ABSTRACT

Yu, Ming. Characterization of cDNAs from *Nicotiana benthamiana* that encode proteins which interact with tomato golden mosaic virus AL2 protein in the yeast two-hybrid system. (Under the direction of Dr. I.T.D. Petty).

The AL2 protein of tomato golden mosaic virus (TGMV) is multifunctional. It is required for derepression of the TGMV *AR1* gene in phloem tissue, and for *trans*-activating the *AR1* and *BR1* genes in mesophyll cells. It also enhances virus susceptibility when expressed in transgenic plants. It is thought that TGMV AL2 protein accomplishes these functions by interactions with unknown host factors. In this study, cDNAs from *Nicotiana benthamiana* plants that encode proteins which interact with TGMV AL2 were characterized. A yeast two-hybrid assay identified two cDNA clones, Nb#51 and Nb#62, that specifically interacted with TGMV AL2, but not with negative control proteins. Sequences of these two cDNA clones were determined by primer walking, which revealed that Nb#51 appears to be a 3'-coterminal truncated version of Nb#62. Inspection of the amino acid sequences encoded by Nb#62 found the presence of both ankyrin-repeats and tetratricopeptide repeats (TPR). Deletion analysis showed that the TPR motif, together with its flanking regions, was sufficient to confer on Nb#62 the ability to interact with TGMV AL2, whereas the ankyrin-repeats were not required for this interaction. Nb#62-specific mRNAs were detected in *N. benthamiana* plants by northern hybridization in potato virus X (PVX) infection experiments, but not in heat-shock experiments. Virus-induced gene silencing (VIGS) assays were used to investigate the possible function(s) of the Nb#62-encoded protein in normal plants and in the context of a TGMV infection. When PVX carrying a 618-bp fragment from the 5'-end of the Nb#62

cDNA was used as a silencing trigger in *N. benthamiana* plants, VIGS effectively targeted the transcripts which contained sequence similarity with the trigger fragment. However, the infected plants didn't have difference in the phenotype when compared to the PVX vector infection. When TGMV carrying a 93-bp fragment from the 5'-end of the Nb#62 cDNA infected plants, viral DNA accumulation in the upper leaves was reduced, when compared to wild-type TGMV or a control TGMV construct containing a 95-bp fragment from the tobacco *Sulfur* gene.

Characterization of cDNAs from *Nicotiana benthamiana* that encode proteins which interact with tomato golden mosaic virus AL2 protein in the yeast two-hybrid system

by

Yu, Ming

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APPROVED BY:

Dr. Eric S. Miller	Dr. Fred J. Fuller

Chair of Advisory Committee
Dr. Ian T.D. Petty

BIOGRAPHY

I was born on August 21st, 1975 in Wuxi, P.R.China. Came to United States in 2000. Attended the Department of Microbiology at North Carolina State University in 2001. I will attend the oral biology Ph.D. program, at UNC-CH School of Dentistry, starting from August 2003.

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List of Abbreviations

aa	amino acid
ACMV	African cassava mosaic virus
Ank	ankyrin-repeats
BGMV	bean golden mosaic virus
BLAST	basic local alignment search tool
bp	base pair
CabLCV	cabbage leaf curl virus
CMV	cucumber mosaic virus
CP	coat protein
dpi	day post-inoculation
dsDNA	double stranded DNA
dsRNA	double stranded RNA
GFP	green fluorescent protein
HA tag	hemagglutinin tag
HC-Pro	helper component-proteinase
HSP70	heat shock protein 70
kb	kilobase
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PDS	phytoene desaturase

poly A	polyadenylated
PTGS	post-transcriptional gene silencing
PVX	potato virus X
5'- RACE	5'- rapid amplification of cDNA ends
RdRP	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-PCR	reverse transcriptase-polymerase chain reaction
siRNA	small interference RNA
ssDNA	single stranded DNA
ssRNA	single stranded RNA
su	tobacco *Sulfur* gene
TrAP	*trans*-activation protein
T-DNA	transfer DNA from *Agrobacterium tumefacians*
TGMV	tomato golden mosaic virus
TPR	the tetratricopeptide repeats
TRV	tobacco rattle virus
3'- utr	3'- untranslated region
VIGS	virus-induced gene silencing
wt	wild type

INTRODUCTION

Post-transcriptional gene silencing and virus-induced gene silencing in plants

Post-transcriptional gene silencing (PTGS) in plants is a sequence-specific, RNA-degrading process that has recently been related to plant defense responses against invasive or mobile nucleic acids. PTGS-related pathways have also been found in fungi (called quelling) and animals (called RNA interference).

A unifying feature of these processes in different organisms is to form a high level of double stranded RNA (dsRNA). In plants, the high level of dsRNA comes from a broad range of silencing inducers including transgenes that express self-complementary, single-stranded hairpin RNA (hpRNA), replicating viruses and dsRNA molecules. If a virus carrying sequences homologous to a nuclear gene is used as a trigger, silencing will cause the cytoplasmic degradation of the corresponding mRNA. This phenomenon is termed virus-induced gene silencing (VIGS) (Kjemtrup *et al*., 1998; Ruiz *et al*., 1998)

Our current understanding of the mechanisms underlying PTGS comes from diverse experimental systems including genetic studies in plants and *Caenorhabditis elegans,* and biochemical studies of drosophila extracts (Bernstein *et al*., 2001; Dernburg *et al*., 2000; Pal-Bhadra *et al*., 1997). The convergence of observations suggests that there are common elements of silencing pathways in different organisms and that dsRNA is a potent trigger of silencing in many, if not all, organisms. Exactly how the dsRNA is produced remains unsolved. In plants, it has been proposed that a cellular RNA-dependent-RNA polymerase (RdRp)-like protein may recognize 'aberrant transcripts' derived from highly expressed loci and convert these into dsRNA. In arabidopsis plants,

the RdRp homologue SDE1/SGS2 is required for transgene silencing, but not for RNA virus induced silencing (Dalmay *et al*., 2000). Therefore, VIGS mediated by RNA viruses might use an SDE1/SGS2-independent pathway to produce dsRNA. Recent results suggest that viral RdRp could provide dsRNA in VIGS mediated by RNA viruses (Mourrain *et al*., 2000).

Once produced, the dsRNA intermediates are recognized by an RNA digesting enzyme and cleaved into small interfering RNAs (siRNAs) 21-25 nucleotides in length (Figure 1). These siRNAs are proposed to pair up with complementary single stranded target RNA, and direct RNA-degradation machinery to the target RNA in a sequence-specific manner. This model reflects the fact that PTGS is highly sequence-specific and the specificity is dictated by the silencing trigger itself. Recently, Bernstein *et al*. (2001) identified a silencing-associated dsRNase in drosophila, named Dicer. This protein was proposed to contain RNase III-like, helicase and dsRNA binding domains. It potentially might bind and untwist dsRNA, and cleave it into siRNAs of 21-25 nucleotides in length. These siRNAs could then associate with Dicer, or other RNA-degrading nuclease(s), to form the sequence-specific silencing complex that degrades target sequences containing high sequence similarity with the trigger. This complex, known as RISC (RNA-induced silencing complex), has recently been purified from drosophila S2 cells in which silencing had been triggered with dsRNA *in vivo* (Hammond *et al*., 2000)

An intriguing property of PTGS in plants is that silencing can be triggered locally, then spread throughout the plant. There are several ways to initiate PTGS. Apart from transgenes and viruses, DNA delivered by bombardment, agrobacterium infiltration and grafting of unsilenced scions onto silenced rootstock can also initiate PTGS under some

conditions (Palauqui *et al*., 1997; Voinnet *et al*., 1998). Silencing spreads from these localized delivery points into most distant tissues. The mobile silencing signal has not been identified, but is expected to contain a nucleic acid component because of the sequence specificity of PTGS. Two types of signal transmission have been identified. First is short-range, cell-to-cell transmission through plasmodesmata, the cytoplasmic bridges connecting plants cells. Second is long-distance transmission via the phloem. Notably, spreading of silencing signals exploits the same routes as those of viruses for cell-to-cell and systemic movement (Wolf *et al*., 1991; Carrington *et al.*, 1996). For a virus to cause systemic infection, it may have to be capable of interfering, or competing, with the intercellular silencing signal. Alternatively, fast moving viruses may be able to spread before PTGS can be triggered.

The biological role of PTGS has been proposed to serve as a plant defense system against virus infection. The silencing-associated siRNAs are targeted toward the viral genome, which results in viral sequence-specific RNA degradation. In this way, plants are able to limit virus propagation. An increasing number of observations have risen in support of the proposed relationship between PTGS and plant antiviral defense. In the case of tomato black ring virus, silencing causes a 'recovery' phenotype, in which new leaves are free of virus and lack symptoms. Recovered plants are immune to infection by a second virus that contains sequence homology to the silencing triggering virus. This is also termed cross protection (Ratcliff *et al*., 1997).

In support of the link between PTGS and plant antiviral defense, viruses are found to encode proteins that counteract silencing. These silencing suppressors can be identified with a silencing-reversal assay, in which a transgene encoding green fluorescent protein

(GFP) is used as a reporter. This assay showed that completely silenced GFP transgenes in plants could be reactivated after the plants were infected with specific viruses, or transfected with viral genes encoding a silencing suppressor protein.

Several suppressors of PTGS have been reported in RNA viruses. Helper component-proteinase (HC-Pro) in potyviruses was identified as a highly effective suppressor. HC-Pro targets the maintenance of silencing triggered by either transgenes or viruses (Llave *et al*., 2000). The silencing-associated siRNAs from the target gene are absent after HC-Pro is induced, and an already established silencing of a transgene could be reversed in cells that express HC-Pro (Mallory *et al*., 2001; Brigneti *et al*., 1998). Recent studies indicated that the PTGS suppression effect of HC-Pro also involves its interaction with a tobacco gene *rgs-CaM*, the first identified cellular suppressor of silencing (Anandalakshmi *et al*., 2000). In contrast to the effects of HC-Pro, the 2b protein of cucumber mosaic virus (CMV) appears to interfere with the silencing initiation or signaling step. Silencing reversal of a reporter gene occurred only in plant tissues that newly emerged after induction of 2b protein or infection by CMV, but not in previously silenced old tissues (Brigneti *et al*., 1998).

DNA viruses likely encode PTGS suppressors as well. In particular, the AC2 protein of the geminivirus African cassava mosaic virus (ACMV) produced a similar pattern in a silencing reversal assay as HC-Pro of potyviruses did. Voinnet *et al*. (1999) showed that ACMV AC2 protein carried by a potato virus X (PVX) vector reactivated GFP reporter gene expression in previously silenced GFP-transgenic plants.

On the other hand, plant viruses that do not encode strong suppressors of PTGS can be used as vectors to trigger silencing. In practice, VIGS has been developed into an

efficient functional tool for genomic research in plants. The underlying mechanism is that if a recombinant virus carrying fragments from exons of a plant host gene triggers PTGS, the silencing would also target transcripts of the host gene and mimic a knockout phenotype in plants. Compared to conventional approaches for analysis of gene function in plants, there are several distinct advantages of VIGS. First, conventional techniques like insertional mutagenesis cannot target genes which are essential, either for housekeeping functions, or for normal development. VIGS overcomes these problems because it can be applied to mature plants. Second, compared to transgenic plant technology, which is laborious and time-consuming, VIGS is rapid and can be suited to high-throughput analysis. Starting with the partial sequence of a target gene, a virus vector carrying a fragment from the target gene can be constructed within days. The construct may be infectious as directly inoculated DNA if the vector is a DNA virus, or as *in vitro* transcript of an RNA virus.

Several VIGS vectors have been constructed from the genomes of RNA or DNA viruses for applications in plants. VIGS systems that use the RNA viruses PVX, or tobacco rattle virus (TRV) have been developed to silence endogenous genes as well as transgenes in tobacco and tomato plants (Ruiz *et al*., 1998; Liu *et al*., 2002). Geminiviruses, which have DNA genomes, have also been developed as VIGS vectors. Tomato golden mosaic virus (TGMV) is an efficient VIGS trigger in *N. benthamiana* plants (Kjemtrup *et al*., 1998; Peele *et al*., 2001), while cabbage leaf curl virus (CabLCV) can be used to trigger gene silencing in arabidopsis plants (Turnage *et al*., 2001).

Geminiviruses and their AL2 proteins

Members of the family *Geminiviridae* are small plant-infecting viruses characterized by a circular single stranded (ss) DNA genome, which is encapsidated in geminate particles. Three genera have been distinguished among the family members based on their genetic and biological features: they are the mastreviruses, the curtoviruses and the begomovirus (van Regenmortel *et al*., 2000). Although both the mastreviruses and the curtoviruses have a monopartite genome and are transmitted by leafhoppers or treehoppers, the mastreviruses infect monocotyledonous plants while the curtoviruses infect dicotyledonous plants. Begomoviruses may have either a monopartite or bipartite genome, but they are all transmitted by whiteflies, and infect dicotyledonous plants (van Regenmortel *et al*., 2000). Viruses of the genus *Begomovirus* are of particular interest. Examples of bipartite begomoviruses, which are the most intensively studied geminiviruses, are TGMV and bean golden mosaic virus (BGMV). Although they have very similar genome organization, number of genome components and mechanisms of DNA replication, they exhibit distinct host specificity. Both of them can establish an infection in *N. benthamiana.* However, BGMV is not well adapted to this host in comparison with TGMV. BGMV causes asymptomatic systemic infection and accumulates 25-fold less viral DNA than TGMV (Petty *et al*., 1995).

The bipartite geminiviruses contain two 2.6-kb circular ssDNA components called A and B, which harbor six well characterized viral genes. Each DNA component is separately encased in a twinned icosahedral capsid (Figure 2). There are two genes located on the DNA B component, *BR1* and *BL1*. Both *BR1* and *BL1* encode movement proteins that are required for local and systemic movement of TGMV in infected leaves

(Brough *et al.,* 1988; Jeffrey *et al.,* 1996). The BR1 protein is thought to transport viral ssDNA from the nucleus to the cytoplasm, whereas BL1and BR1 function cooperatively in trafficking of the viral genome from cell to cell (Noueiry *et al*., 1994; Sanderfoot *et al*., 1995).

Four genes (*AL1, AL2, AL3* and *AR1*) are located on DNA A. The *AL1* and *AL3* genes encode products that are involved in viral DNA replication. The AL1 protein provides replication origin recognition and enzymatic functions (Fontes *et al*., 1994; Hanson *et al*., 1995; Hoogstraten *et al*., 1996; Orozco *et al*., 1998), whereas AL3 enhances viral DNA accumulation up to 50-fold by an unknown mechanism (Sunter *et al*., 1990). More details on the functions of *AL2* will be presented below. The *AR1* gene encodes the viral coat protein (CP) (Kallender *et al*., 1988; Azzam *et al*., 1994). The *AL4* open reading frames of BGMV and TGMV are either non-functional or encode a product that is completely dispensable in infected plants (Elmer *et al*., 1988; Pooma and Petty, 1996; Hoogstraten *et al*., 1996).

The *AL2* gene encodes a multifunctional regulatory protein with remarkable features, which is essential for infection of plant hosts by TGMV. Begomoviruses adopt the general strategy of DNA virus transcription strategies that early gene products activate the expression of viral genes required later in the replication cycle. In TGMV, it is the AL2 protein that activates the transcription of the *AR1* and *BR1* genes (Sunter and Bisario, 1992). The AL2 protein is sometimes called TrAP (*Trans*-Activation Protein), because of this *trans*-activation effect. Consistent with this function, this small protein (15 kDa) has been shown to contain a carboxyl-terminal acidic region responsible for transcription activation (Hartitz *et al.,* 1999). Recently, compelling data suggest another

role for AL2 in the suppression of host defenses against virus infection, which is distinct from its *trans*-activation function. Data obtained from transgenic *N. benthamiana* plants suggested that the N-terminal part of the AL2 protein could antagonize a host defense system *in vivo* (Sunter *et al*., 2001). Expression of a truncated *AL2* transgene lacking the activation domain, or its paralogue, the *L2* gene from beet curly top virus, sensitized the plant to infection with both DNA and RNA viruses (Sunter *et al*., 2001). The data suggested that the begomoviruses and curtoviruses use the products of the *AL2* and *L2* genes to suppress the plant stress response and this suppression function doesn't require the transcriptional activation activity of the AL2 protein.

