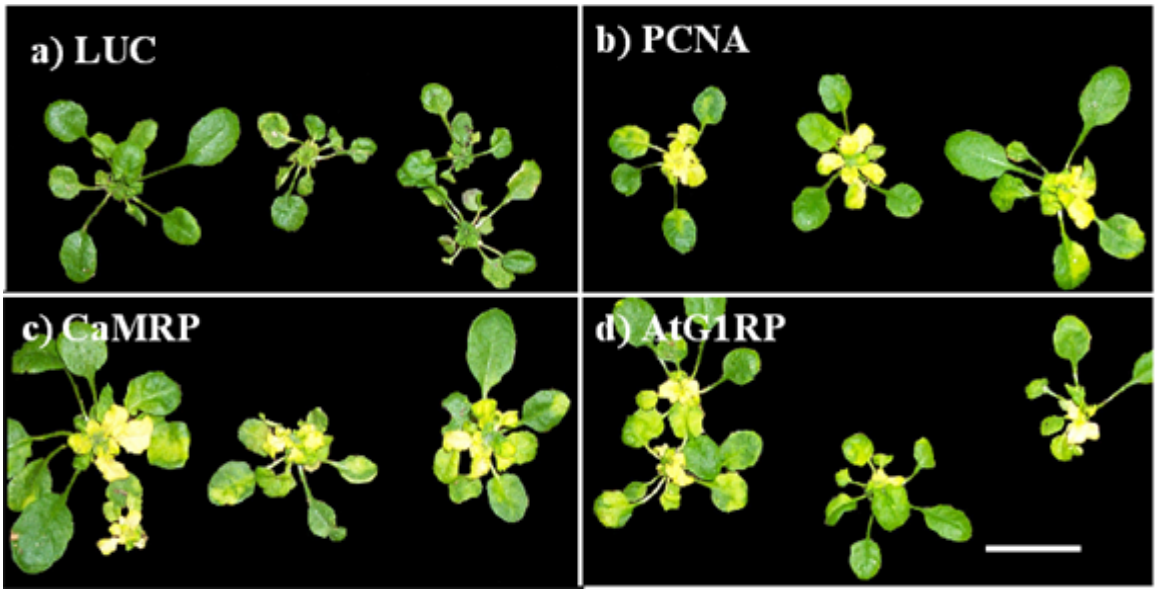

Figure 2. **VIGS of *PCNA, CaMRP,* and *AtG1RP* endogenous genes.**
Wild type Arabidopsis plants four-weeks-old were inoculated with CbLCV::luc or CbLCV carrying a fusion of *CH42* fragment and one of these three genes: *PCNA, CaMRP,* or *AtG1RP*. CH42 silencing, yellow tissue, was observed in PCNA (b), CaMRP (c) and AtG1RP (d) plants. Photographs were taken at 25 dpi. The scale bar = 2 cm.
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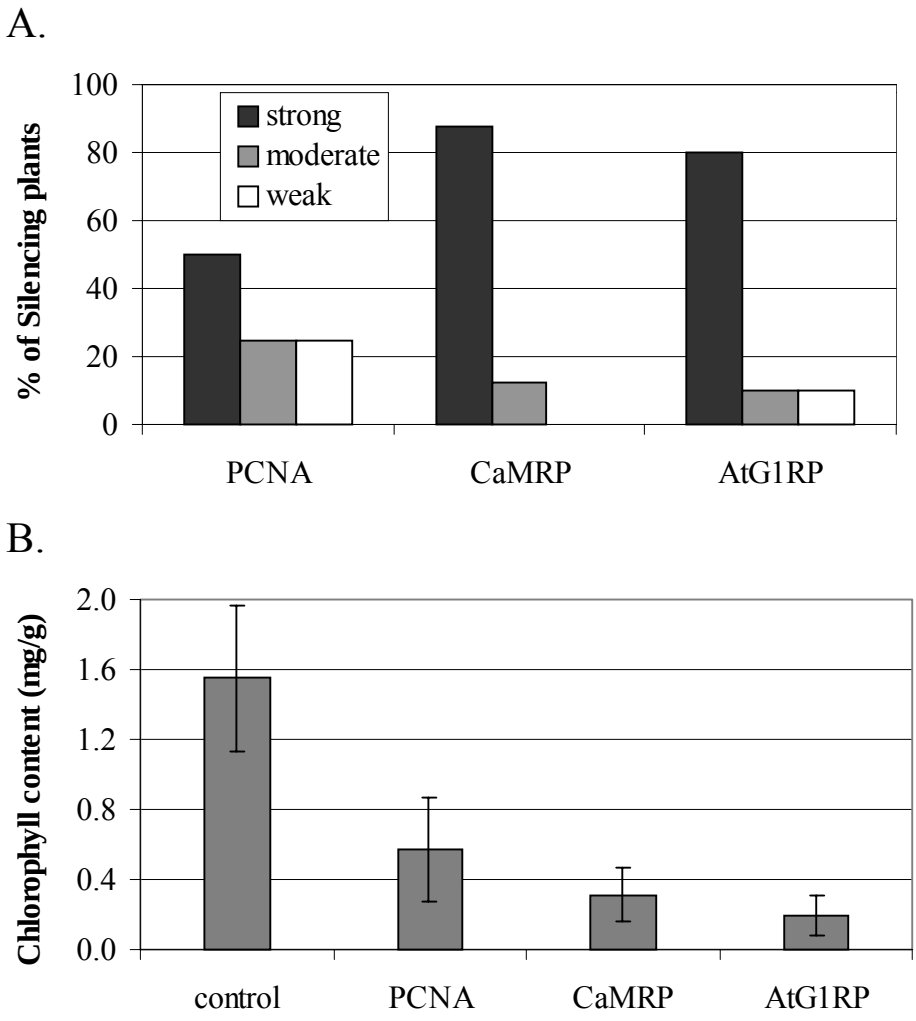

Figure 3. **Silencing levels and chlorophyll contents in the CbLCV::PCNA, CbLCV::CaMRP, and CbLCV::AtG1RP plants.**

A. Silencing phenotypes were sorted into three categories: strong, moderate, and weak. "Strong", most or all systemically infected leaves were yellow; "moderate", a few systemically infected leaves were yellow; "weak", most or all systemically infected leaves were light green or green.

B. Total chlorophyll content was determined at 25 dpi from 10 infected plants. Control corresponds to CbLCV::luc infected plants. Error bars indicate one standard deviation from the mean.