There are several remarkable features of transcriptional activation by the AL2 protein. First, the regulation mechanism of the TGMV *AR1* gene by AL2 varies in different cell types. AL2 activates the *AR1* promoter in mesophyll cells, but de-represses it in phloem cells, which suggests that there exist distinct cellular factors with which AL2 protein interacts to carry out the transcription activation function (Sunter *et al*., 1997).

More interestingly, studies on transcription activation with other begomoviruses indicate that AL2 doesn't function in a virus specific way. Analysis showed that the AL2 protein from ACMV, Texas pepper virus, squash leaf curl virus, and tomato yellow leaf curl virus can complement a TGMV *al2* mutant in tobacco protoplasts (Sunter *et al*, 1994). Additional evidence showed that AL2 protein is functional interchangeably between BGMV and TGMV (Hung and Petty, 2001). Further, TGMV AL2 protein binds preferably to ssDNA, but shows no sequence specificity *in vitro* (Hartitz *et al*., 1999). The lack of sequence specificity, again, suggests that host factors are highly involved to accomplish AL2 protein functions.

MATERIALS AND METHODS

Microbiology protocol

Yeast Manipulations

Standard microbiological techniques (see following protocols) and media were used. Media designations are as follows: YPD is yeast extract (1%) plus peptone (2%) with 2% glucose. Minimal dropout media are designated by the component that is left out (e.g., -Trp-His medium lacks tryptophan and histidine). Each minimal dropout medium contains 2% glucose (Glu) or 2% galactose (Gal) plus 1% raffinose (raf).

Yeast Strains and Plasmids

EGY48 contains a chromosomal *LEU2* gene with its upstream regulatory elements replaced with three high-affinity LexA operators. Activation-domain fusion proteins (preys) were conditionally expressed from the *GAL1* promoter on plasmid pB42AD (Figure 4), which contains the yeast 2-μm origin and the *TRP1*gene. The LexA fusions (baits) were expressed from the *ADH1* promoter on one of the bait expression vectors, pLexA (Figure 3) or pNLexA (Figure 3). Both contain the yeast 2-μm origin and the *HIS3* gene. The plasmid pRFHM contains amino acids 2-160 of *Drosophila melanogaster* Bicoid fused to LexA. The pLexA-53 contains amino acids 72-390 of murine tumor suppressor p53 fused to LexA. The plasmid pEG202-max contains the human Max protein lacking its C-terminal 15 amino fused to LexA. The plasmid pEG202-Lam contains the amino acids 110-273 of human lamin C protein.

Competent bacterial cells

A single colony of *Escherichia coli* strain DH5αF' was inoculated into 3 ml of LB broth (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, pH7.5) and incubated overnight with shaking at 37°C at 250 rpm. 25 µl of overnight culture was sub-cultured into 7.5 ml of LB broth in a sterile plastic tube and then incubated at 37°C with shaking for 4 hours. The culture was centrifuged in a clinical centrifuge at approximately 5000 rpm for 3 minutes. The supernatant was decanted and the cell pellet was suspended in 7.5 ml of cold 50 mM $CaCl_2$ and incubated on ice for 20 minutes. The cells were then centrifuged again in a clinical centrifuge at approximately 5000 rpm for 3 minutes and the pellet was resuspended in 1 ml of cold 50 mM $CaCl_2$.

Transformation of *E. coli*

200 µl of competent *E. coli* cells were added to each Eppendorf tube containing the DNA (0.2 µl of purified plasmid DNA or a complete ligation reaction for each transformation). The tube was incubated on ice for 40 minutes, and heat shocked in a 42 °C water bath for 3 minutes. They were put back on ice for another 5 minutes. 0.8 ml of 2xTY broth was added to the tube and incubated at 37 °C for at least 10 minutes (Transformation for a ligation reaction usually needs more than 45 minutes.). 200 µl aliquots of the cell suspension were spread on a LB plate containing 100 µg/ml of ampicillin. Plates were air-dried and incubated at 37 °C overnight.

Transformation of yeast

A yeast colony (strain EGY48) was inoculated into 5 ml of YPD and incubated at 30°C with shaking at 250 rpm overnight. The overnight culture was diluted into 1:10 and 1:100 in sterile deionized water (dH_2O). 10-µl aliquot of the 1:100 dilutions was placed

in a hemocytometer for cell counting. A portion of the overnight culture containing $2.5X10^8$ cells was added to 50 ml of YPD and incubated at 30 ºC with shaking at 250 rpm for 4 hours. After the 4-hour incubation period, the cells were counted again to ensure that the cell population had doubled.

The culture was then transferred to a sterile 50ml falcon tube and spun in a clinical centrifuge at approximately 3000 rpm for 5 minutes. The supernatant was poured off and the cell pellet was resuspended in 25 ml of sterile dH_2O. The cell pellet was resuspended and spun again for 5 minutes. The supernatant was removed and the cell pellet was resuspended in 1ml of 100 mM lithium acetate. The suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged to pellet the cells. The cell pellet was resuspended in 450 µl of 100 mM lithium acetate. 50 µl of the suspension was aliquoted for each transformation in 1.5 ml Eppendorf tubes. The tubes were spun down and the supernatant was removed. The following reagents were added into the cell pellet in each tube in the following order: 240 µl of 50% polyethylene glycol 3350, 36 µl of 1.0 M lithium acetate, 25 µl of sheared and denatured (2 mg/ml) salmon sperm DNA, 50 µl of sterile dH_2O containing plasmid DNA. The cell pellet was resuspended by vigorous mixing, then incubated for 30 minutes at 30 ºC and heat shocked in a 42ºC water bath for 25 minutes. Then the cell pellet was gently resuspended in 1ml sterile dH_2O. 200 µl of the transformation mix was plated onto selective minimal media. Plates were air-dried and incubated at 30 ºC for three days.

Molecular biology protocol

Polymerase chain reaction

5 ng of plasmid DNA was used as template for amplification. The template was added to the following reagents: 5 µl of 10X thermo-buffer (500 mM KCl, 100 mM Tris-HCl pH 9, 1% Triton® X-100), 5 µl of 10X $MgCl_2$ (25 mM), 1 µl of 50X dNTPs (50 mM of dATP, 50 mM of dGTP, 50 mM of dCTP and 50 mM of dTTP), 250 ng of each primer, 0.25 µl Taq DNA polymerase (5 u/ml, Fisher) and filled to 50 µl with dH_2O. The sample was covered with 50 µl of mineral oil. The samples went through required cycles in a PCR machine (Perkin-Elmer). 50 µl of chloroform was added to extract PCR product from oil. 5 µl of PCR product was checked on an agarose gel (BRL, electrophoresis grade).

Topoisomerase-mediated cloning of PCR products

The following agents were added in order into an Eppendorf tube: 1 µl of PCR product, 1µl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) 3 µl of sterile water, and 1 µl of Topoisomerase-activated pCR2.1 vector. The tube was incubated at room temperature for 5 minutes and 2 µl of the reaction mix were added to one vial of one-shot competent *E. coli* Top10F'cells. The vial was incubated on ice for 5 minutes, heat shocked in a 42 ºC water bath for 30 seconds and back on ice immediately. 250 µl of SOC media (2% bactotryptone, 5% yeast extract, 1% 1 M NaCl, 1% 2 M glucose, 1% 2 M $MgCl_2$, 0.25% KCl) were added. The tube was capped tightly and incubated at 37 ºC for 1 hour with shaking horizontally at 200 rpm. Aliquots of 10 µl and 50 µl of the transformation mixture, plus 190 µl of dH_2O or 150 µl of dH_2O, were spread on LB plates (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 2% bactoagar, pH 7.5) containing 50 µg/ml kanamycin. The plates were air-dried and incubated overnight at 37 ºC.

Reverse transcription-PCR

1 μg of total RNA was mixed with 500 ng of Oligo(dT)$_{12-18}$ primer (Pharmacia) to a total volume of 13.5 μl in an Eppendorf tube. Reverse transcription (RT) reaction was performed in a total volume of 40 μl containing the RNA and the primer mixture: 1 X RT buffer (New England Biolab); 1 mM of dNTP mixer (50 mM of dATP, 50 mM of dGTP, 50 mM of dCTP and 50 mM of dTTP); 10 mM DTT; 1 unit of RNase inhibitor (Promega) and 0.8 μl of MMLV Reverse Transcriptase (New England Biolab) for 0.5 hours at 37°C. 0.8 μl of fresh enzyme were added and the tube was incubated for another 0.5 hours at 37°C. The PCR reactions were performed as previous described, in a total volume of 50 μl consisting of 2 μl of the RT reaction. Control reactions without reverse transcriptase were used to assess the presence of any contaminating DNA.

Restriction endonuclease digestion

Restriction digestions were performed according to the manufacturer's directions. The appropriate buffers and reagents were added for different enzymes. Most digestion reactions were incubated for 2 hours at the appropriate temperature, while some require overnight incubation. Digests were analyzed on agarose gels in either TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM disodium EDTA) or TAE buffer (400 mM Tris base, 50 mM sodium acetate, and 10 mM disodium EDTA, pH 7.8).

Photography

Agarose gels and yeast plates were photographed with the Polaroid camera, film type 667 or the GELDOC video system (BIO-RAD). A Kodak digital camera, DC290, was used to take the photographs of plants. Images were processed with Adobe PHOTOSHOP software.

Small-scale preparation of double-stranded plasmid DNA (miniprep)

A single colony was picked from a transformation plate and used to inoculate 3 ml of LB broth containing 100 μg/ml ampicillin. The tube was incubated overnight at 37°C with shaking at 200 rpm. 1.5 ml of culture was transferred to a 1.5 ml Eppendorf tube and centrifuged for 1 minute at maximum speed. The supernatant was poured off and the cell pellet was resuspended in 100 μl of TEG (25mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose). 200 μl of freshly prepared alkaline-SDS (200 mM NaOH, 1% SDS) were added into the tube. The tube was mixed by inversion, and incubated on ice for 5 minutes. 150 μl of 3 M KOAc (pH4.8) were added, vortexed vigorously and centrifuged for 5 minutes in the cold. The supernatant was transferred to a clean Eppendorf tube, and 0.5 ml of 1:1 phenol:chloroform were added (phenol was saturated with 0.1M Tris plus 0.1% 8-hydroxyquinoline). The tube was vortexed vigorously to form emulsion and centrifuged at room temperature for 2 minutes. The aqueous phase was transferred to a fresh tube. 1 ml of 100% ethanol was added into the tube. This tube was mixed by inversion, incubated at room temperature for 2 minutes, and centrifuged for 5 minutes at 4°C. The supernatant was removed and the pellet was dried under vacuum for 20 minutes. The pellet was then resuspended in 50 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 50 μg/ml DNase-free RNase A and incubated at 37°C for 30 minutes. 1 μl of 10 mg/ml proteinase K was added and incubated at 37°C for another 30 minutes. 50 μl of 1:1 phenol:chloroform were added, vigorously vortexed and centrifuged for 3 minutes at maximum speed to separate the phases. The aqueous phase was transferred to a clean Eppendorf tube, and 50 μl of dH_2O were added to the

remaining phenol phase. The two aqueous phases were pooled, and 100 µl of chloroform were added. The tube was vortexed and centrifuged for 1 minute and the aqueous phase was transferred to a clean Eppendorf tube. 25 µl of 10 M ammonium acetate and 250 µl of 100% ethanol were added. It was stored at -20°C overnight (or at least two hours) to precipitate the DNA. The precipitated DNA was centrifuged for 10 minutes at 4°C, the supernatant was removed and the pellet was dried under vacuum for 30 minutes. The pellet was resuspended in 50 µl TE buffer.

Purification of DNA fragments from agarose gel (Gene Clean™)

Restriction digest fragments were separated by electrophoresis through a 1% agarose gel in TBE buffer gel containing 1 µg/ml ethidium bromide. DNA fragments were removed from the gel with a razor blade under long-wave UV transillumination. The DNA was eluted from the agarose using the GENECLEANII® kit (BIO 101 Inc). The agarose slices containing the DNA were mixed with an aliquot of 6 M NaI and 'TBE modifier' (4.5 times and 0.5 times of the volume of the gel slice, respectively) in an Eppendorf tube. This mixture was vortexed and incubated in a 50°C water bath for 5 minutes or until the agarose was completely dissolved. 5 µl of thoroughly vortexed GLASSMILK was added to the mixture and incubated at room temperature for 5 minutes. The mixture was centrifuged for approximately 30 seconds to pellet the GLASSMILK and the supernatant was removed. The GLASSMILK with the bound DNA was washed 3 times with 500 µl of NEW wash. After the final wash the pellet was air-dried for 10 minutes. The pellet was then resuspended in 10 µl of dH_2O and incubated at 50°C for 5 minutes. The mixture was centrifuged for 1 minute and the elution was

removed. A second elution was pooled with the first one. 5 μl of the final volume was analyzed by agarose gel electrophoresis.

Biochemistry

Extraction of nucleic acid from plant tissue -- phenol-chloroform method

0.3 g of systemically infected leaf tissue or 1.0 g of inoculated leaf tissue was placed in a mortar with 1 ml of extraction buffer (2XSTE (0.5 M Tris-HCl, pH 8.0, 1 M NaCl, 0.01 M EDTA), 1% SDS, 1% 2-mercaptoethanol, pH 8.0) and 1 ml of 1:1 phenol:chloroform mixture. The tissue was ground into slurry with a pestle, and transferred to an Eppendorf tube and kept on ice. Samples were centrifuged for 5 minutes at 4°C. The supernatant was removed to a clean Eppendorf tube containing 0.6 ml of 1:1 phenol:chloroform mixture. Samples were vigorously vortexed and centrifuged for 5 minutes at 4°C. 0.5 ml of the supernatant was transferred to a clean Eppendorf tube and 50 μl of 3M sodium acetate (pH 5.2) and 1 ml of 100% ethanol was added. The mixture was mixed by inversion and kept at -20°C at least 1 hour for precipitation. Samples were centrifuged for 10 minutes at 4°C, the supernatant was removed and the pellet was dried under vacuum for 30 minutes. The pellet was resuspended in 50 μl of TE buffer.

Purification of total RNA from total nucleic acid-LiCl precipitation

The total nucleic acid was resuspended in TE buffer. A certain volume of 6 M LiCl solution was added to obtain the final 2 M LiCl in the tube. The tube was incubated on ice for 4 hours. Then it was centrifuged for 10 minutes at 4°C. The supernatant was removed and the precipitant was resuspended to TE buffer. The whole process was repeated for twice. The concentration of the purified total RNA was determined with

GENEQUANT *pro* RNA/DNA Calculator (Amersham Pharmacia Biotech). 1 μg of total RNA was analyzed by 1.5% agarose gel electrophoresis. The total RNA was transferred to a clean Eppendorf tube, mixed with 3 M of sodium acetate (pH 5.2) and 100% ethanol. The tube was kept at –20 °C for precipitation of the DNA.

Purification of poly $(A)^+$ RNA from total RNA---Oligotex^{TM} suspension

The total RNA was centrifuged for 10 minutes at 4°C. The supernatant was removed and the pellet was dried under vacuum for 30 minutes. The pellet was resuspended into the amount of the RNase-free water recommended by the manufacturer's directions. The appropriate amount of binding buffer OBB (20 mM Tris.Cl, pH 7.5, 1 M NaCl, 2 mM EDTA and 0.2% SDS) and Oligotex suspension (Qiagen) were added for different amount of total RNA. The contents were mixed thoroughly by flicking the tube. The tube was incubated for 3 minutes at 70°C, then at room temperature for 10 minutes. The tube was centrifuged for 2 minutes at maximum speed (14,000 X g) at room temperature. The supernatant was removed to a fresh tube, labeled 'poly $(A)^-$ RNA'. The pellet was resuspended in 1 ml wash buffer OW2 (10 mM Tris.Cl, pH 7.5, 150 mM NaCl and 1 mM EDTA). The wash was repeated for three times and the supernatant was removed. The pellet was resuspended into 100 μl hot (70 °C) elution buffer OEB (5 mM Tris.Cl, pH 7.5). The tube was centrifuged for 2 minutes at room temperature at maximum speed. The supernatant containing eluted poly $(A)^+$ RNA was transferred to an RNase-free tube. The elution was repeated twice and the eluates were pooled. The concentration of the purified poly $(A)^+$ RNA was determined with GENEQUANT *pro* RNA/DNA Calculator and 1μg of total RNA was analyzed by 1.5%

agarose gel electrophoresis. The poly $(A)^+$ RNA was transferred to an RNase-free tube, mixed with 3 M of sodium acetate (pH 5.2) and 100% ethanol. The tube was stored at – 20 °C for precipitation of the DNA.

Transfer of nucleic acids to nylon membranes by Southern blotting

The concentration of each DNA sample was estimated fluorometrically with Hoechst 33258 dye (Polysciences) using FluoStar software. Equal amount of each sample was transferred to an Eppendorf tube containing 1 μg/ml RNase A and incubated at room temperature for 30 minutes. These samples were loaded onto a 1.5% TBE agarose gel. The gel ran at 80 volts until the blue dye was at the bottom of the gel, and the picture of the agarose gel was taken. The gel was soaked for 15 minutes in a 0.25 M solution of HCl, rinsed in tap water, then in 200 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH) with gentle agitating. The gel was rinsed in tap water, and soaked for 30 minutes in 200 ml of neutralizing solution (1.5M NaCl, 1M Tris-HCl, pH 7.4) with gentle agitating and the gel was ready for transfer. The area above the wells was cut and the trimmed gel was placed onto a 10X30 cm piece of 3MM Whatman paper over a glass plate across a plastic tray containing 300 ml of 2XSSPE (0.02 M NaH_2PO_4, 0.3 M NaCl, 0.02 M EDTA, pH 7.4). An 8.5X15 cm piece of nylon membrane (Schleicher and Schuell, Nytran) was cut and soaked in dH_2O. The nylon membrane was cut in the right corner and placed over the gel. Any bubbles between the gel and the paper were removed by rolling a glass pipette across the surface. On top of the nylon membrane, three pieces of 8.5X15cm 3MM Whatman paper were placed, along with a stack of paper towels. A heavy book was placed on the glass plate as a weight. The transfer was allowed overnight and the membrane was removed for crosslinking. The membrane was placed into a UV

crosslinker (Fisher Scientific) with DNA face up and irradiated with UV light on the optimal crosslink setting (energy dose 120mJ/cm^2).

Transfer of nucleic acids to nylon membranes by northern blotting

RNA samples were prepared in an RNase-free environment. The procedures of running RNA gel were similar to those of running DNA gel, with the exception of the incubation of RNaseA. After running for about 2 hours or until the blue to the bottom, the TBE gel was photographed and ready for transfer. After this point, all the steps for the transfer of RNA to a nylon membrane were the same as that of DNA.

Probe preparation and hybridization

The nylon containing the DNA (RNA) was placed in a hybridization bottle with 10 ml of hybridization solution (2XSSPE, 1% SDS). The hybridization bottle was put into a hybridization oven at 65°C for 1 hour. Radioactively labeled probes were made from DNA (RNA) template, with a Prime-It II kit (Stratagene) and α-^{32}P-dCTP (5 μCi/ μl). 5 μl of 5 ng/μl DNA (RNA) template, 23 μl of dH_2O and 10 μl of random primers were mixed and boiled for 5 minutes. The sample was then allowed to cool. 10 μl of 5X primer buffer plus 1 μl of Klenow DNA plymerase were added to the mixture. The tube was then quick centrifuged. 5 μl of α-^{32}P-dCTP were added and incubated at 37°C for 10 minutes. 2 μl of stop mix was added to the tube and the tube was boiled for another 5 minutes and placed on ice for a snap cool down. Then the tube was centrifuged for 30 seconds and added to the hybridization bottle. The bottle was incubated at 65°C overnight.

When the incubation was complete, the hybridization solution in the bottle was poured into the radioactive liquid-waste container and 100 ml of the wash solution

(0.5XSSPE, 1% SDS) were added into the bottle to wash the blot. The first wash was incubated at room temperature for 5 minutes, and the wash solution was discarded into the radioactive liquid-waste container. The second and third washes were carried out at 65 °C for 30 minutes. The blot was taken out of the bottle and rinsed in dH_2O, then placed onto a paper towel to dry. The dry blot was wrapped in Saran wrap, and exposed to an X-ray film (FUJI) with an intensifying screen at -80°C for about 6 hours. It was alternatively exposed to a Phosphorimager screen (Molecular Dynamics) for 2-3 hours. Relative RNA accumulation was digitally analyzed with the ImageQuant software.

Protein gel electrophoresis and western blotting

Yeast transformants (strain EGY48) were incubated overnight in a 10 ml of minimal selective media at 30 ºC with shaking at 250 rpm. The cell density was determined by counting serial dilutions on a hemacytometer and $2.5X10^8$ cells was transferred into 50 ml of YPD (2 % peptone, 1 % yeast extract, 2 % glucose) media and incubated for about 4 hours at 30 ºC with shaking at 250 rpm until the population had doubled at least twice. The culture was then poured into a 50 ml centrifuge tube and centrifuged for 5 minutes at 3000 rpm. The cell pellet was resuspended in 1 ml of sterile dH_2O. The mixture was transferred into an Eppendorf tube and stored at –80 ºC.

The casting apparatus was assembled and the following reagents were added to make a 12 % polyacrylamide separating gel: 3.3 ml of dH_2O, 2.5 ml of 1.5 M Tris HCl (pH 8.8), 4 ml of 30% acrylamide stock (30 acrylamide:0.8 bis-acrylamide), 100 µl of 10% SDS, 100 µl of ammonium persulfate (APS) and 5 µl of tetramethylethylenediamine (TMED). 100 µl of tertiary-amyl alcohol were added to get rid of the air bubble. During the gel solidification, the following reagents were added in order to make 4%

polyacrylamide stacking gel: 4.33 ml of dH_2O, 0.75 ml of 1 M Tris HCl (pH 6.8), 0.8 ml of 30% acrylamide stock, 60 µl of 10% SDS, 60 µl of 10% APS and 5 µl TEMED. After the separating gel was polymerized, the tertiary-amyl alcohol was removed and the stacking gel was poured on the top. Resuspended yeast cell pellets were removed from the -80°C freezer and 0.5 ml of Laemli 2X reagent (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) was added. The samples were vortexed vigorously until resuspended, then boiled for 5 minutes. After boiling the samples were mixed vigorously for 30 seconds and centrifuged for 5 minutes. 20 µl of the protein supernatant was loaded onto the gel. The protein gel running buffer (1.92 M glycine, 250 mM Tris base, 0.1% SDS) was poured .The gel ran at 100 volts until the bromophenol blue reached the bottom of the gel. The western blot apparatus was assembled and immersed in 1X western transfer buffer (1.92 M glycine, 250 mM Tris base, 20% methanol). The transferring went overnight at 33 volts. The following day the nitrocellulose was removed from the western blot assembly and placed in a sterile tray.25 ml of 5% milk blocking solution (2.5 g dried milk, 125 µl 10% Tween, and 50 ml TBS (2 M NaCl and 0.5 M HCl)) were added and the tray was rotated for 1 hour. The milk blocking solution was recycled and 50 ml of primary antibody (1:200 dilution of anti- monoclonal antibody (Santa Cruz Biotechnology) or 1:200 dilution of anti-HA epitope tag monoclonal tag antibody F-7 (Santa Cruz Biotechnology) in TBS with 5% milk, 0.05% Tween-20) was added and the tray was rotated for 1 hour. The blot was then washed with TBS for 5 times, 5 minutes each time. A 1:2000 dilution of anti-mouse IgG horseradish peroxidase conjugate in blocking solution was added and the tray was rotated for 1 hour. After washing in TBS 5 times, 5 minutes each time, 5 ml of supersignal

chemiluminescent substrate stable peroxide solution and 5 ml of supersignal chemiluminescent substrate luminol enhancer (Pierce) were combined and added to the blot. This blot was rotated for 5 minutes at maximum speed. The nitrocellulose membrane was transferred onto Saran wrap. In the dark room it was exposed to an X-ray film (Fuji) for 30 seconds and again for 5 minutes. The film was placed in a developer solution for 4 minutes, then in a fixer solution for another 4 minutes. The film was rinsed with tap water and air-dried.

Plant Inoculation

Particle Bombardment – DNA virus

Preparation of Gold particles:

1 ml of 100% ethanol was added into 60 mg of 1.6 μm gold in an Eppendorf tube, then vortexed vigorously for 1 minute. The tube was centrifuged briefly to pellet the gold particles, and the ethanol was removed. The process was repeated for 3 times. After the final wash, the gold pellet was resuspended in 1ml of sterile dH_2O, centrifuged briefly and the supernatant was removed. This process was repeated once. The particles were then resuspended in a final volume of 1 ml of dH_2O and stored at room temperature.

Coating Gold particles

10 μg of DNA (5 μg of plasmid A and 5 μg of plasmid B or 10 μl of miniprep plasmid) were mixed with 20 μl of 60 mg/ml of 1.6 μm gold particles. 20 μl of 2.5 M $CaCl_2$ were added, and the tube was vigorously vortexed, 8 μl of 100 mM spermidine were added, and vortexed constantly for 3 minutes at full speed. 250 μl of 100% ethanol were added to the mixture, vortexed, centrifuged for 30 seconds, and the supernatant was

removed. This wash step was repeated twice. The pellet was then resuspended in 25 µl of 100% ethanol.

Particle Delivery System Operation

The macrocarrier was placed into a holder. 12.5 µl of the DNA-coated gold particle ethanol suspension were pipetted in a spiral onto the center of the installed macrocarrier, and allowed to dry completely. A stopping screen was placed in a holder and the macrocarrier was assembled with the gold particles facing down. The macrocarrier coverlid was assembled in the second slot from the top. A 1550-psi rupture disk was placed into the retaining cap and the cap was then replaced into the apparatus directly under the macrocarrier support to be inoculated. The system power and the vacuum pump were turned on, and the helium tank pressure was set to approximately 1650 psi. The vacuum switch was turned on. When the vacuum reached approximately 10 mmHg, the fire button was turned on and held until the pressure reached 1550 psi. After the gold particles have been fired onto the plant, the 'fire' button was released and the vacuum switch was set to the vent position. After the vacuum was released, the plant was taken out. The inoculated *N. benthamiana* plants were placed into a growth chamber in the Phytotron (NC State University).

Inoculation of PVX

Potato virus X (PVX) constructs were lineraized by digestion with *Spe*I (New England Biolab). 5 µl of linearized DNA template, 10 µl of 10X transcription buffer, 10 µl of capping cocktail (5 mM of ATP, 5 mM of CTP, 5 mM of TCP and 0.75 mM of GCP), 8 µl of 10 mM diguanidine triphosphate, 4 µl of T7 RNA polymerase (New England Biolab) were mixed in a tube, filled to 100 µl with dH_2O. The tube was

incubated at 37°C for 60 minutes and checked by agarose gel electrophoresis. The resulting RNA transcripts were purified by digestion with Proteinase K, followed by extraction with phenol:chloroform, and ethanol precipitation. The cleaned RNA transcripts were ready for mechanical inoculation of *N. benthamiana* plants. For one plant, 25 µl of the transcription reaction was mixed with 150 µl of GKP mix (50 mM glycine, 30 mM dipotassium hydrogen-phosphate, pH 9.2, 1% bentonite, 1% celite). The mixture was aliquoted into three drops on a small piece of Parafilm. The mixture was rubbed onto the plant with forefinger, youngest leaves first. The inoculated plants were placed into a growth chamber in the Phytotron.

RESULTS

1. Identification of candidate *N. benthamiana* cDNA clones that interact with TGMV AL2 protein in the yeast two-hybrid system

The yeast two-hybrid system has been a standard and powerful tool to detect specific protein-protein interactions. It is based on the basic property of multi-domain proteins that the function of the intact protein can be reconstituted by bringing individual domains into intimate contact. For example, combining a DNA-binding domain with a transcriptional activation domain can generate a transcriptional activator (Fields and Song, 1989).

The main components in a yeast two-hybrid system are bait, prey and reporter gene in *Saccharomyces cerevisiae* cells. In the LexA-based system, the bait vector expresses the DNA-binding domain of the *E. coli* LexA protein, and has a *HIS3* selectable marker (Figure 3). The protein of interest is fused to either the N-terminus or the C-terminus of the LexA DNA-binding domain. The bait protein should be expressed stably in the nucleus and it should not significantly activate the reporter gene without the presence of prey protein, a phenomenon known as autoactivation. The prey vector, pB42AD, expresses an artificial activation domain together with a hemagglutinin (HA) epitope tag, and has a *TRP1* selectable marker (Figure 4). The *GAL1* promoter in pB42AD drives expression of the fusion protein only when galactose is present as the carbon source in the medium. An interaction between the prey and bait fusion proteins will reconstitute a transcription activator for the reporter gene, *LEU2*, which allows the yeast to grow on medium lacking leucine.

In this study, the yeast two-hybrid system was used to identify host cDNAs encoding proteins which interact with the TGMV AL2 protein when fused with the LexA DNA-binding domain. It was also used to map the regions that mediate interaction with TGMV AL2 protein in such host cDNAs.

Previously, two bait proteins based on TGMV AL2 (TAL2) have been used in yeast two-hybrid screens of a cDNA library from *N. benthamiana*. One is LexA-TAL2ΔC; in which a truncated TGMV AL2 protein lacking its C-terminal 13 amino acids (the minimal activation domain) is fused to the C-terminus of the LexA DNA-binding domain. The other is TAL2 (W124A)-LexA, which consists of an N-terminal fusion to LexA of TGMV AL2 containing a tryptophan to alanine substitution mutation at residue 124 (W124A). These two bait proteins did not autoactivate although they accumulated in yeast cells efficiently (J.K.Jordon, G.Thomas and I.T.D.Petty, unpublished results). From sequential library screenings, four candidate cDNA clones, representing three genes, were isolated (J.K.Jordon and I.T.D.Petty, unpublished results). Clones Nb#26 and Nb#39 were identified using LexA-TAL2ΔC as bait, whereas Nb#51 and Nb#62 were identified using TAL2 (W124A)-LexA as bait.

To further characterize these cDNA clones from *N. benthamiana*, the specificity of their interaction with TGMV AL2 was tested. As controls, each cDNA clone expressed from the pB42AD vector was paired with the pLexA vector expressing LexA alone, or with pLexA-Lam, a C-terminal fusion of the LexA DNA-binding domain with human lamin C protein, an irrelevant bait expected not to interact with plant proteins. Combinations of these plasmids were transformed into yeast and plated on minimal medium lacking histidine and tryptophan to select for the pLexA- and pB42AD-based

plasmids, respectively. Serial dilutions of each yeast culture were then spotted onto minimal medium lacking histidine, tryptophan and leucine with galactose/raffinose as carbon source (Gal/raf-His-Trp-Leu). Galactose in the medium induces expression of the cDNA under the control of the *GAL1* promoter. If the bait and prey proteins interact, the reporter gene *LEU2* is activated. As a result, the yeast can grow on medium lacking leucine.

As shown in Figure 5, the yeast two-hybrid assay indicated that yeast containing cDNA Nb#26 in combination with LexA-TAL2ΔC grew to the same degree as yeast containing Nb#26 and the vector pLexA. Although slightly reduced, growth of yeast was also seen when they contained cDNA Nb#26 and the irrelevant bait pLexA-Lam. This suggested that the protein-protein interaction between Nb#26 and LexA-TAL2ΔC was not specific for the TGMV AL2 moiety. A similar result was obtained for cDNA Nb#39 (Figure 5). Therefore, Nb#26 and Nb#39 were eliminated as false positives from candidate cDNAs pools because of their lack of specific interactions with the TGMV AL2 protein.