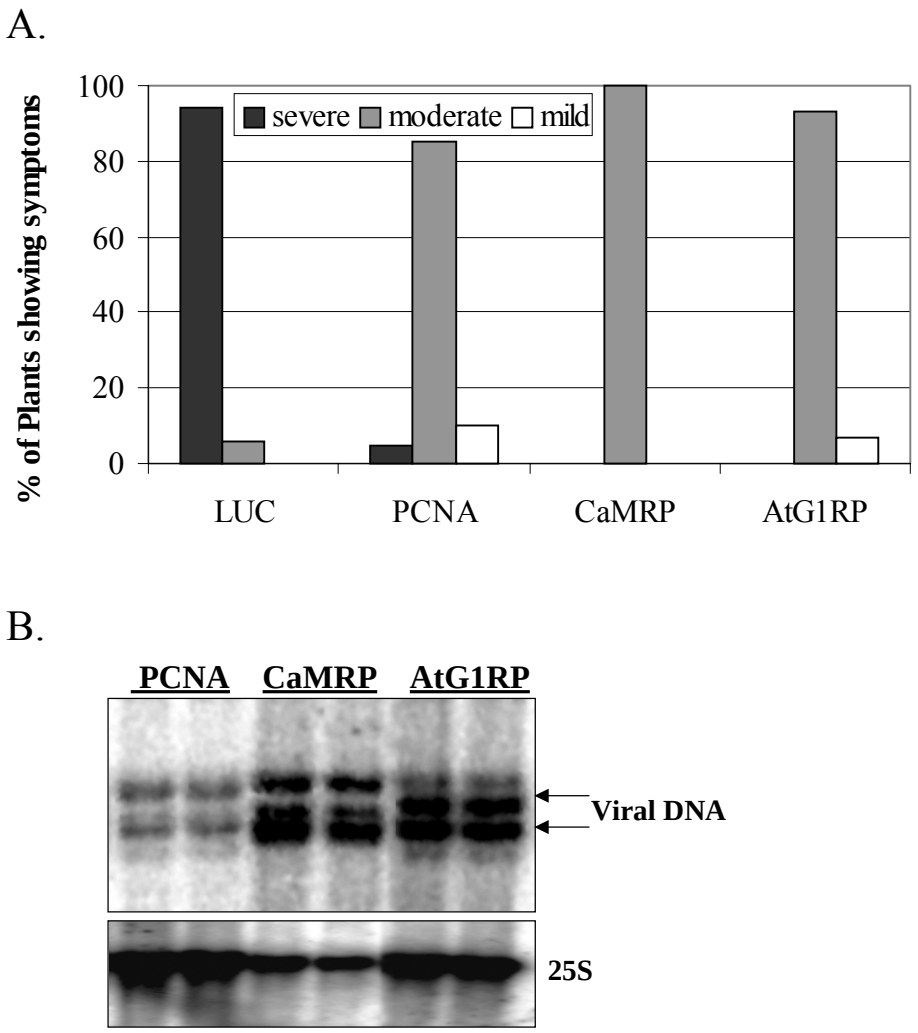

Figure 4. **Symptom severity and viral DNA accumulation.**
A. Symptoms were classified into three groups: severe, moderate, and mild. "Severe", small new growth with very small and curly systemic leaves; "moderate", more new growth with less curly and small leaves; "mild", more new growth with large and normal leaves".
B. Southern blot analysis for the presence of viral DNA accumulation. Total DNA was isolated from a pool of five plants. Five µg per sample was digested with *Acc*651 (fivefold excess) overnight, loaded in duplicate, and transferred to Hybond-N membrane. The blot was probed with *AL1* or 25S probes.