Since cDNAs Nb#51 and Nb#62 only interact with TGMV AL2 when it is fused to the N-terminus of LexA (Jernigan, 2003), to test the specificity of those interactions required one or more irrelevant baits in the same configuration.

2. Construction and testing of irrelevant baits fused to the N-terminus of LexA for use as negative controls in the yeast two-hybrid system

To confirm whether the observed interaction of cDNAs Nb#51 and Nb#62 was specific for the TGMV AL2 protein, N-terminal irrelevant baits were made to act as

negative controls. These irrelevant baits would be expected not to interact with the prey of interest.

The strategy for cloning and expression of the irrelevant bait proteins was similar in each case. The corresponding open reading frame (ORF) was amplified by PCR from the plasmids pRFHM, pEG202-max, pEG202-Lam and pLexA-53 respectively (see Materials and Methods). Unique restriction sites for *Eco*RI and *Xho*I were incorporated into the 5' and 3' primer for subsequent cloning. The PCR products were cloned into an intermediate plasmid vector pCR2.1-TOPO (see Materials and Methods) and the inserts were sequenced. Cloned inserts without any PCR-induced mutations were excised with restriction enzymes *Eco*RI and *Xho*I, and then inserted into the 5'-end of the bait vector, pNLexA (Figure 3). Proteins encoded by the resulting plasmids were named Bicoid-LexA, max-LexA, Lam-LexA and p53-LexA, which encode the drosophila bicoid homeodomain (amino acids 2-160), the human Max protein lacking its C-terminal 15 amino acids, the amino acids 110-273 of human lamin C protein and amino acids 72-390 of murine tumor suppressor p53, respectively.

These four plasmids were transformed into yeast and plated on minimal medium lacking histidine, with glucose as carbon source (Glu-His). Accumulation of the fusion proteins was analyzed by western blotting with a monoclonal antibody directed against the LexA DNA-binding domain, using whole-cell protein extracts from yeast transformants (Figure 6). The western blot results showed that the max-LexA and Lam-LexA fusion proteins could be expressed well in yeast and accumulated efficiently, while neither Bicoid-LexA nor p53-LexA could be detected.

To be used as baits, neither max-LexA nor Lam-LexA should autoactivate the transcription of the *LEU2* reporter gene. To test for autoactivation, serial dilutions of yeast cell suspensions were spotted onto minimal medium lacking histidine to select for the bait plasmid, or lacking both histidine and leucine to test for autoactivation of the *LEU2* reporter gene. As well as the test strains, yeast cultures containing either pLexA-Pos, which encodes a fusion of LexA DNA-binding domain with the activation domain of the yeast transcription factor Gal4p, or containing pNLexA were spotted onto the plates to act as positive and negative controls, respectively (Figure 7). The positive control strain containing pLexA-Pos grew equally well on medium lacking histidine alone, or lacking both histidine and leucine. However, none of the test plasmids allowed yeast growth on medium lacking leucine. For max-LexA and Lam-LexA, each of which was shown by western blot to accumulate in yeast efficiently, these results indicated that neither fusion protein autoactivates. For Bicoid-LexA and p53-LexA, failure to grow could be due to the fact that these two fusion proteins were unable to accumulate in the yeast cells, and no conclusion can be drawn about their autoactivation potential. Based on the western blot analysis and autoactivation test results, both max-LexA and Lam-LexA were suitable for use as N-terminal fusion irrelevant baits.

3. Confirmation of the specific interaction of TGMV AL2 with Nb#51 and Nb#62

To test whether the interaction of TAL2(W124A)-LexA with Nb#51 and Nb#62 was specific for the TGMV AL2 moiety, each cDNA clone fused to the B42 activation domain was also paired with the N-terminal fusion irrelevant bait max-LexA, as well as unfused LexA. In a further set of negative controls, each bait plasmid was also paired

with the prey vector pB42AD without a cDNA insert. Combinations of plasmids were transformed into yeast, and yeast growth on selective media with, or without, added leucine was determined (Figure 8).

The yeast two-hybrid assay indicated that both the Nb#51 and Nb#62 allowed the expression of the reporter gene *LEU2* when co-expressed with TAL2(W124A)-LexA. The growth of yeast containing the combination of TAL2(W124A)-LexA with Nb#51 or Nb#62 was efficient on medium lacking leucine. No yeast growth was observed when either of these cDNAs was co-expressed with either the irrelevant bait max-LexA, or with LexA alone. As expected, expression of pB42AD without a cDNA insert didn't support the yeast growth on medium lacking leucine. Similar results were obtained with Lam-LexA as irrelevant bait (data not shown).

Based on these results, both Nb#51 and Nb#62 were shown to specifically interact with TGMV AL2 protein in the yeast two-hybrid system. Further work was focused on characterization of these two cDNAs.

4. Determination of nucleotide sequences for cDNA clones Nb#51 and Nb#62

As a first step to characterize the Nb#62 and Nb#51 cDNA clones, their sequences were determined. Figure 9 illustrates the sequencing strategy and primers used for Nb#62. A similar strategy was used for sequencing Nb#51. Overlapping regions of clone Nb#62 were sequenced, proceeding from the 5'-end toward the 3'-end of the cDNA insert. The first fragment was sequenced using a forward primer based on the sequence of cloning vector pB42AD upstream from the insertion site. The information obtained from the first sequencing step was used to design primers for downstream sequences. All

subsequent steps were based on previous sequencing results. Four consecutive forward sequencing steps were taken before reaching the 3'-end of the sequence. Six additional backward sequencing steps were necessary to provide information for checking correctness of forward sequencing results and minimize sequencing errors. 100% of the sequence was obtained on both strands. It was found that cDNA clone Nb#62 contains 1700 nucleotides, and cDNA clone Nb#51 contains 1105 nucleotides.

5. Bioinformatic analysis of Nb#51 and Nb#62

The sequences of Nb#51 and Nb#62 were compared with each other, using the BLAST2 program (Altschul *et al*., 1997). The results revealed that Nb#51 appears to be a 3'-coterminal truncated version of Nb#62, and the 5'-end of Nb#51 is located at nucleotide 595 of Nb#62 (shown schematically in Figure 10a).

The conceptually translated sequence of Nb#62 was compared with the non-redundant GenBank database using the BLASTX program (Altschul *et al*., 1997). The results revealed a hit with 64% amino acid sequence similarity (BLAST expect value e^{-165}) between the conceptual translated sequences of Nb#62 and an ankyrin-related protein AT3g04710.1 (GenBank accession number gi15229331), encoded by Arabidopsis gene AT3g04710, a gene of unknown function. It's very likely that AT3g04710 is the arabidopsis orthologous gene of *N. benthamiana* Nb#62. For brevity, I will refer to the cDNA and the corresponding plant gene as Nb#62 unless it causes confusion to do so.

The most striking feature of the protein encoded by Nb#62, as revealed by using NCBI conserved domain search program BLASTP (Altschul *et al*., 1997), is the presence of at least four consecutively repeated modules of about 33 amino acid which are known

as ankyrin-repeats (Ank) (BLAST expect value $2 \times e^{-22}$) in combination with 3 copies of a 34 amino acid motif which is known as the tetratricopeptide repeat (TPR) (BLAST expect value $6 \times e^{-12}$). The conceptually translated sequence of Nb#62 was also submitted to the ScanProsite, a protein pattern and profile search server (Gattiker *et al*., 2002). The ScanProsite program predicted that the ankyrin repeats were between residues 64 and 292, and TPR motif was between residues 357 to 460. The whole TPR motif and part of the ankyrin-repeats are present in both Nb#62 and Nb#51(Figure 10b). Then the nucleotide sequences of Nb#62 were compared with full-length cDNA of AT3g04710, using the BLAST2 program (Altschul *et al*., 1997). The alignment of AT3g04710 and Nb#62 revealed three regions of significant similarities. The three pieces, a short piece in the 5'-end (with 78% similarity), a piece in the central part which encodes part of the ankyrin-repeat region (with 68% similarity), and a piece in the 3'-end which encodes the whole TPR motif (with 72% similarity), are more highly conserved than the rest of the sequences (Figure 11). Both the ankyrin-repeat and TPR domain are thought to mediate protein-protein interactions (Sedgwick *et al*., 1999; Blatch *et al*., 1999).

6. Identification of regions in Nb#62 required for interaction with the TGMV AL2 protein in the yeast two-hybrid system

To approximately identify the region(s) in Nb#62 required for its interaction with TGMV AL2 protein, a series of Nb#62 deletion mutants was constructed and tested. Representative results are summarized in Figure 12, 13 and 14**.**

As shown above, both Nb#62 and Nb#51 could specifically interact with TGMV AL2 protein. Therefore, the N-terminal region (amino acids 1-198) missing from Nb#51

was not required for the interaction with TGMV AL2. Also, as expected, deletion of the 3'-untranslated region (3'-utr, nucleotides 1468-1723) did not affect the ability of Nb#51 to interact with TGMV AL2.

Within amino acids 199-489, deletion analysis was carried out to test whether the remaining ankyrin-repeats (amino acids 199-292) are responsible for interaction with TGMV AL2. As shown in Figure 13a and Figure 14, the ankyrin-repeats alone conferred no ability to interact with TGMV AL2 although the fusion protein accumulated abundantly in yeast, whereas the C-terminal region lacking the ankyrin-repeats (amino acids 293-489) exhibited a strong interaction. These results suggest that the ankyrin-repeats in Nb#62 are not involved in its interaction with TGMV AL2 protein.

Deletion analysis narrowed down the contributing regions to amino acids 293-489 of Nb#62, which contains the TPR motif. To determine whether the TPR motif mediated the interaction with TGMV AL2, the region of amino acids 293-489 was divided into three blocks: (1) C1 comprising amino acids 293-356, which corresponds to the upstream TPR-flanking region; (2) C2 comprising amino acids 357-460, which corresponds to the TPR motif; (3) C3 comprising amino acids 461-489, which corresponds to the downstream TPR-flanking region. Regions of cDNA Nb#62 encoding each of these three blocks were inserted into pB42AD using the same strategy as described previously, and co-transformed into yeast expressing TAL2(W124A)-LexA. However, no significant interaction with TGMV AL2 was observed for any of these three regions, C1, C2 or C3, although each fusion protein was able to accumulate efficiently in yeast (Figure 13b).

In summary, the ankyrin-repeats of Nb#62 are not involved in its interaction with TGMV AL2 in the yeast two-hybrid system. In contrast, the TPR motif, together with its

flanking regions, was sufficient to confer on Nb#62 the ability to interact with TGMV AL2.

7. Heat shock did not detectably induce the expression of Nb#62.

The BLAST results revealed that the conceptually translated sequences of Nb#62 and the rice putative stress-inducible protein (GenBank accession number gi12656815) have sequence similarity in the C-terminal region containing the TPR motif (BLAST expect value e^{-103}). Because the TPR motif has been found to mediate interactions of molecular chaperone complexes involving heat shock protein 70 and 90 (HSP70 and 90) (Smith *et al*., 1993; Chang *et al*., 1994), and because most stress-inducible genes are rapidly induced during heat shock, it was investigated whether heat shock could induce the expression of Nb#62 mRNA.

To determine whether the expression of Nb#62 was heat inducible, 5-week-old *N. benthamiana* plants were incubated at 42 °C for 1 hour, or 2 hours for heat shock treatment. Control plants were maintained at 23 °C. Total nucleic acid was extracted from control and heat shocked plants immediately after each heat treatment. The poly $(A)^+$ RNAs were purified from the total nucleic acids. Equal amounts of the poly $(A)^+$ RNAs (3.0 μg) were analyzed by agarose gel electrophoresis, followed by northern hybridization. As well as with Nb#62-specific probe, the poly $(A)^+$ RNAs from plants under each heat treatment were also hybridized with two other probes at the same time. One was a specific probe for the *N. benthamiana* β-actin gene, a housekeeping gene whose expression is not affected by heat stress, and acted as a negative control. The other was a specific probe for the *N. benthamiana HSP70* gene, which encodes a heat shock

protein HSP70 and act as a positive control. A dilution series of *in-vitro* transcripts corresponding to the Nb#62-specific probe was also included to determine the detection threshold of the northern hybridization.

As shown in Figure 15, the detection threshold of the northern hybridization was 1 pg of the Nb#62 mRNA. However, the Nb#62 mRNA was not detected, either in control plants maintained at 23 °C, or in heat-stressed plants. The results suggested that the Nb#62 mRNA level in 3.0 μg poly $(A)^+$ RNA from control plants was lower than 1 pg, and the expression of Nb#62 in heat-stressed plants was not high enough to be detected in the northern analysis. The mRNA level of actin gene was shown to be at similar levels under each treatment. The mRNA level of *HSP70* gene was not detected in plants maintained at 23 °C by the *HSP70*-specific probe, whereas a distinct band corresponding to *HSP70* mRNA was detected in poly $(A)^+$ RNA from heat-stressed plants.

8. Virus-induced gene silencing assays directed against Nb#62

Virus-induced gene silencing (VIGS) takes place when plants are infected by viruses containing fragments of host genes. It reduces the mRNA level of the target gene via a post-transcriptional gene silencing (PTGS) mechanism.

Here, a VIGS assay was used to study Nb#62, which has been shown to encode a protein that specifically interacts with TGMV AL2 in the yeast two-hybrid assay. The intention was to suppress gene expression of Nb#62 by VIGS, and to observe the effect (if any) on the plants, and on TGMV infection.

8.1 Evaluation of PVX as a VIGS vector in *N. benthamiana* plants

Potato virus X (PVX) has been studied extensively as an RNA virus vector system to express genes and carry out functional analysis in various plants, such as tomato and tobacco (Rommens *et al.,* 1995; Ruiz. *et al*., 1998). Its cDNA clone form is called pP2C2S (Baulcombe *et al*., 1995). The recombinant PVX for the expression system contains a duplicate promoter that allows the foreign gene to be expressed along with the viral genes (Chapman *et al*., 1992). Figure 16 shows the genomic organization of PVX used in the VIGS assay. In this study, a cDNA fragment of the tobacco *Sulfur* gene (*su*) was used as a marker for silencing experiments. Silencing of the *Sulfur* gene causes loss of chlorophyll, which can be readily recognized by the appearance of white leaves (Peele *et al*., 2001).

To test the ability of PVX to trigger gene silencing in *N. benthamiana* plants, a 519-bp DNA fragment was amplified from the tobacco *su* cDNA (Figure 17a), with primers containing *Cla*I and *Eco*RV restriction sites on the 5' and 3'ends, respectively. The PCR product was cloned into an intermediate plasmid and propagated in *E. coli*, then subcloned into the PVX expression plasmid pP2C2S to create pP2C2S::su. Since PVX is an RNA virus, the cDNA clone needs to be transcribed before inoculation onto *N. benthamiana*. The pP2C2S derivatives were linearized with *Spe*I, which cuts the cDNA immediately 3' of the poly (A) tract, and transcribed *in vitro* with T7 RNA polymerase, resulting in the infectious transcripts of PVX and PVX::su. They were rub-inoculated onto 5-week-old *N. benthamiana* plants (see Materials and Methods).

The plants infected with wild-type PVX exhibited symptoms at 7 days post-inoculation (dpi) including chlorotic lesions on the inoculated leaves, as well as rugosity and distortion of systemic infected leaves. In *N. benthamiana* plants that were infected

with PVX::su, a mosaic of green and white extended throughout the plant including stems and flowers. The results indicated that PVX vectors could initiate VIGS, as reported previously for PVX vectors targeting an endogenous phytoene desaturase (PDS) in *N. benthamiana* (Ruiz *et al*., 1998).

8.2 Nb#62-specific transcripts were silenced in *N. benthamiana* plants by PVX-based VIGS assay in a sequence-specific manner

In an effort to suppress Nb#62 expression in plants, a 618-bp fragment from the 5'-end of the cDNA Nb#62 (Figure 17b) was cloned into the PVX vector to create PVX::62. *N. benthamiana* plants were then inoculated with infectious transcripts of wild-type PVX and PVX::62. PVX::su was rub-inoculated onto the plants at the same time as a positive control. Mock inoculation by buffer alone was included as a negative control.

The results showed that the PVX vector, PVX::su and PVX::62 all caused infection in plants over a similar time course. In addition, the phenotype of PVX::62 infection was not readily distinguishable from that of PVX vector infection. At 21 dpi, total nucleic acids were extracted from systemically infected leaves. The poly $(A)^+$ RNA was purified from total nucleic acids, and analyzed by agarose gel electrophoresis and northern hybridization (Figure 18). To assay the plant Nb#62 mRNA levels independently of the PVX::62 RNA, the 3'-end of the Nb#62 sequence not present in PVX::62 was first used as a probe (3'-probe, Figure 18a) in the northern blot analysis. As shown in Figure 18b, multiple bands hybridizing to the 3'-probe appeared in the mRNA from mock-inoculated plants. The hybridization pattern of the mRNA from plants infected with PVX vector was similar to that from mock-inoculated plants, although

some bands appeared slightly shifted. In contrast to PVX vector infection, there was only one distinct band (band I) which hybridized to the 3'-probe in the mRNA from PVX::62 infected plants. Band I appeared to be the faster migrating band of the two major bands seen in mRNA from mock-inoculated plants and PVX vector infected plants.

To further characterize these potential transcripts, the 5'-end of the Nb#62 sequence, which was cloned into PVX::62 to trigger silencing, was used as a probe (5'-probe, Figure 18a) in another northern blot analysis. In this case, only the mRNA from mock-inoculated plants could be analyzed. As shown in Figure 18c, there was a unique band (band II) that hybridized to the 5'-probe.

When aligning these two northern blots, band II was found to migrate more slowly than band I and appeared to be one of the multiple bands in RNA from mock-inoculated and PVX vector infected plants that hybridized with the 3'-probe. However it apparently was not present in RNA from PVX::62 infected plants.

Based on these facts, it was conceivable that bands I and II corresponded to two different RNA transcripts, transcript I and II, from the same *N. benthamiana* gene. Transcript I (shown as band I) contains only the 3'-end of cDNA Nb#62, and therefore could only hybridize to the 3'-probe. In contrast, transcript II, the longer one (shown as band II), contains both the 5'- and 3'-end of Nb#62 and could hybridize to both the 5'- and 3'- probes. Transcript II, which contains sequences present in the silencing trigger fragment, was eliminated in RNA from PVX::62 infected plants while the level of transcript I was not affected. Band A and B (indicated in figure 18b) were probably the electrophoresis artifacts since the RNA samples were separated by electrophoresis through a non-denaturing gel. They could be resolved by running a denaturing gel.

The results indicated that, when PVX::62 carrying the 5'-end of cDNA Nb#62 was used as a silencing trigger, VIGS effectively targeted the transcripts which contained the 5'-end of cDNA Nb#62, but not the Nb#62-related transcript which didn't contain sequence similarity with the silencing trigger. Therefore, the transcripts were silenced in *N. benthamiana* by a PVX-based VIGS vector in a sequence-specific manner. However, PVX::62 infection didn't produce additional symptoms over a PVX vector infection of *N. benthamiana*. Therefore, it's difficult to speculate the function of Nb#62 from the results of the PVX-based VIGS assay.

8.3 TGMV-mediated silencing of Nb#62 was correlated with reduced viral DNA accumulation in systemically infected leaves

TGMV has been developed as a VIGS vector to trigger gene silencing in *N. benthamiana* (Kjemtrup *et al*., 1998; Peele *et al*., 2001). It has been reported that both TGMV A and TGMV B can be used as silencing vectors. However, the silencing phenotype is more extensive if the TGMV B component is used as the VIGS vector, co-inoculated with the wild-type TGMV A component. The TGMV B component has been engineered to contain an *Xba*I restriction site immediately downstream from the *BR1* gene that can be used for inserting foreign DNA (Figure 19, Schafer *et al*., 1995). It has also been reported that when the TGMV B component was used as a vector, there is a size limitation for DNA inserts. Silencing of an endogenous plant gene was effective in *N. benthamiana* with a TGMV B vector carrying a 92-bp fragment of the target gene. Larger DNA inserts tended to be deleted during virus propagation (Peele *et al*., 2001).

To investigate if silencing of Nb#62 affects TGMV infection, a 93-bp fragment of cDNA Nb#62 (Figure 17b) was inserted into the TGMV B vector at the *Xba*I site (Figure 19) in either the sense or antisense orientation. A 95-bp fragment of the *su* gene (Figure 17a) cloned into the TGMV B vector was used as a positive control for silencing experiments (Peele *et al*., 2001). The resulting plasmids containing TGMV/B::62(+), TGMV/B::62(-) and TGMV/B::su , as well as the wild-type TGMV DNA B, were co-inoculated with TGMV DNA A into *N. benthamiana* plants. Mock inoculation by bombardment with gold particle alone was included as a negative control.

When TGMV B carrying a 93-bp fragment of cDNA Nb#62 was bombarded into *N. benthamiana* plants with TGMV DNA A component, a unique symptom was observed. The whole plant had the same height as control plants that had been mock inoculated instead of being stunted which is characteristic of the wild-type TGMV infection. The leaves immediately above the inoculated leaves were bumpy and curled, and exhibited the strongest symptoms of virus infection in the plant. The upper leaves of plants infected with TGMV A/B::62 recovered from the virus infection and were symptom-free. Both the sense (+) and antisense (-) inserts (TGMV/B::62 (+) and TGMV/B::62(-)) had this effect on TGMV symptom development. In contrast to plants infected with TGMV A/B::62, plants infected with TGMV A/B::su were stunted. The leaves immediately above the inoculated leaves were not distinguishable from the typical symptoms of the wild-type TGMV infection. Leaf bleaching was exhibited in the upper parts in plants. These observed symptoms indicated that silencing of the *Sulfur* gene was not triggered in the leaves immediately above the inoculated leaves, but it was established in the upper leaves. It was also noticed that in plants infected with wild-type TGMV or TGMV

A/B::su, the upper leaves were less bumpy or distorted than the leaves immediately above the inoculated leaves, consistent with a partial recovery by the host.

At 21 dpi, nucleic acids were extracted from leaves that were immediately above the inoculated leaves and showed the strongest symptoms, and also from upper, symptom-attenuated leaves. DNA samples were analyzed by agarose gel electrophoresis and Southern hybridization (Figure 20). The relative accumulation of viral DNA in leaves immediately above the inoculated leaves, and in upper leaves, was determined by Phosphorimager analysis (Table 2).

The overall accumulation of viral DNA A in the leaves immediately above the inoculated leaves in the plants infected with TGMV A/B::62 (+), TGMV A/B::62(-) and TGMV A/B::su was 112%, 92% and 137% , respectively, of that achieved by wild-type TGMV. Therefore, in the leaves immediately above the inoculated leaves where silencing was not triggered, the viral accumulation in plants infected with TGMV-based silencing vectors was at about the same level as wild-type TGMV. However, the viruses accumulated much less in the upper leaves. There was 50.8% and 60.8% of that level in the upper leaves in plants infected with wild-type TGMV and TGMV A/B::su. The overall viral DNA A accumulation was reduced to 14.7% and 24.6% in the upper leaves in the plants infected with TGMV A/B::62 (+) and TGMV A/B::62(-), respectively. Therefore, in upper leaves where silencing was presumably established, the viral accumulation in the plants infected with TGMV A/B::su was at the same level as wild-type TGMV, but viruses accumulated much less in the plants infected with TGMV A/B::62(+) or TGMV A/B::62(-). The overall viral DNA B accumulation in those plants fit the same trend as viral DNA A, but with a more drastic effect (Figure 20b, Table 2).

DISCUSSION

It has been previously determined that AL2 protein in TGMV is a multifunctional protein. TGMV AL2 protein was initially found to be a viral *trans*-activator (Sunter and Bisaro, 1997). It de-represses the *AR1* promoter in phloem tissue, and *trans*-activates the *AR1* and *BR1* promoters in mesophyll cells (Sunter and Bisaro, 1997). It has also been found that AL2 protein binds preferentially to ssDNA and its binding lacks sequence specificity *in vitro* (Hartitz *et al*., 1999). It's likely that TGMV AL2 protein interacts with cellular factors that are capable of recognizing responsive elements in promoters, in a fashion similar to the adenovirus E1A protein and Epstein-Barr virus EBNA-2 protein. These proteins are also *trans*-activators and they appear to function via protein-protein interactions with host factors, rather than through direct contact with specific DNA sequences (Liu and Green, 1994; Heish and Hayward, 1995; Gerster and Roeder, 1988).

Subsequent studies have shown that besides its transcriptional activation activity, the TGMV AL2 protein is actively involved in host defense suppression and causes enhanced virus susceptibility in transgenic plants (Sunter *et al*., 2001). The host defense suppression activity of TGMV AL2 requires only the N-terminal 100 amino acids of the protein. In contrast, the transcription activation function of TGMV AL2 requires a C-terminal acidic activation domain. It has been reported that the interaction between TGMV AL2 and a homologue of SNF1, a global metabolic regulator in eukaryotes, mainly contributes to enhanced virus susceptibility in transgenic plants (Hao *et al*., 2003).

Therefore, to identify and characterize other host factors that interact with TGMV AL2 protein would yield novel insight into the molecular details of how geminiviruses

manipulate cellular machinery for their own use and how plants respond to virus infection.

Identification of host cDNAs that interact with TGMV AL2 protein using a yeast two-hybrid system: the specificity of interactions and the confidence of results

In this study, a yeast two-hybrid system was used to identify host cDNAs encoding proteins that interact with TGMV AL2 protein. Although the yeast two-hybrid system has proven to be a powerful tool for detecting protein-protein interactions *in vivo*, the problem of false positives exists. In this study, cDNAs Nb#26 and Nb#39 were screened out from the *N. benthamiana* cDNA library as being likely to interact with TGMV AL2 protein using LexA-TAL2ΔC as bait. However, further tests on the specificity of their interaction with TGMV AL2 protein revealed that when paired with either LexA-Lam or the LexA alone, these cDNAs allowed yeast growth in media lacking leucine. Therefore, the protein-protein interactions that activated the *LEU2* gene were not specific for the TGMV AL2 moiety. The results indicated that Nb#26 and Nb#39 were artifacts of the screening process and they were discarded as false positives.

Several factors may cause the problem of false positives. One possibility is that perhaps the fusion proteins B42AD-Nb#26 and B42AD-Nb#39 interact directly with the LexA protein, which activates the *LEU2* gene without an actual interaction with TGMV AL2 moiety.

To confirm whether the observed interactions of cDNAs Nb#51 and Nb#62 were specific for the TGMV AL2 protein, two N-terminal irrelevant baits, max-LexA and Lam-LexA were tested as negative controls. These irrelevant baits encode human Max

and lamin C proteins and are expected not to interact with proteins encoded by plant cDNAs. A strong interaction was observed when either Nb#51 or Nb#62 was co-expressed with TAL2(W124A)-LexA, but not with any of the negative control proteins, or with LexA alone. Based on these results, both Nb#51 and Nb#62 were shown to specifically interact with TGMV AL2 protein in the yeast two-hybrid system.

To confirm whether there is direct physical interaction between TGMV AL2 and the protein encoded by Nb#51 or Nb#62, several biochemical approaches could be used. One approach is to use an immunocapture assay. An immunoaffinity column can be made with the anti-LexA monoclonal antibody. Western blot results indicated that the fusion protein B42AD-Nb#51 could accumulate abundantly in cells (Figure 13b). Cell lysates prepared from yeast strains that express TAL2(W124A)-LexA and B42AD fused with Nb#51 or Nb#62 would be passed over the anti-LexA column. After washing, the potential presence of prey protein with TAL2(W124A)-LexA in the eluate would be evaluated by western blots with an anti-HA tag antibody that binds to the B42AD fusion proteins. The proteins that are detected in the western blot would have to be physically complexed with TAL2(W124A)-LexA. To confirm the specificity of the interaction, the same strategy would be used on negative control strains that express irrelevant baits (max-LexA or Lam-LexA) and fusion proteins B42AD-Nb#51 or B42AD-Nb#62, or TAL2(W124A)-LexA and B42AD vector alone. The successful detection of specific physical interactions in yeast would initiate further experiments in plant cells.

To evaluate whether the specific interactions observed in the yeast two-hybrid system have biological relevance for TGMV infection, some reverse genetics approaches could be used to create a knock-out phenotype of Nb#62. In this thesis, PVX- or TGMV-

based VIGS assays have been attempted. VIGS assays have distinct advantages, which have been reviewed in the Introduction part of this thesis. The results of these assays will be discussed in detail later. One major disadvantage of using a replicating virus to infect a plant and induce gene silencing of Nb#62 should be mentioned. Both TGMV and PVX cause symptoms that could mask the phenotype caused by the silencing of Nb#62. Other than VIGS, silencing of Nb#62 could be achieved by transforming *N. benthamiana* plants with transgenes that express self-complementary hpRNAs corresponding to Nb#62.

Regardless of how to disrupt the function of Nb#62, if the interactions between Nb#62 and the TGMV AL2 protein are biologically relevant, knockouts of Nb#62 would have some effect on TGMV infection. Supporting data might also be obtained from analysis of arabidopsis gene AT3g04710 orthologous to Nb#62, since *Arabidopsis thaliana* with its known genome sequences is a model system for plant genomics research. For arabidopsis gene AT3g04710 and the AL2 homologue in CabLCV, a begomovirus which can infect both *N. benthamiana* and arabidopsis plants, it's likely that AT3g04710 would also interact with CabLCV AL2 and this interaction would be biologically relevant to CabLCV infection of arabidopsis plants, if the hypothesis is correct. Analysis of arabidopsis / CabLCV system would also allow the use of other gene disruption techniques such as transferred DNA (T-DNA), or transposon-tagging, and a number of large public arabidopsis stock centers, such as the Arabidopsis Biological Resource Center, and The Arabidopsis Information Resource (TAIR).

Sequence and functional analysis of Nb#62

BLAST search indicated that AT3g04710 appears to be an arabidopsis orthologous gene of *N. benthamiana* Nb#62. At this time, there is no experimental information available for the function of AT3g04710. However, one can still learn something about Nb#62 by comparing to AT3g04710. The nucleotide sequence alignment of full-length cDNA of AT3g04710 and cDNA Nb#62 revealed that the 5'-end sequences, the sequences encoding part of the ankyrin-repeats and the whole TPR motif are more highly conserved than the rest (Figure 11). The ankyrin-repeats and the TPR motif are known to be important functional domains. It's not clear how the 5'-end sequences, or the part of the protein they encode, would take part in the function of Nb#62. Nevertheless, the results suggest that more work is needed to investigate the 5'-end of Nb#62, since Nb#62 and AT3g04710 have their highest percent similarity (78%) in this region.

BLASTP search for homologues of the protein encoded by Nb#62 have identified over 100 significant hits, most of which contain ankyrin repeats. Ankyrin repeats are known to mediate protein-protein interactions in a diverse family of proteins (Sedgwick *et al*., 1999). However, they apparently are not involved in interaction between Nb#62 and TGMV AL2 protein, as shown in deletion analysis in the yeast two-hybrid system. It's likely that they mediate interactions with different plant proteins and these interactions could be involved in one or more of the variety of functions known for ankyrin-repeat containing proteins, including transcription regulation, signal transduction and protein transport. A yeast two-hybrid assay could be used to screen the *N.*

benthamiana cDNA library using the ankyrin-repeat part of Nb#62 as bait, to identify plant proteins that potentially interact with Nb#62 through the ankyrin-repeats. It also could be tested whether Nb#62 interacts with itself through the ankyrin-repeats. The results would provide information about possible function(s) of Nb#62 in healthy plants.