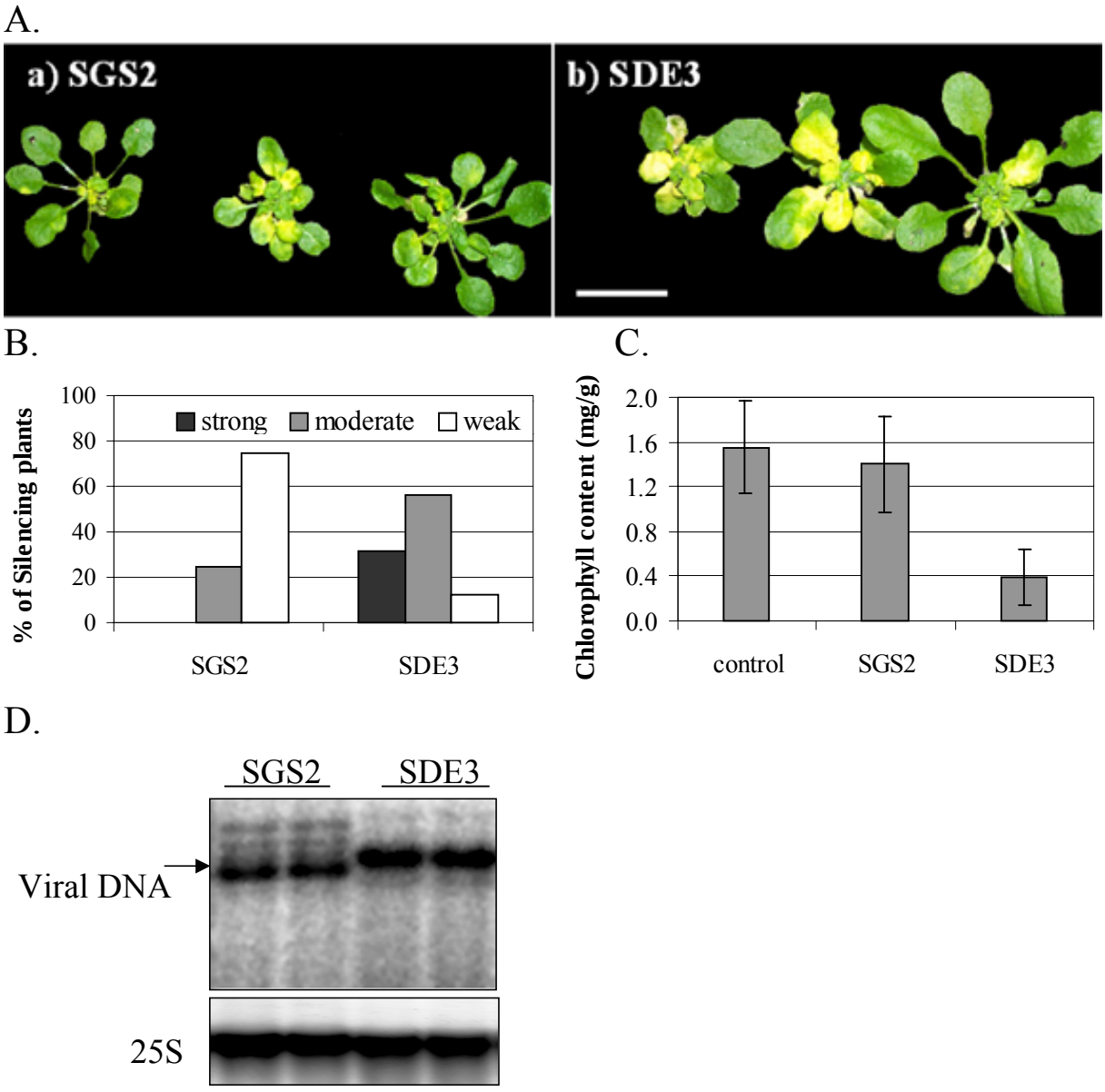

Figure 5. VIGS of *SGS2* and *SDE3* endogenous genes.
A. Silencing phenotypes in the CbLCV::SGS2 and CbLCV::SDE3 plants at 25 dpi.
B**.** Silencing phenotypes were sorted into three categories: strong, moderate, and weak as described in Fig. 3. The scale bar = 2 cm.
C. Total chlorophyll content was determined at 25 dpi from 10 infected plants. Control corresponds to CbLCV::luc infected plants. Error bars indicate a standard deviation from the mean.
D. Southern blot analysis for the presence of viral DNA accumulation was described as in Fig. 3.

Table 1. Describes the CbLCV A and CbLCV B plasmids used for the experiments herein. CP, Chip Peele; MT, Michael Turnage; NM, Nooduan Muangsan

Name	Description
pCPCbLCVA.007	A pUC-based vector of CbLCVA, AR1 ORF deleted
pMTCbLCVA::CH42	360 bp fragment with 360 bp of homology to the *ch42* gene, antisense orientation
pNMCbLCVA::CH42 (sense)	406 bp fragment with 374 bp of homology to the *ch42* gene, sense orientation
pNMCbLCVA::luc	630 bp fragment with 618 bp of homology to the *luciferase* gene, sense orientation
pNMCbLCVA::PCNA	412 bp fragment, corresponding to nt 115-526 of *PCNA1* cDNA, antisense orientation, accession No. NM100611
pNMCbLCVA::CaMRP	400 bp fragment, corresponding to nt 21-420 of *CaMRP c*DNA, antisense orientation, accession No. AY117325
pNMCbLCVA::AtG1RP	398 bp fragment, corresponding to nt 1026 to 1421 of *AtG1RP* cDNA, antisense orientation, accession No. AY054498
pNMCbLCVA::SGS2	434 bp fragment, corresponding to nt 2211-2644 of *SGS2* cDNA, antisense orientation, Accession No. AF239718
pNMCbLCVA::SDE3	437 bp fragment, corresponding to nt 1591-2027 of *SDE3* cDNA, antisense orientation, accession No. AF339908
pCPCbLCVB.002	A pUC-based vector of CbLCV B
pNMCbLCVB::CH42 (antisense)	144 bp *Bam*HI/*Eco*RV fragment of the *ch42* cDNA, antisense orientation
pNMCbLCVB::CH42 (sense)	144 bp *Bam*HI/*Eco*RV fragment of the *ch42* cDNA, sense orientation

Chapter 6

New Perspectives and Directions

Advantages of VIGS for gene silencing

Virus-induced gene silencing (VIGS) has become one of the most rapid and powerful tools for reverse functional genomic studies. It is similar to post-transcriptional gene silencing (PTGS) that results in reduced mRNA levels of endogenous expressing plant genes that have a sequence similarity of about 80% or more with the sequences carried by the virus [1, 2]. Viruses themselves can also be the targets of PTGS machinery [3]. Several viruses, including RNA and DNA viruses, have been successfully used to create a gene-knockout phenotype.

There are a few advantages of the VIGS technique over competing techniques. First, VIGS is a quick method to determine the loss-of-function phenotype for a gene while a great deal of time is consumed using the *Argrobacterium* transformation technique. Second, only portions of a gene are required. Small cDNA fragments (92 bp-500 bp) appear to be sufficient to induce silencing [1, 2]. However, synthetic 21-nt dsRNAs with two nucleotides 3'overhange have been used to trigger gene silencing in mammalian cells [4]. Finally, essential genes that cannot be studied by T-DNA knockouts can be studied using this method.

DNA geminiviruses have provided some other advantages as silencing vectors. They have small genomes that make them suitable for cloning, they do not integrate into plant chromosomes, and they are not seed transmitted. Moreover, viruses are excluded from meristematic areas, but the mobile signals are not. Therefore, they are useful for meristematic gene studies [1].

The utilities of TGMV and CbLCV as silencing vectors

A and B components of TGMV and CbLCV both can be used as silencing vectors by either replacing the AR1 coding sequence for a gene fragment of up to 800 bp for the A component, or by inserting a fragment of up to about 100-150 bp downstream of the BR1 stop codon and before a polyadenylation site. TGMV B is a better silencing vector than TGMV A [1, 5]. In contrast, the CbLCV A component is better ([6], Chapter 3). TGMV B has a size limitation for propagation of foreign DNA insertion and approximately 150 bp is close to the optimal size. A combination of both components can be used to obtain extensive silencing.

Summary of the parameters and limitations of G-VIGS

CbLCV infects mainly the Brasicaceae family (e.g. cabbage and Arabidopsis [7]. The use of CbLCV-derived vectors needs the following considerations:

1. CbLCV A is a better silencing vector than CbLCV B, producing extensive silencing and fewer symptoms, while CbLCV B–derived vectors give weak silencing and severe symptoms ([6], Chapter 3).

2. The AR1 coding sequence of the A component can be replaced with an insert up to 800 bp. However, deletion forms are randomly observed in infected plants. This is also seen naturally in wt geminiviruses [8]. The optimal size for propagation of a foreign DNA insert remains to be determined.

3. Seed germination can be done either on a flat or on sterile medium. It is more convenient for bombardment if sterile plates are used, especially if there is a large number of plants but might interfere with uniform silencing. Ten to fifteen seeds per plate should be used and placed at the middle of the plate. Infected plants are then transferred from plates to pots of

soil three days later. If using 3”x3”pots for the bombardment, 3-4 plants per pot should be used. The infected plants can be then transferred to pots of soil later. In both cases, 50%-75% of plants get infected.