The TPR motif is also a consensus sequences involved in protein-protein interactions in a variety of organisms from bacteria to humans (Blatch *et al*., 1999). Deletion analysis showed that the TGMV AL2 protein interacts with the protein encoded by cDNA Nb#62 via the C-terminal portion which contains the TPR motif. The participation of the TPR motif in the function(s) of Nb#62 will be discussed later. Deletion analysis showed that the TPR motif is important, but not sufficient to confer on Nb#62 the ability to interact with TGMV AL2 protein in the yeast two-hybrid system, and that the flanking regions are also required. Similar results have also been found in other systems. One example is in the Hsp90-interacting immunophilins Cyp40 and FKBP52, where both flanking regions at the ends of the TPR motif are required for Hsp90 binding (Ratajczak *et al*., 1996). Another example is that the co-chaperone Hip, an Hsp70-interacting protein, requires the TPR motif together with its flanking regions to accomplish the interaction with the ATPase domain of Hsp70 (Irmer *et al*., 1997). The folded structure of the TPR motif could provide an explanation for the participation of the TPR motif flanking regions in the interaction. The first crystal structure of a TPR containing protein, the human protein phosphatase 5, has revealed that each TPR motif folds into a similar tertiary structure, a pair of antiparallel α-helices (Das *et al*., 1998). The correct folding of the TPR motif is critical for its proper function. The regions flanking the TPR motif contain charged residues that could help the TPR motif to fold

correctly and stabilize the TPR motif three-dimensional structure. Also, the acidic and basic residues in the flanking regions may stabilize the protein-protein interactions through hydrophobic and/or electrostatic forces.

Right now, one cannot rule out the possibility that the motif analysis tools were not able to give the precise location of the TPR motif. Or maybe when the cDNA sequences are inserted into the B42AD vector to make a fusion protein, the linker amino acids joining the B42AD adversely affect the interaction, or maybe the designed fusion protein could not fold correctly. Modeling of the Nb#62 amino acid sequences on the crystal structure of the TPR motif would provide information to distinguish among these possibilities, and to further investigate how the TPR motif mediates protein-protein interactions between Nb#62 and TGMV AL2.

Is Nb#62 a stress-inducible gene?

The mRNA level of Nb#62 in the heat-shock experiment was too low to be detected by northern blot. However, that level was considerably higher in mock-inoculated plants in the silencing experiment and was detected by northern blot. The disparate results may reflect the differences in ages of plants used in the two experiments (5-week-old plants in the heat-shock experiment and 8-week-old plants in the silencing experiment), differences in stresses imposed on plants (heat stress in the heat-shock experiment and wound stress during rub-inoculation in the silencing experiment), or differences in growing environment (growth chambers in the lab or in the Phytotron). It is not known if Nb#62 is expressed in all tissues, or if the promoter of Nb#62 might be active only in cells of a particular developmental stage, or whether Nb#62 responds to

wound stress, or is induced under certain environmental conditions (nutrition, light, water etc.). All of these parameters should be investigated in future experiments.

Because the PVX genome and subgenomic mRNAs contain poly $(A)^+$ tails, a large proportion of the poly $(A)^+$ RNA extracted from plants inoculated with the PVX-based vectors was actually PVX-specific, and only a small proportion was from plant poly $(A)^+$ mRNAs. If Nb#62 expression was not induced by PVX infection, in the equal amount of poly $(A)^+$ RNA to be loaded on the gel, the proportion of the poly $(A)^+$ RNA made up by Nb#62 from PVX-infected plants would be expected to be much less than that from mock-inoculated plants. However, as shown in Figure 17b, the level of Nb#62-specific transcript II was higher in PVX infected plants than mock-inoculated plants. Also, considering the existence of PVX in the poly $(A)^+$ RNA pool, the proportion of Nb#62-specific transcript I in the plant poly $(A)^+$ mRNA pool was more in PVX infected plants than mock-inoculated plants, although the amount of transcript I was similar in two samples. Therefore, the PVX infection is very likely to induce the expression of Nb#62.

To further confirm that the mRNA level of Nb#62 is induced during PVX infection, the mRNA level of Nb#62 should be quantified. It could be normalized using the mRNA level of *N. benthamiana* β-actin, a constitutively expressed housekeeping gene that was used as a negative control in the heat-shock experiment.

Data have been obtained from arabidopsis GeneChip microarrays (Affymetrix) to identify changes in gene expression in response to diverse viruses (Whitham *et al*., 2003). However, the arabidopsis gene AT3g04710 orthologous to Nb#62 was not included on these microarrays, and currently, nothing is known about its mRNA expression profile. It would be an interesting gene to study in the future.

PVX-based VIGS assay revealed transcription mechanisms of Nb#62

The northern analysis of RNA from mock-inoculated plants with two distinct Nb#62-specific probes provided evidence that there are at least two transcripts of Nb#62. These two transcripts of different length, transcripts I and II, are identical in the 3'-end, while the 5'-end of cDNA Nb#62 is missing in the transcript I.

Alternative splicing might account for the existence of multiple transcripts of Nb#62. Actually, about 5% of eukaryotic pre-mRNAs can be alternatively spliced, generating multiple mRNAs that encode multiple proteins (Weaver, 2002). According to sequence and northern blot data, it's possible that transcripts I and II might represent two alternatively spliced transcripts from a common pre-mRNA (shown schematically in Figure 21). According to this model, transcript I and II would have identical 5'-end sequences, which were truncated during cloning procedures. Two 5'-end splice sites (GTT) in different downstream locations could compete for the same upstream 3'-splice site. Normally, the 3'-splice site would prefer the closest 5'-splice site and form transcript II, corresponding to cDNA Nb#62. In some cases, the more distant 5'-splice site could be chosen and thus transcript I could be formed.

This hypothesis could explain why some Nb#62-specific transcripts were not affected by PVX::62 inoculation. VIGS is a homology-dependent RNA degradation process. The silencing trigger fragment was in the 5'-end of cDNA Nb#62, which was absent in transcript I. Therefore, the silencing elicited by this fragment could only suppress the mRNA level of transcript II, leaving transcript I unaffected. The protein encoded by transcript I could carry out part of, if not all, the gene function. Therefore, the

target gene function may not have been totally disrupted by VIGS, and this may explain why there was no difference in the phenotype of PVX::62 infection when compared to the PVX vector infection.

The fact that arabidopsis gene AT3g04710 contains 13 exons supports the alternative splicing hypothesis. It's speculated that the gene encoding Nb#62 also has a similar number of exons. However, more work needs to be done to test the alternative splicing hypothesis. 5'- rapid amplification of cDNA ends (5'- RACE) could be done to obtain the precise 5'-end sequences of transcripts I and II. Under this alternative splicing hypothesis, both transcripts would be expected to have the identical 5'-end sequences.

Another possible explanation for the presence of at least two Nb#62-specific transcripts could be the presence of two promoters, and transcripts I and II could be expressed using these alternative promoters. The putative downstream promoter might be located in an intron, which would be spliced out of the longer transcript post-transcriptionally. Under this hypothesis, transcripts I and II would have distinct 5'-end sequences determined by the transcription start sites of the two promoters and Nb#51 might represent the full-length transcript I. Genomic sequence data from the Nb#62 locus, as well as detailed transcript mapping will be needed to study the gene expression regulation of Nb#62.

A TGMV-based VIGS assay to evaluate the role of Nb#62 in TGMV infection

There are some advantages of using TGMV as a silencing vector. It has a wide host range. Its small genome is easy to manipulate. It can be readily delivered into plants either by particle bombardment, agrobacterium infiltration, or mechanical abrasion. And it allows direct observation of the effect of silencing on TGMV infection.

However, the interpretation of VIGS assays using TGMV as the silencing vector is complicated by the fact that TGMV is a target as well as the trigger of VIGS. Because the silencing trigger fragment is inserted in the 3'-utr of the TGMV *BR1* gene, silencing would be directed not only against Nb#62, but presumably also the *BR1* gene. Reduction of viral DNA accumulation in the TGMV A/B::62 infected plants could be indirectly due to the degradation of Nb#62 mRNA. However, we could not rule out the possible explanation that TGMV *BR1* expression was affected. Moreover, it's unlikely that the 93-bp fragment from the 5'-end of #62 could target all Nb#62-speicifc transcripts.

In this study, the tobacco *Sulfur* gene (*su*) was used as a positive control in the silencing experiment. Silencing of the *su* gene could be readily recognized by the appearance of white leaves. A similar sized Nb#62 fragment was inserted into the same location of TGMV DNA B and this construct was inoculated onto plants in parallel with the positive control. Therefore, by comparing TGMV B::62 to the TGMV B::su positive control, any impact on TGMV infection that was due to the size of inserts or the location of insertion, or an adverse effect on TGMV *BR1* expression, could be ruled out definitely. The appearance of white leaves in plants infected with TGMV A/B::su indicated that silencing of the *su* gene was not initiated shortly after inoculation, but that it took some time to establish. Leaf bleaching was exhibited in the upper leaves, but not in leaves immediately above the inoculated leaves. Except for leaf bleaching in the upper leaves, plants infected with TGMV A/B::su had the typical symptoms of wild-type TGMV infection, with similar signs of recovery in the upper leaves. Consistently, the viral DNA accumulation in plants infected with wild-type TGMV or TGMV A/B::su were at the same level and they showed a similar decrease in the upper leaves. Plants infected with

TGMV A/B::62(+) or TGMV A/B::62(-) exhibited a similar pattern, with severe symptoms in the leaves immediately above the inoculated leaves and attenuated symptoms in the upper leaves. However, compared to plants infected with TGMV A/B::su or wild-type TGMV, there was a stronger effect of attenuation in the upper parts of plants. The plants were not stunted and the upper leaves were symptom-free. Consistent with this symptom pattern, TGMV A/B::62 DNA accumulation was much greater in the leaves immediately above the inoculated leaves than in the upper leaves. Compared to plants infected with TGMV A/B::su, the accumulation of TGMV A/B::62 was at similar level in the middle leaves where silencing was not initiated. However, TGMV A/B::62 showed a more pronounced reduction in the upper leaves where silencing was presumably established, in comparison to TGMV A/B::su. The effect on viral DNA accumulation observed in plants infected with TGMV A/B::62 (+) or TGMV A/B::62(-) was very likely due to the effect of silencing toward Nb#62.

Data are presented in this thesis that plants infected with the wild-type TGMV showed partial recovery from symptoms in the upper leaves with concomitantly less viral DNA accumulation. The results suggest that *N. benthamiana* plants can initiate a gene silencing-like mechanism to restrict TGMV accumulation. This phenomenon is consistent with the proposed biological role of PTGS as a plant defense system against virus infection.

VIGS assays of Nb#62 provide new perspectives for silencing experiment design

In the future silencing experiment design, to achieve an extensive silencing of the target gene by VIGS and to be able to observe the effect of gene knock-out phenocopy on the TGMV infection, two major points should be taken into consideration.

(a) The design of silencing triggers. Before designing the silencing trigger, it would be necessary to collect data from comparison of Nb#62 gene expression patterns in the absence or presence of the TGMV infection. Since there appears to be multiple transcripts from a single gene, detailed transcription mapping should be employed to characterize the transcripts and uncover the exon structures. The most useful silencing trigger fragments should be homologous to all transcripts, or target the important functional domains. For example, since TGMV AL2 interacts specifically with the C-terminal portion of the protein encoded by cDNA Nb#62, sequences from the 3'-end of cDNA Nb#62 would be expected to be a more informative silencing trigger than the 5'-end sequences used in this study.

(b) The choice of virus vector. A different virus could be used to trigger VIGS in *N. benthamiana* plants, after which the plants would be challenged with wild-type TGMV to observe any effect on superimposed TGMV infection. It would be desirable that the silencing trigger virus alone should be able to successfully replicate and elicit silencing while produce asymptomatic infection in the plants. Moreover, it should not interfere with TGMV infection. In this sense, using PVX as the VIGS trigger in *N. benthamiana* plants does not seem to be ideal because the PVX vector alone causes severe symptoms, and it has not been determined if PVX would interfere with TGMV infection. Another

disadvantage of using PVX is that the poly (A) tails on the PVX genome and subgenomic mRNAs complicated the data analysis. Attempts to use BGMV, which like TGMV is a begomovirus, as a silencing trigger were made in this study, but were not successful (see Appendix). Another RNA virus, TRV (see Introduction) could be a potential VIGS vector for analysis of gene function in *N. benthamiana* plants. Its genome and subgenomic mRNAs don't contain poly (A) tails. It causes a very mild and yet extensive infection in *N. benthamiana* plants. Alternatively, a non-virus-induced gene silencing system also could be used, which could circumvent some of the problems associated with using VIGS. *N. benthamiana* plants could be transformed with transgenes that express self-complementary hpRNAs corresponding to Nb#62 to trigger silencing.

Possible roles for Nb#62 in the context of TGMV infection

TGMV AL2 protein affects cellular machinery in at least two aspects: it targets and/or activates cellular transcription machinery for expression of late viral genes; and it counteracts a system of plant defense against viral infection.

Unfortunately, it's difficult to directly deduce the function of Nb#62 from the results of the PVX-based VIGS assay conducted in this study. However, provided that the interaction between Nb#62 and TGMV AL2 protein has biological relevance, based on the data presented in this thesis, one can propose at least two hypotheses for the role(s) of the protein encoded by Nb#62 in TGMV infection.

(1) Hypothesis I: Nb#62 is a defense-associated gene. TGMV AL2 protein interacts with Nb#62 to antagonize host defense responses mounted against the viral invasion (shown schematically in Figure 22a).

Virus invasion, which is perceived as a kind of stress by plants, could increase the expression of a number of host genes (Whitham *et al*., 2003). In healthy plants, Nb#62 may interact with components of plant defense systems via the TPR motif. Its expression may be induced upon virus infection. TGMV AL2 protein, which is thought to be expressed shortly after infection, could compete with cellular proteins for binding to the TPR motif of Nb#62, and consequently interfere with the plant defense response. Therefore, if Nb#62 expression is suppressed in TGMV A/B::62 infected plants, TGMV AL2 could not have impact on the plant defense response and one would expect a normal (if not even more effective) plant defense against TGMV in presumably silenced tissue. The lack of symptoms observed in upper leaves accompanied with the markedly reduced viral DNA accumulation in TGMV A/B::62 infected plants is consistent with this hypothesis.

Although whether Nb#62 expression responds to heat stress is not known, northern hybridization analysis indicated that the mRNA level of Nb#62 was likely elevated in response to PVX infection. Whether the Nb#62 mRNA is induced in response to TGMV infection would be a key question to answer in future studies.

One way to test the defense-associated hypothesis would be to determine if the N-terminal 100 amino acids residues of the TGMV AL2 protein could interact with Nb#62 in the yeast two-hybrid system, or by other assays, since this region of TGMV AL2 is required for host defense suppression *in vivo* (Sunter *et al*., 2001). If the hypothesis is correct, one should expect a strong positive result which can be confirmed by different approaches.

(2) Hypothesis II: Nb#62 is involved with the cellular transcription machinery and is hijacked by TGMV to direct viral gene expression. Under this hypothesis, one would also expect that silencing of Nb#62 would affect TGMV infection adversely. The putative Nb#62 silencing-related recovery seen in upper leaves in the TGMV-based VIGS assay is consistent with Hypothesis II.

The finding of three copies of the TPR motif downstream of the ankyrin-repeats may provide insight into the function of the protein encoded by Nb#62. TPR-containing proteins are involved in a variety of cellular functions including transcription, cell-cycle control and protein transport (Blach and Lassle, 1999). The deletion analysis data showed that the TPR motif, together with its flanking regions, mediated the interaction between Nb#62 and TGMV AL2 in the yeast two-hybrid system. In plant cells, Nb#62 may be a component of a transcription regulation complex. The TPR motif of Nb#62 could be involved in repression of viral late gene promoters by functioning as an adaptor to mediate interaction with a specific cellular transcription factor and to recruit other elements in the transcription repression complex. Perhaps the most relevant model is the interaction of the Ssn6p and Tup1p proteins in *S. cerevisiae* cells. Ssn6p, a TPR-containing protein, interacts with Tup1p, a transcription repressor, to form an Ssn6p-Tup1p complex. This complex doesn't bind DNA directly. Rather, it recruits a promoter-specific DNA-binding protein via the TPR motif in Ssn6p to accomplish repression activity (Tzamarias *et al*., 1995).

Alteration of host cellular machinery for their own use is a general strategy adopted by most viruses. For example, the major function of the p53 protein in kidney cells is to monitor cellular replication and prevent abnormal cell proliferation (Pipas and

Levine, 2001). The T antigen from SV40 blocks p53 function and drives cells into S-phase to produce viral progeny. TGMV AL2 protein is thought to be expressed early after TGMV infection. It's known that the mechanisms of *trans*-activation of viral late genes by TGMV AL2 protein have cell and tissue specificity. In phloem tissue, the TGMV *AR1* promoter is de-repressed, whereas in mesophyll cells it is *trans*-activated (Sunter and Bisaro, 1997). Conceivably, Nb#62 could be involved in either of these processes. For example, in phloem tissue, AL2 could interact with the Nb#62 adaptor protein via the TPR motif, and then bind a promoter-specific transcription repressor X to de-repress the promoter (shown schematically in Figure 22b). In mesophyll cells, alternatively, AL2 could interact with the Nb#62 protein, which recruits a promoter-specific DNA-binding protein Y, and thereby activates the viral promoter (shown schematically in Figure 22c). Several cellular transcription factors have been identified that can behave as activators or repressors depending on the presence or absence of viral regulatory proteins (Hsieh and Hayward, 1995; Shi *et al*., 1991).

To continue investigation in this direction, the *N. benthamiana* cDNA library could be screened to identify the host factors that interact with Nb#62 in the yeast two-hybrid system, by using Nb#62 as bait. Regardless of the outcome of future studies of Nb#62, the results are likely to be important and novel, in the light of the fact that only one plant protein that interacts with TGMV AL2 protein has been identified so far (Hao *et al*., 2003), and it apparently is not involved in transcription regulation.

Table 1. Primers used for sequencing Nb#62 by PCR walking.

Sequencing step	Primer	Sequences (5'-3')	Orientation (+/-)*
1	F184	5'-CCAGCCTCTTGCTGAGTGGAGATG	+
2	F227	5'-GCATCATGCTGCAGGAATGGGACATGT	+
3	F260	5'-GTGTTCCAGAATGGAGTGTGG	+
4	F239	5'-GCCTATCCTATCTGAAACTAGGATAAACC	+
5	B252	5'-TTAAAGAATTCAAACGGAGGATAA	-
6	B261	5'-GCCATGAAATTCTCTACCAGC	-
7	B251	5''-GAGCTCCATATTTTCAGGGTCGA	-
8	B250	5'-TCAGATAGGATAGGCTCAGATTG	-
9	B267	5'-GCAGCCCATATAAGTGGTGT	-
10	B268	5'-ATCATCCAGTTGCTTTGCCAA	-

* The '+' symbol means the mRNA sense and the '-' symbol means the complementary sense.

Table 2. Relative viral DNA accumulation in *N. benthamiana* plants inoculated with TGMV A/B::62(+) and TGMV A/B::62(-), compared to wild-type TGMV or TGMV A/B::su.

	Inoculum [a]	Relative viral DNA accumulation (%)[b]	
		DNA A	DNA B
Middle leaves[c]	TGMV wt	100.0	100.0
	TGMV A/B::62(+)	112.4	63.2
	TGMV A/B::62(-)	92.0	33.0
	TGMV A/B::su	132.5	106.1
Upper leaves	TGMV wt	50.8	18.4
	TGMV A/B::62(+)	14.7	1.9
	TGMV A/B::62(-)	24.6	2.0
	TGMV A/B::su	60.8	17.0

(a) Inoculum included 5 μg of a plasmid containing TGMV-based DNA B silencing vectors TGMV /B::62(+), TGMV/B::62(-), TGMV/B::su, or wild-type TGMV DNA B, together with 5 μg of a plasmid containing wild-type TGMV DNA A component. They were inoculated into *N. benthamiana* by microprojectile bombardment.

(b) The viral DNA accumulation was quantified by Phosphorimager analysis of Southern blots (shown in Figure 19). The relative overall viral DNA A or DNA B accumulation (dsDNA and ssDNA) in each sample was estimated by comparison with wild-type TGMV, and given as a percentage of wild-type TGMV DNA A or DNA B accumulation.

(c) The middle leaves were leaves that were immediately above the inoculated leaves in plants with strongest symptoms.

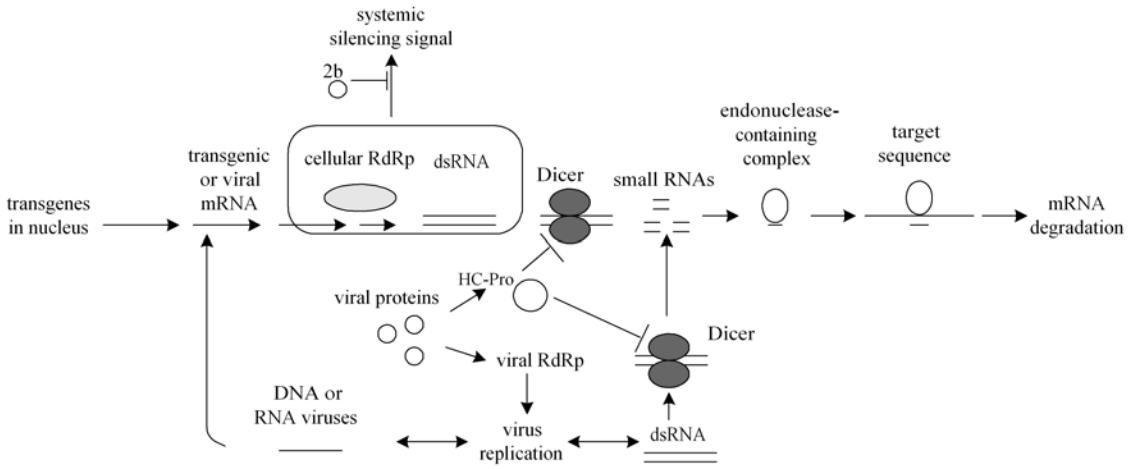

Figure 1. A current model for PTGS in plants. Silencing of an endogenous gene by a viral vector or by overexpressed transgenes is shown schematically. Double stranded RNA (dsRNA) produced by either a cellular RdRp-dependent reaction or viral RNA replication is proposed to initiate RNA silencing. Small RNAs processed by dsRNase Dicer mediate sequence-specific RNA degradation. Some proteins involved in RNA silencing are indicated. The virus-encoded silencing suppressors 2b and HC-Pro are shown. The boxed segment indicates the cellular RdRp-dependent part that leads to production of the systemic silencing signal.

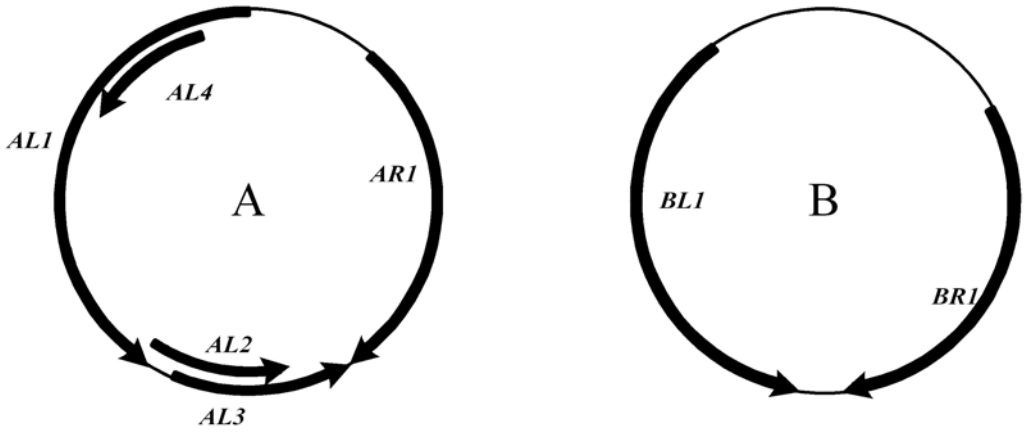

Figure 2. Schematic illustration of begomovirus genome organization. The solid arrows define the positions of open reading frames (ORFs) in each DNA component.

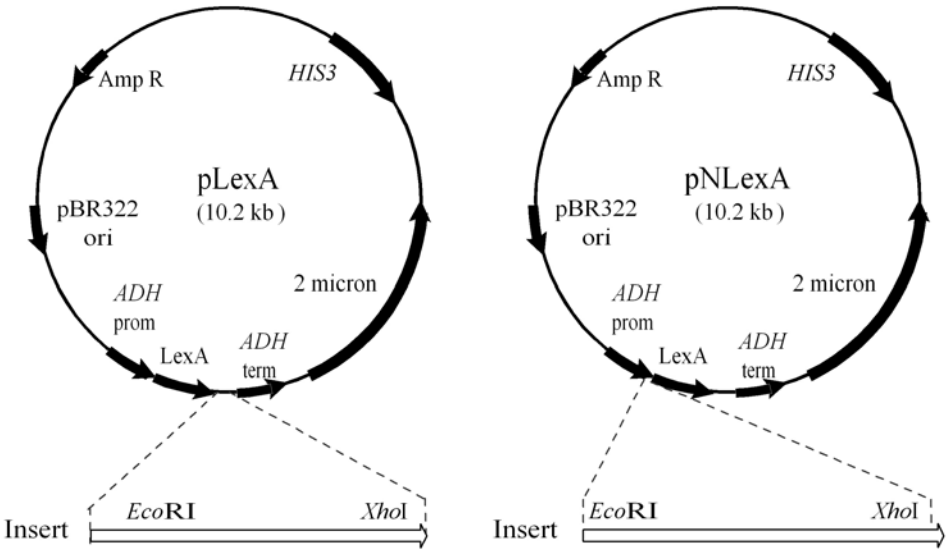

Figure 3. Illustration of bait plasmids pLexA and pNLexA. The insertion site is different in these two plasmids. pLexA expresses cDNAs or other coding sequences inserted into the unique *Eco*RI and *Xho*I sites as translational fusions to the C-terminus of the LexA DNA-binding domain, while the protein of interest is fused to the N-terminus of LexA in pNLexA. The *HIS3* transformation marker is for selection in yeast. The 2-micron origin of replication is for high copy number in yeast. The *ADH* promoter is for expression of fusion protein in yeast. The pBR322 origin of replication is for maintenance in *E. coli*, and AmpR is for selection in *E. coli*.

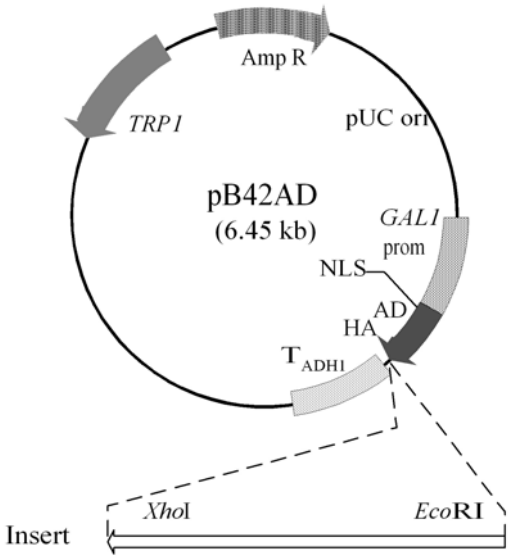

Figure 4. Illustration of prey plasmid pB42AD. pB42AD expresses cDNA or other coding sequences inserted into the unique *Eco*RI and *Xho*I sites as translational fusions to a cassette consisting of the SV40 nuclear localization sequence (NLS), the 88 residue B42 activation domain (AD), and the hemagglutinin epitope tag (HA). Fusion protein expression is under the control of the *GAL1* inducible promoter. The *TRP1* marker is for selection in yeast. The pUC origin of replication is for maintenance in *E. coli*, and AmpR is for selection in *E. coli*.

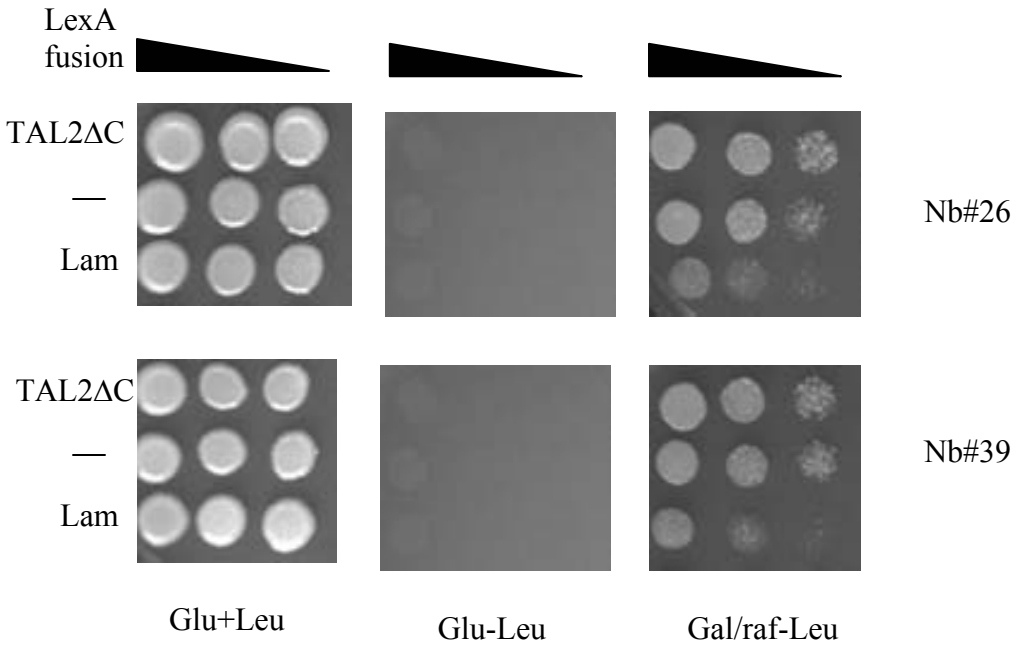

Figure 5. The interaction of cDNA Nb#26 and Nb#39 with LexA-TAL2ΔC was not specific for the TGMV AL2 moiety. Serial dilutions of each yeast strain (10^{-1}, 10^{-2}, 10^{-3}) were spotted onto three selective plates: minimal medium with leucine or without leucine, with glucose as the carbon source (Glu+Leu and Glu-Leu), or without leucine with galactose and raffinose as the carbon source (Gal/raf -Leu). The yeast growth on medium lacking leucine is Gal-dependent. The plates were photographed after 3 days of incubation at 30°C. Yeast strains were EGY48 containing combination of plasmids shown in the graph. The growth of yeast containing Nb#26 in combination with LexA-TAL2ΔC was as much as that of yeast containing Nb#26 and the vector pLexA. The same results were obtained with Nb#39.

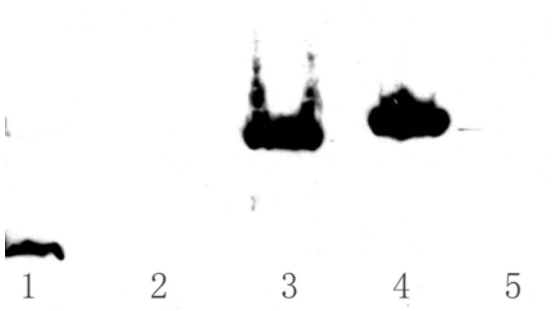

Figure 6. Potential irrelevant baits Lam-LexA and max-LexA accumulated efficiently in yeast. Whole cell extracts were fractionated on a denaturing 12% polyacrylamide gel, transferred to a nitrocellulose membrane and detected by anti-LexA monoclonal antibody. Protein was prepared from yeast strains containing the positive control LexA-TAL2ΔC (lane 1), and test strains Bicoid-LexA (lane 2), Lam-LexA (lane 3), max-LexA (lane 4) and p53-LexA (lane 5). The accumulation of Lam-LexA and max-LexA were shown, while neither Bicoid-LexA nor p53-LexA was detected.