4. The age of seedlings used for the bombardment should be taken in consideration. Infected young seedlings show silencing faster and silencing is extensive, but the plants develop severe symptoms. Indeed, similar phenotypes were observed regardless of the age of seedlings, but symptoms are more pronounced if younger seedlings are used for the bombardment. I found that 10 to 12-leaf stage seedlings were effective for silencing.

5. Growth condition (short-day and long-day) could be an important factor depending upon what gene is used and where that gene is expressed in plants. Short-day growth condition promotes rosette leaves whereas long-day condition promotes bolting.

6. It is possible to achieve the simultaneous silencing of two or more genes by cloning the sequences of the individual genes into one vector or by inoculating plants with several constructs. At least two genes have been simultaneously silenced using geminiviruses-derived vectors ([6], and Chapter 5). However, the length of all inserts should not be longer than 800 bp or should be less. For example, if using pCPCbLCVA.007 as a cloning vector, 400 bp-800 bp can be used. If an insert fragment is obtained by PCR or RT-PCR, PCR products flanked by restriction enzymes facilitate cloning. I used *Acc*651 and *Xba*l located at the 5’ end and 3’of the PCR product, or vice versa, depending on what orientation of the insert is preferred. Silencing produced from antisense transcripts derived from the viral promoter was more extensive than from sense transcripts (Chapter 5). However, extensive silencing was obtained regardless of the insert orientation after 25 dpi.

7. Because plants infected with CbLCV-derived vectors have severe symptoms and the symptoms could affect the silencing phenotype of a gene of interest, modified vector producing attenuated symptoms is required before functional studies can be performed.

Future directions

Although geminivirus-derived vectors have the ability to suppress or silence gene expression in plants by activating sequence-specific RNA degradation, a better understanding of this viral-mediated silencing is necessary. It would be interesting to know what other gene components are required for G-VIGS, transgene and endogenous genes, and which of these genes are inhibitors or activators of G-VIGS. In addition, it is also interesting to know if geminiviruses contain silencing inhibitors, what they are, and what the targets of the viral inhibitors of G-VIGS are. Indeed, CbLCV could inhibit PTGS of a GUS transgene but the inhibition was weak. It remains to be determined how geminiviruses themselves escape silencing.

Several lines of evidence have shown that DNA methylation of the silenced genes is associated with PTGS. Unfortunately, we were unable to detect methylation changes using methylation-sensitive restriction enzymes methods within the region of homology due to the high level of viral DNA. Viral DNA should be eliminated from total genomic DNA isolated from infected plants before subjected to methylation-sensitive restriction enzymes digestion.

Clearly, an exciting and challenging direction for this field is dissecting the factors that associate with G-VIGS and applying this technology for gene discovery in plants.

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Appendix A.

Virus-induced gene silencing in silencing defective (*sde*) mutants

Materials and Methods

Plant materials

Seeds of *sde1, sde2, sde3* (silencing defective) mutants and Amp 243 plants were kindly provided by Dr. Baulcombe (Sainsbury Laboratory, Norwich, U.K.). Amp243 plants and *sde* mutants were previously described by Dalmay et al. [1]. In brief, the Amp243 transgenic plants contain a 35S-PVX::GFP, and *sde* plants contain both the 35S-PVX::GFP and the 35S-GFP transgene with a mutation.

Plasmid vectors and bombardment

pMTCbLCV A::CH42GFP contains a fusion of a 360 bp *CH42* fragment and a 388 bp GFP fragment transcribed by the AR1 promoter [2]. pCpCbLCV B.002 is a PUC-based vector containing an 1.5 half copy of CbLCV B. Transgenic Arabidopsis seeds were geminated on a flat for five weeks. Seedlings were then transferred to a 3'x3' pot with 3-4 seedlings per pot, the bombardment was then performed a week later. A combination of pMTCbLCVA::CH42GFP and wild type CbLCV B were used to co-bombard the seedlings. The DNA bombardment was described in detail ([2], Chapter 3). 75-100 % of plants were infected using this method. Plants were grown at 22/20 °C under conditions of an 8 hr light/16 hr dark photoperiod, 70% humidity and 120 μEm-2 lighting at two locations during this study.

Southern blot analysis

DNA was extracted 30 dpi from systemically infected leaves of three individual plants of each transgenic lines infected with CbLCV::CH42GFP [3]. Five μg of each DNA sample was digested with *Acc*651 to produce linear CbLCV DNA fragments (~2.7 kb), and loaded in duplicate. Following electrophoresis and transfer to a Hybond-N

membrane, the blot was hybridized with specific CbLCV AL1 cDNA or 25S probes [4]. Gel blot signals were quantified by phosphorimaging (Bio-Rad Molecular Imager FX, CA) and normalized using internal 25S ribosomal rRNA signals.

Measurement of chlorophyll content

At 30 days after bombardment, systemically infected leaves were assayed for total chlorophyll content according to Moran [5], as previously described in Chapter 4.

Results and discussion

SDE1, SDE2, and SDE3 have previously been shown to be required for transgene induced post-transcriptional gene silencing (PTGS) [1]. SDE1 (identical to SGS2 which was independently identified by Mourrain et al. [6]) and SDE3, encode an RNA-dependent RNA polymerase (RdRP) and RNA helicase-like protein, respectively. In contrast to transgene silencing, SDE1 and SDE3 (as tested) were not required for VIGS by some RNA viruses such as tobacco rattle virus (TRV) [7, 8]. Inoculation of *sde1* or *sde3* mutants with TRV:GFP or TRV:PDS caused the loss of green fluorescence and photobleached symptoms, respectively. These results suggested that viral-encoded proteins might be functionally equivalent to SDE1 or SDE3 in the silencing process [7, 8]. However, geminiviruses, which have a DNA genome, do not encode an RNA polymerase or RNA helicase-like protein [9, 10]. We therefore anticipated that SDE1 and SDE3 might be required in the geminivirus silencing pathway. To investigate whether these genes are required for the geminivirus-induced gene silencing (G-VIGS), *sde1, sde2, sde3* mutants and control transgenic line, Amp243, were inoculated with a combination of CbLCV A::CH42GFP and wt CbLCV B. The Amp243 transgenic

Arabidopsis plants contain a 35S-PVX::GFP, while *sde* plants contain both the 35S-PVX::GFP and the 35S-GFP transgene with a mutation [2].

Silencing of *chlorata42* (*CH42*) is characterized by a yellowish phenotype, due to a loss of chlorophyll production. Initially, light green leaves in new growth were observed in infected *sde1* plants, while infected *sde2, sde3,* or Amp243 plants had yellowed leaves (Fig. 1). Light green leaves persisted throughout the experimental period (30 dpi) in some infected *sde1* plants. Fig. 2 shows percentage of infected plants showing silencing during plant development. The delayed activation of silencing was observed in infected *sde1* plants compared to the others. Indeed, infected *sde1* plants had 10% extensive silencing at 20 dpi while infected *sde2, sde3* and Amp243 plants had extensive silencing ranged between 57% and 80%.

Fig. 3 shows silencing levels and chlorophyll content at 30 dpi. Most CbLCV::CH42GFP infected *sde1* plants had weak silencing (70%) while *sde2*, *sde3* and Amp243 plants had most of their population showing strong and moderate silencing (Fig. 3A). Chlorophyll measurements showed that the loss of chlorophyll was not extensive in infected *sde1* plants compared to other mutants and control plants (Fig. 3B). Taken together, these results suggest that only SDE1 is required for G-VIGS.

It is thought that PTGS is a plant defense mechanism against viruses or invasive nucleic acid [11]. Several silencing mutants such as *sde1* and *sde3* showed enhanced susceptibility to some viruses such as cucumber mosaic virus (CMV), with high viral RNA accumulation and severe symptoms [8]. Similar results were obtained when s*gs2* and *sgs3* mutants were infected with CMV, but not with turnip vein clearing tobamovirus (TVCV) and turnip mosaic potyvirus (TuMV) [6]. When *sde* mutants were infected with

CbLCV::CH42GFP, we found that disease symptoms in new growth were more severe in infected *sde1* and *sde2* plants than in infected *sde3* or Amp243 plants (Fig. 1). The infected *sde1* and *sde2* plants had small new growth with curly leaves while infected *sde3* and Amp243 plants developed more new growth with bigger leaves. We reasoned that the *sde1* and *sde2* mutations lost part of a defense mechanism against CbLCV. Despite the severe symptoms that were observed in *sde1* and *sde2*, there was no correlation between viral DNA accumulation (measured by quantitative analysis of phosphoimager data) and symptom severity among all four genetic backgrounds (Fig. 4). The difference in symptom severity caused by CbLCV infection was not due to amount of viral DNA accumulation. This was consistent with Sunter et al. [12] showing that increased disease symptoms were not always correlated with viral DNA accumulation levels.

VIGS in *sde* mutants is complex. All *sde* plants contain a PVX:GFP amplicon and 35S-GFP transgene, prior to CbLCV::CH42GFP. We cannot rule out that viral proteins (of PVX) could replace the SDE1, SDE2 or SDE3 functions. Based on our results, it seemed that SDE1 was involved in the silencing pathway but not SDE3. SDE2 had slightly effect on the *CH42* silencing but had a major effect on symptoms. It is possible that SDE2 has a role in the plant defense mechanism against viruses. On the other hand, it could be possible that SDE2 is required for cytoplasmic RNA virus silencing. A further investigation of this gene would be necessary.

Acknowledgements

We thank Dr. Baulcombe (Sainsbury Laboratory, Norwich, U.K.) for kindly providing seeds of *sde1, sde2, sde3* (silencing defective) mutants and Amp 243 plants.

We also thank Dr. Arthur Weissinger and his lab for use of the particle gun bombardment device, Dr. Michael Turnage for pMTCbLCV::CH42GFP, and Dr. Kent Burkey for use of a spectrophotometer.

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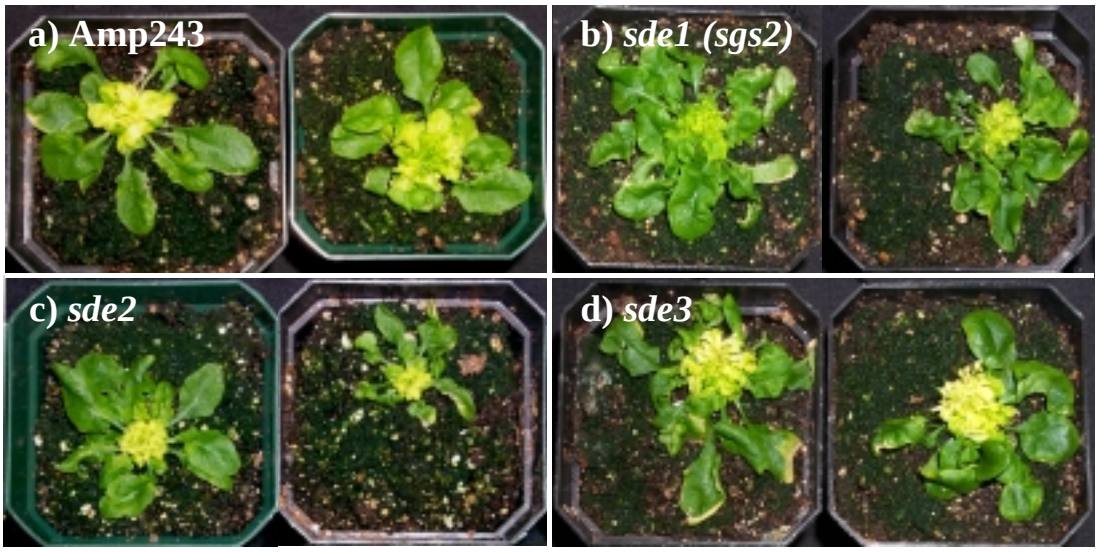

Figure 1. VIGS of a *CH42* endogenous gene in infected *sde* plants.
sde1, *sde2*, and *sde3* (for silencing defective) mutants and control Amp243 plants (PVX amplicon) were inoculated with a combination of CbLCVA::CH42GFP and wt CbLCV B, and photographed at 30 dpi. CH42 silencing was less extensive in infected *sde1* plants (most or all systemically infected leaves were light green). Symptoms were more pronounced in infected *sde1* and *sde2* than in infected *sde3* and Amp243 plants.

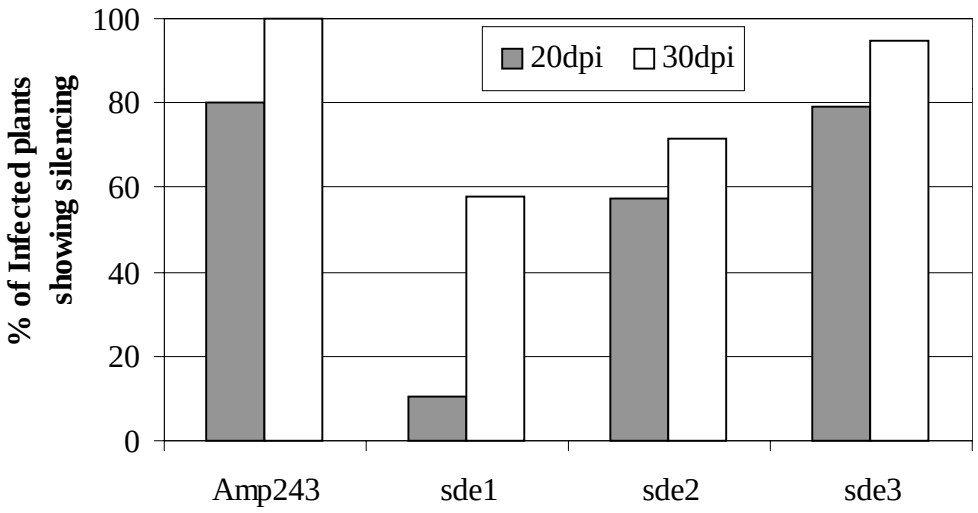

Figure 2. Silencing during plant development.
Percentage of infected plants showing silencing was determined by number of plants showing yellow tissue as a lack of chlorophyll divided by number of symptomatic plants (infected plants). The data were obtained at 20 and 30 dpi from three independent experiments.

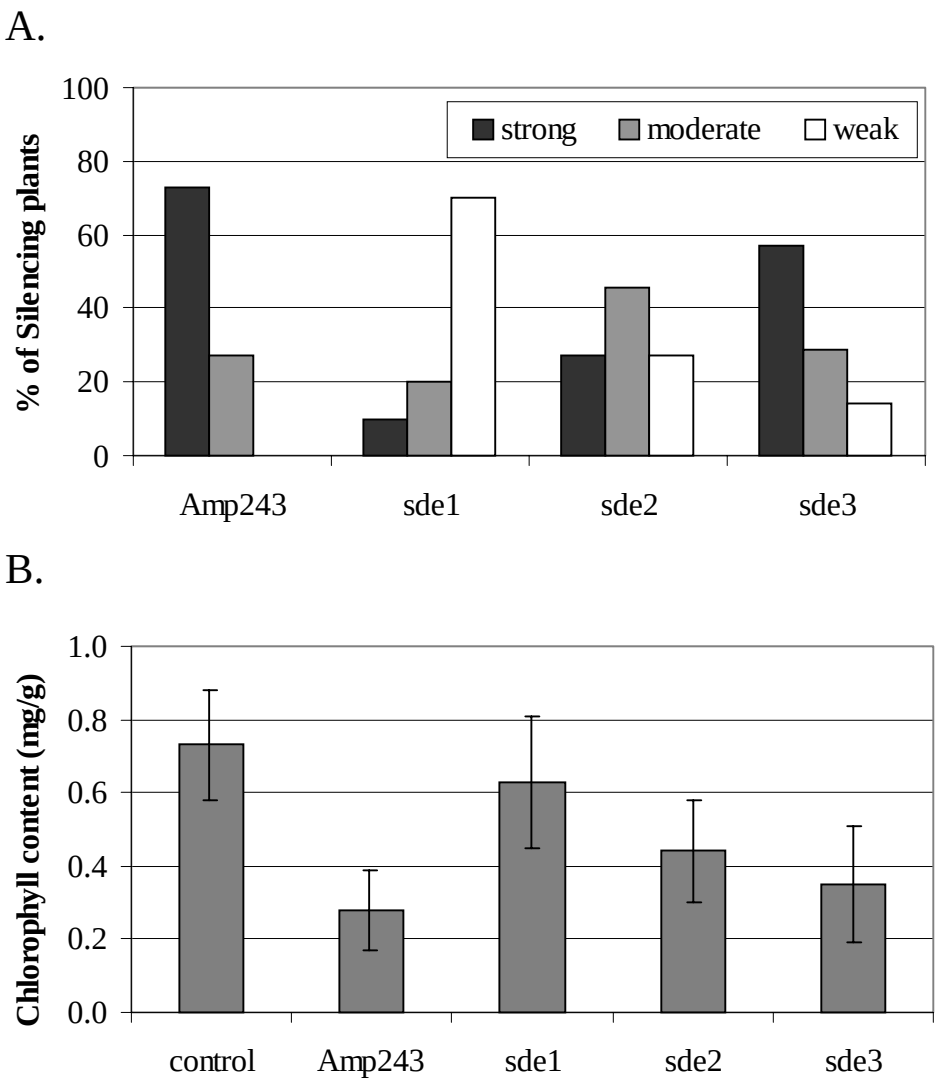

Figure 3. **Silencing levels and chlorophyll content in infected *sde* plants.**
A. Silencing levels were sorted into three categories: strong, moderate and weak. "Strong", most or all systemically infected leaves were yellow; "moderate", a few systemically infected leaves were yellow; "weak", most or all systemically infected leaves were light green or green. This graph presents the result from one experiment.
B. Total chlorophyll content was determined from 10 infected plants demonstrating that the loss of chlorophyll was not efficient in infected *sde1* plants compared to the others. The data present one experiment. Error bars indicate one standard deviation from the mean.

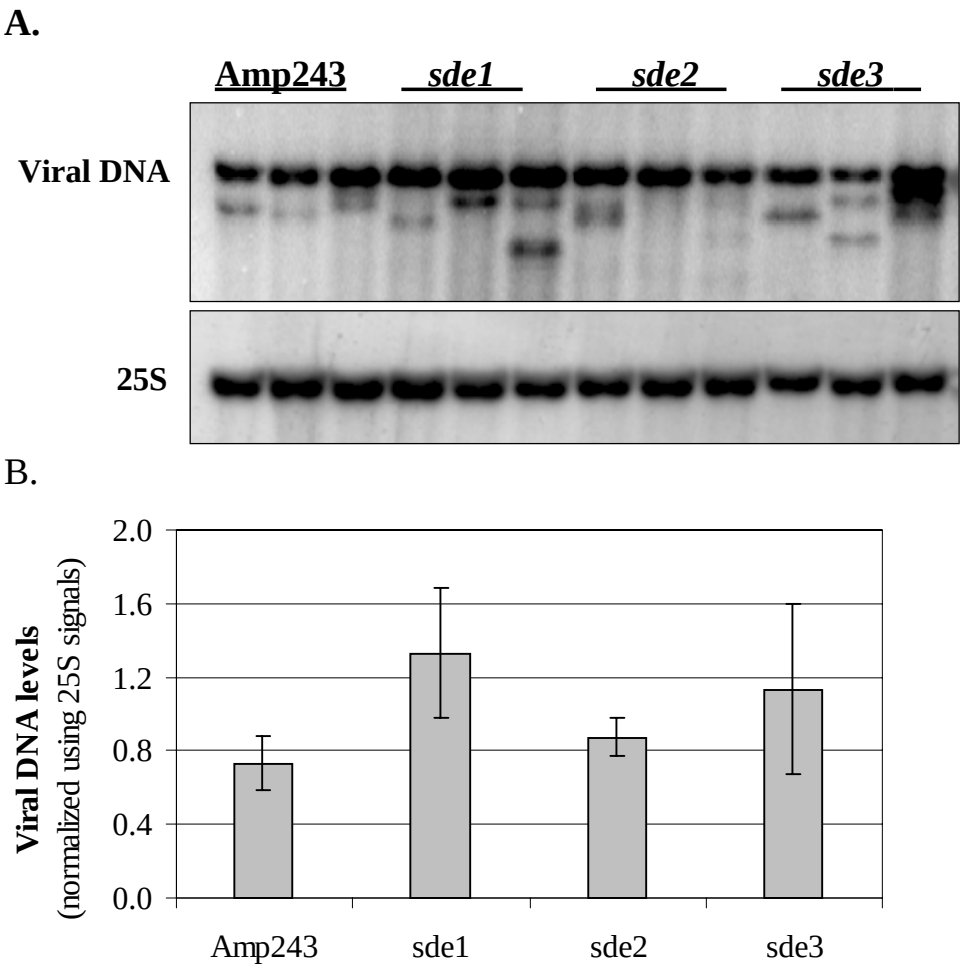

Figure 4. Viral DNA accumulation in infected Amp243 and *sde* plants.
A. Amp243 and *sde* plants were infected with CbLCV::CH42GFP. Total DNA was extracted 30 dpi from systemically infected leaves of three individual plants of each transgenic line. Five µg of each DNA sample was digested with *Acc*651 to produce linear CbLCV DNA fragments (~2.7 kb). Following electrophoresis and transfer to a Hybond-N membrane, the blot was hybridized with specific CbLCV AL1 cDNA or 25S probes.
B. Gel blot signals were quantified by phosphorimaging (Bio-Rad Molecular Imager FX, CA) and normalized using internal 25S ribosomal rRNA signals. Viral nucleic acid signal intensity is an average of three individual plants from each line.

Appendix B.

Supplementary data

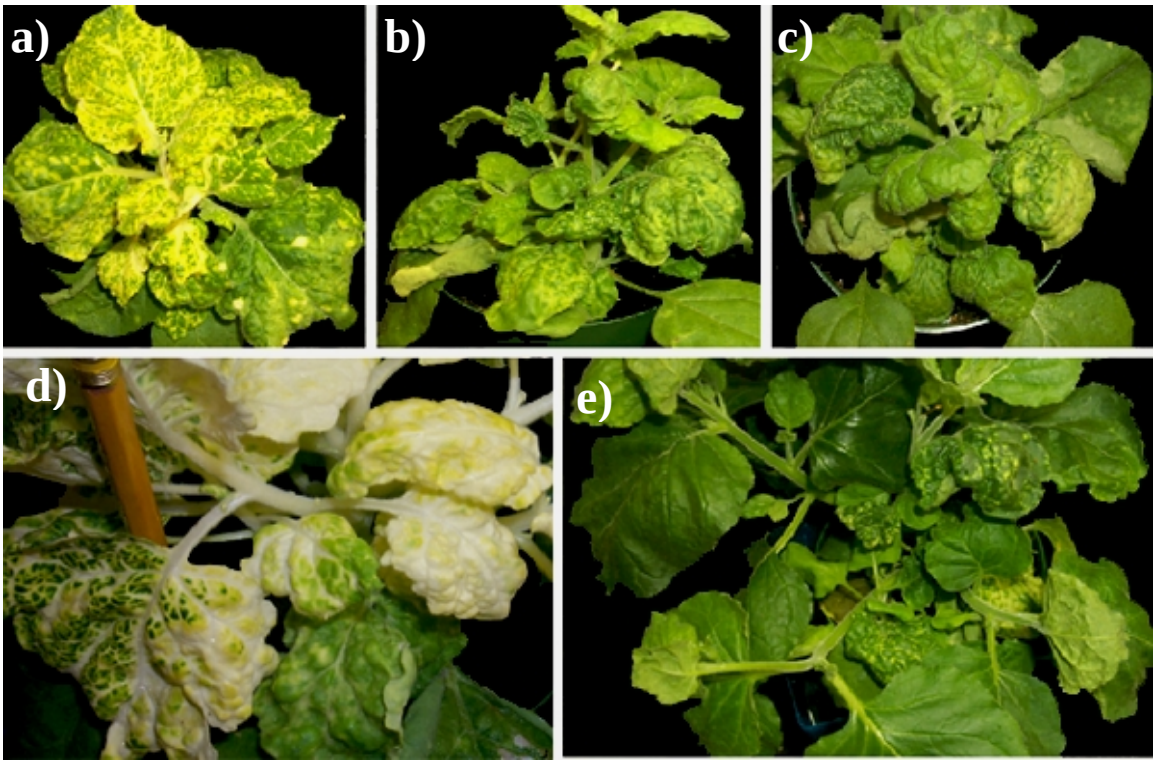

Figure 1. Attenuated symptoms in *Nicotiana benthamiana* plants silenced in *clone 9*. Plants were inoculated with a combination of TGMV A and TGMV B::Clone9 and photographed at two weeks (b-c) and four weeks post inoculation (e). Control plants were inoculated with TGMV A/TGMV B::su at two (a) and four w.p.i (d).

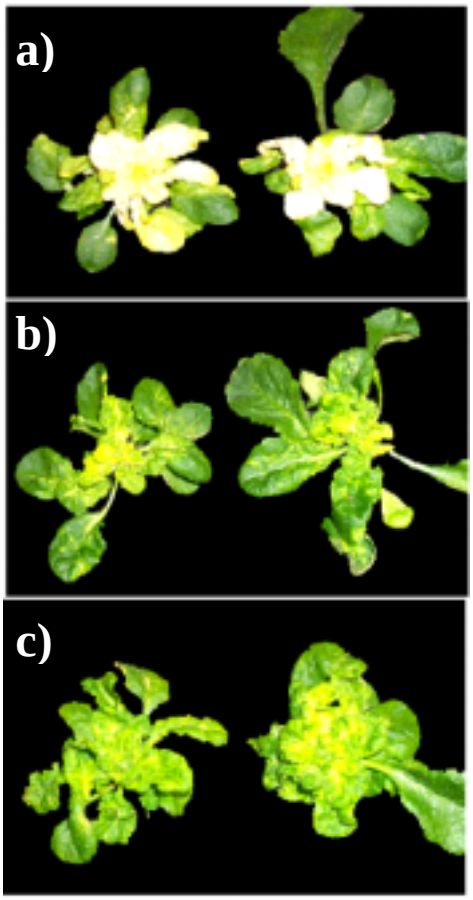

Figure 2. CbLCV B is not as good as CbLCV A as a silencing vector
Silencing of *CH42* was less extensive if CbLCV B was used as a silencing vector, even when bombarded with a combination of CbLCV A empty vector (b) or CbLCV A carrying a 388 bp GFP fragment (c). CbLCVA::CH42–infected plants showed extensive silencing and attenuated symptoms (a).

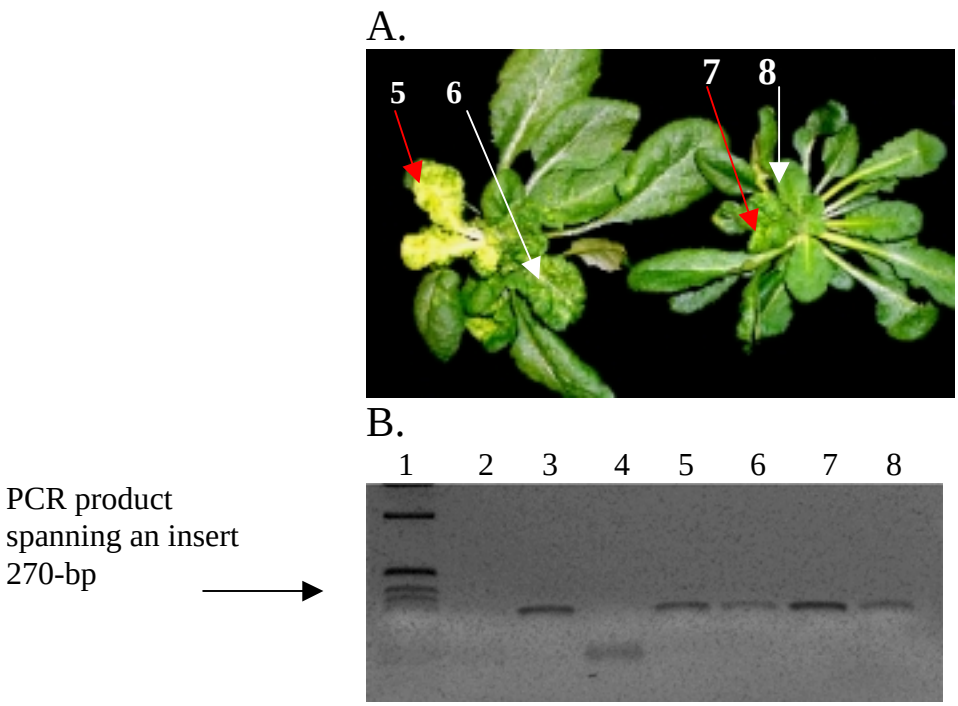

Figure 3. Silencing phenotypes using a CbLCV B-derived vector.
A. Arabidopsis plants ecotype Columbia were inoculated with a combination of CbLCVA and CbLCV B::CH42. Infected plants showed severe symptoms and less extensive silencing.
B. PCR analysis of product spanning the inserted *CH42* fragment demonstrating that the presence of weak silencing (5, 7, 8) was not due to the lost of inserted fragment. PCR products similar in size were obtained from tissues showing extensive or less extensive silencing.
Lane 1, the molecular marker; lane 2, negative control showing that PCR reaction was not contaminated; lane 3, positive control for a plasmid containing a CH*42* fragment; lane 4, negative control of empty vector; lane 5-8, PCR products from tissues of plants above panel as indicated by numbers.

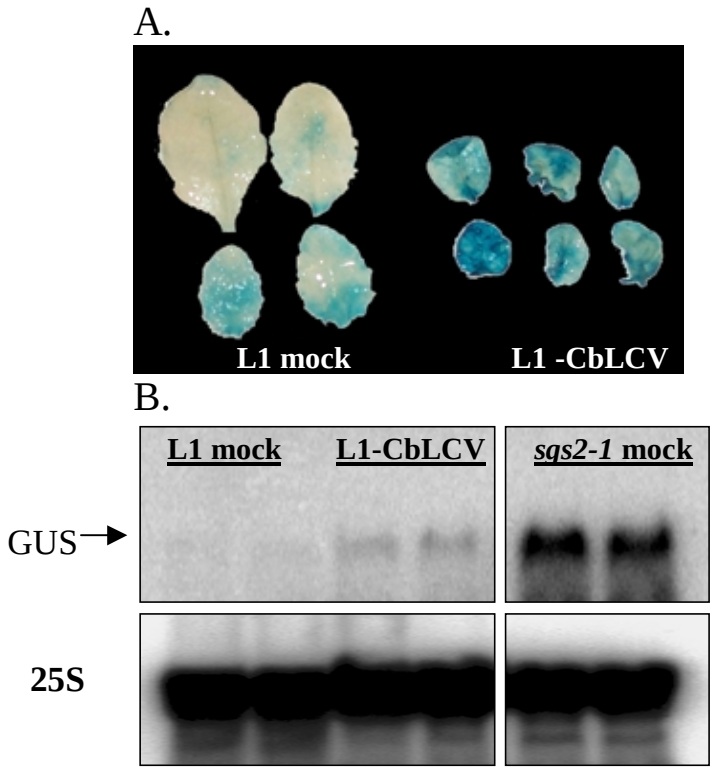

Figure 4. **PTGS of the GUS transgene was partially inhibited by CbLCV infection.**
A. GUS staining of leaves from L1 (GUS silenced) either mocked or inoculated with CbLCV at 25 dpi. The L1 transgenic Arabidopsis plants contained a 35S-GUS transgene and were post-transcriptionally silenced. The *sgs2-1* mutant was impaired with PTGS and was obtained by a mutagenesis of the L1 line. SGS2 encodes for RNA dependent RNA polymerase (RdRP).
B. Northern blot analysis of a steady stage of *uid*A GUS mRNA. 10 µg total RNA per lane was loaded in duplicate and probed with *uid*A GUS or 25S probes. The steady stage of *GUS* mRNA was twofold increased in the L1-CbLCV plants, in comparison to the L1 mock plants.