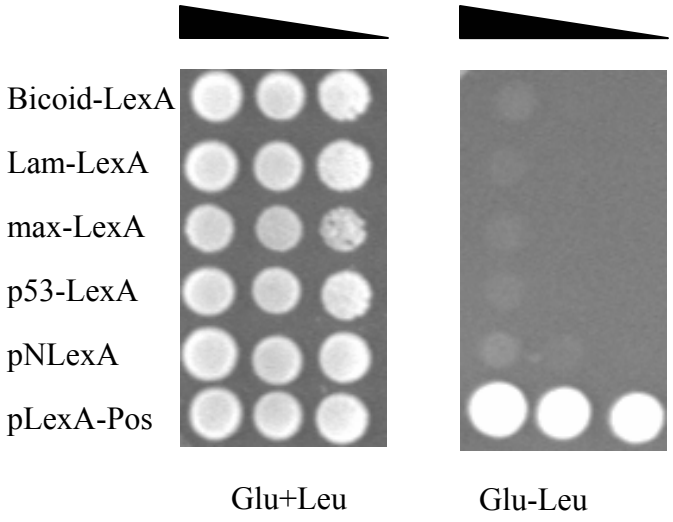

Figure 7. None of the four test irrelevant baits were autoactivators in the yeast two-hybrid assay. Serial dilutions of each yeast strain (10^{-1}, 10^{-2}, 10^{-3}) were spotted onto minimal medium with or without leucine (Glu+Leu and Glu-Leu). The plates were photographed after 5 days of incubation at 30°C. Yeast strains were EGY48 containing plasmids expressing proteins fused to N-terminus of LexA, as indicated. pLexA-Pos is an autoactivation positive control, while pNLexA is a negative control.

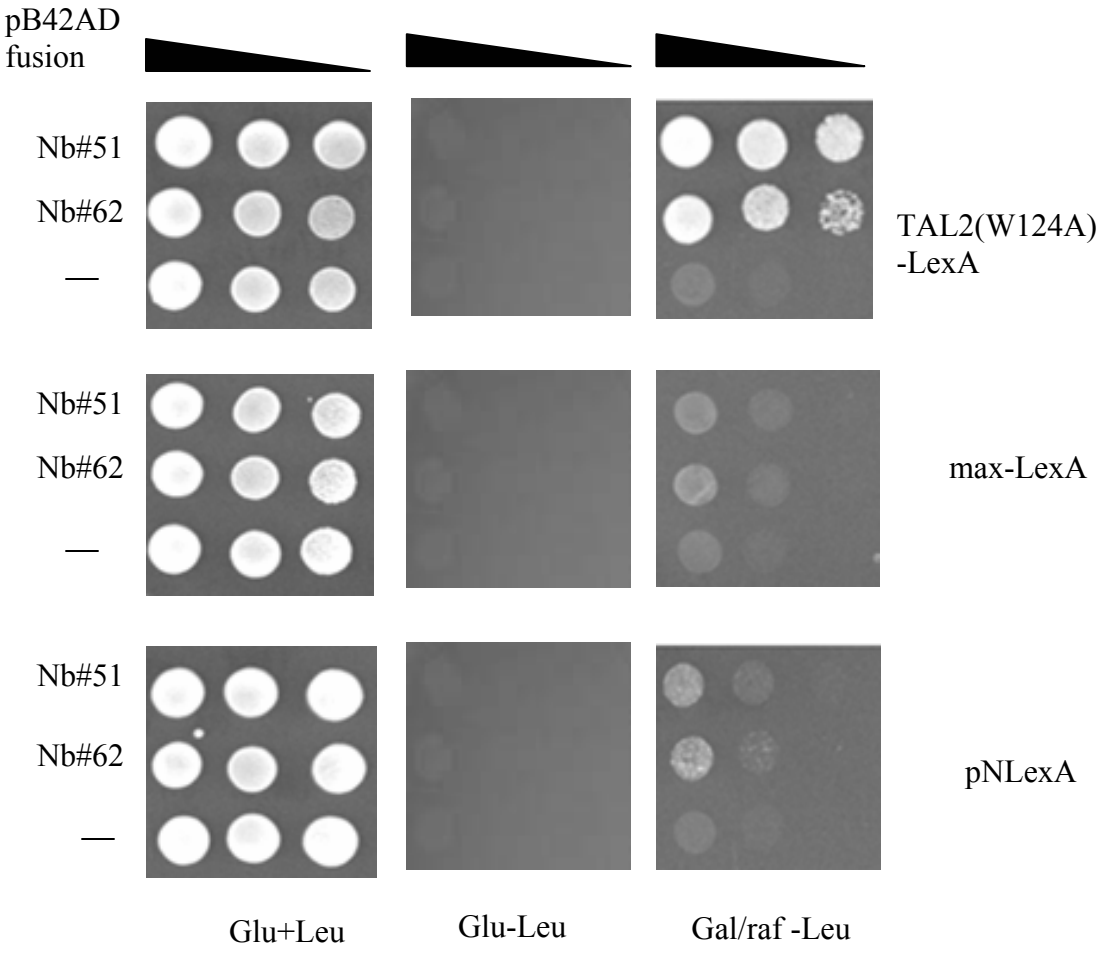

Figure 8. Both Nb#51 and Nb#62 specifically interact with TGMV AL2 in the yeast two-hybrid system, with max-LexA as irrelevant bait. Serial dilutions of each yeast strain (10^{-1}, 10^{-2}, 10^{-3}) were spotted onto minimal medium with leucine or without leucine (Glu+Leu, Glu-Leu), or lacking leucine with galactose and raffinose as the carbon source (Gal/raf -Leu). The yeast growth on medium lacking leucine is Gal-dependent. The plates were photographed after 5 days of incubation at 30°C.

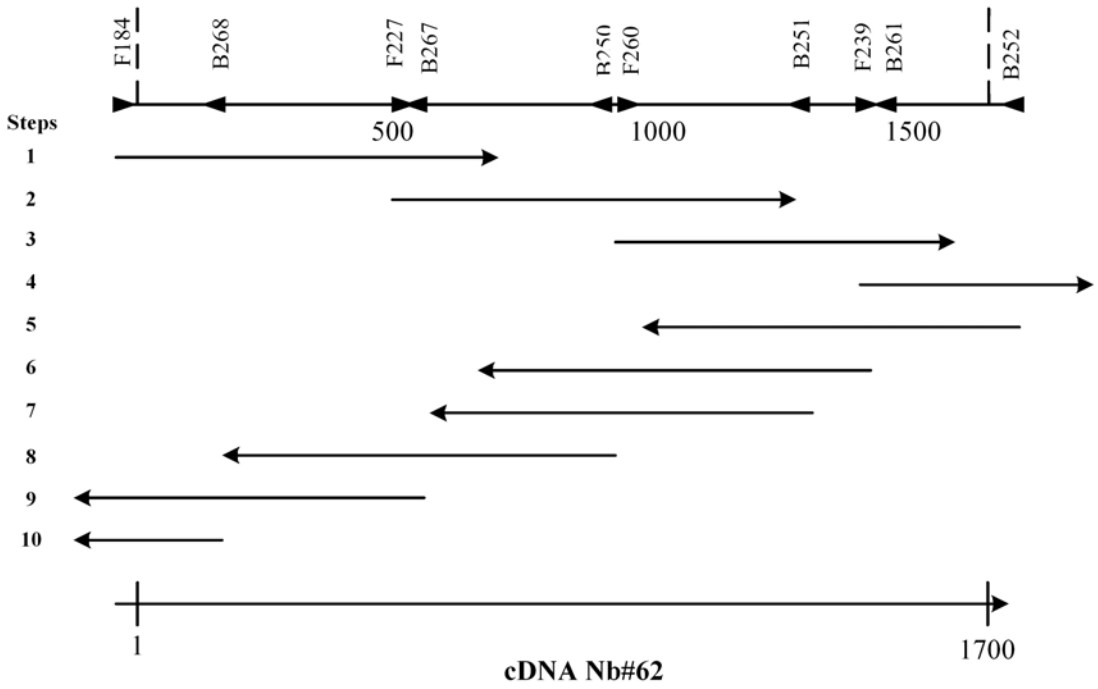

Figure 9. Sequencing the cDNA insert from clone Nb#62 by PCR walking. Triangles indicate the location and direction of primers used (their sequences are given in Table 1). Sequence coordinates are given in nucleotides below the map. Arrows indicate the extent and direction of sequence data obtained from each step.

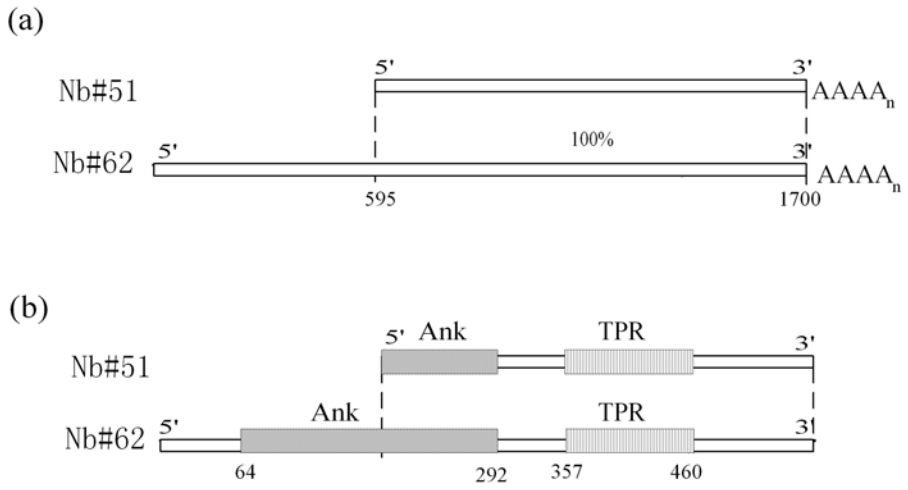

Figure 10. Sequence analysis of Nb#51 and Nb#62. (a) There is 100% similarity in alignment of cDNA Nb#51 and 3'-end of Nb#62. The Nb#51 appears to be a 3'-coterminal truncated version of Nb#62, and the 5'-end of Nb#51 is located at nucleotide 595 of Nb#62. (b) Sequence analysis of the proteins encoded by Nb#62 and Nb#51. The ankyrin repeats were found between amino acid residues 64 and 292, and TPR motif was between amino acid residues 357 to 460. The whole TPR motif and part of the ankyrin-repeats are present in Nb#62 and Nb#51.

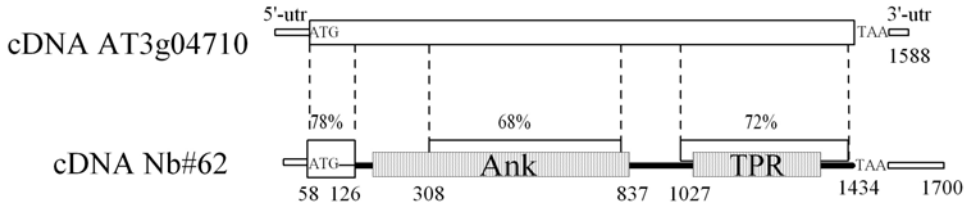

Figure 11. Nucleotide sequence alignment of cDNA Nb#62 and the full-length cDNA of Arabidopsis orthologous gene AT3g04710. The open boxes with the dashed lines indicate the regions with significant similarities between the two sequences. The grid boxes indicate the sequences that encode the ankyrin-repeats (Ank) and the TPR motif. 5'- and 3'-utr indicate the 5'- and 3'-untranslated regions. The start ATG and stop TAA codons are also shown.

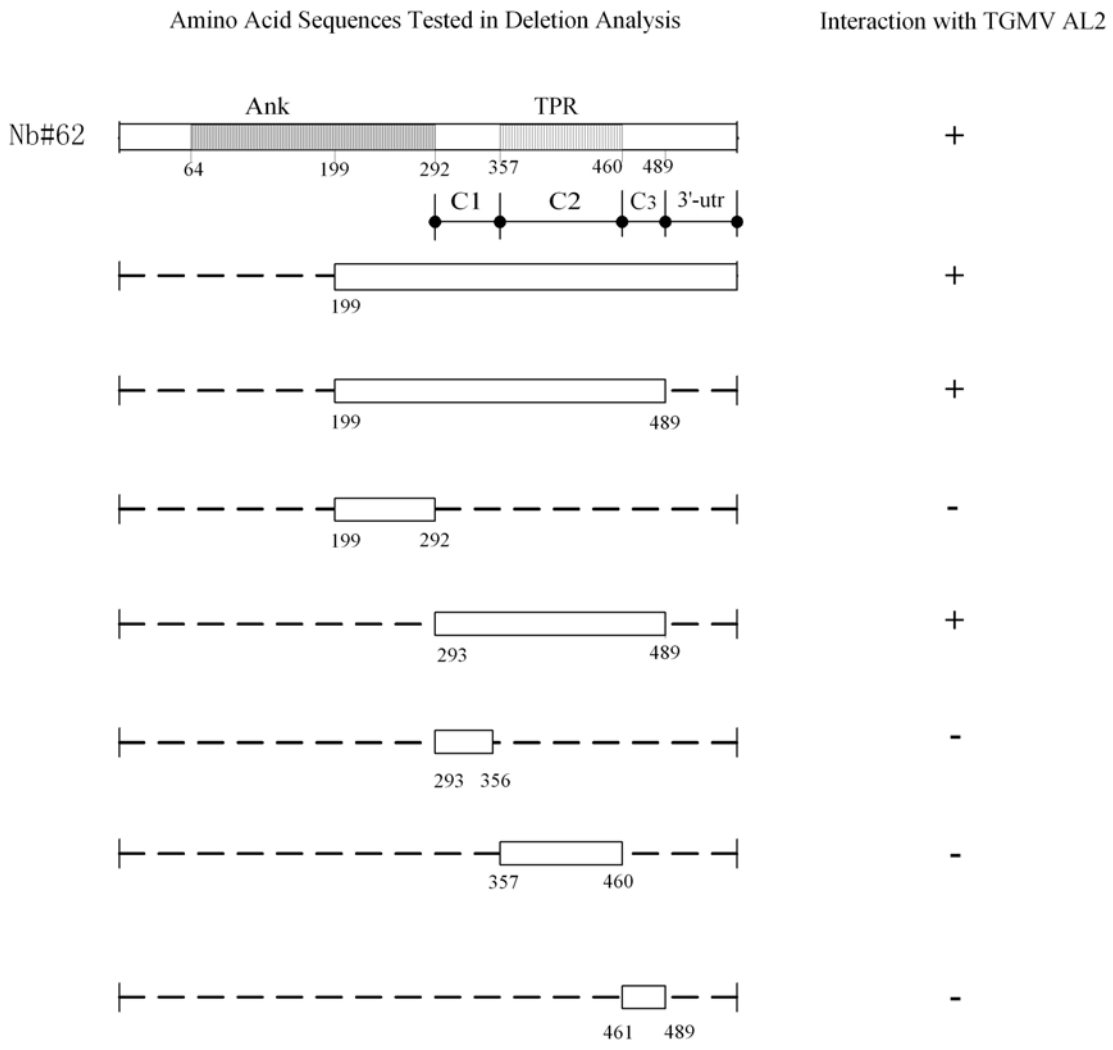

Figure 12. Summary of deletion analysis results. Ankyrin-repeats (Ank) in Nb#62 are not involved in its interaction with TGMV AL2 in the yeast two-hybrid system. In contrast, the tetratricopeptide motif (TPR), with its flanking regions, was sufficient to confer on Nb#62 the ability to interact with TGMV AL2. The locations of Ank and TPR are shown in the graph. The sequences downstream of ankyrin-repeats were divided into C1, C2, C3 and 3'-untranslated regions (3'-utr). The open boxes indicate the amino acid sequences that are fused to pB42AD prey vector, and tested in the yeast two-hybrid system with

TGMV AL2 fused to pNLexA bait vector. The ‘+’ symbol represents significant interaction; and the ‘–’symbol represents no significant interaction.

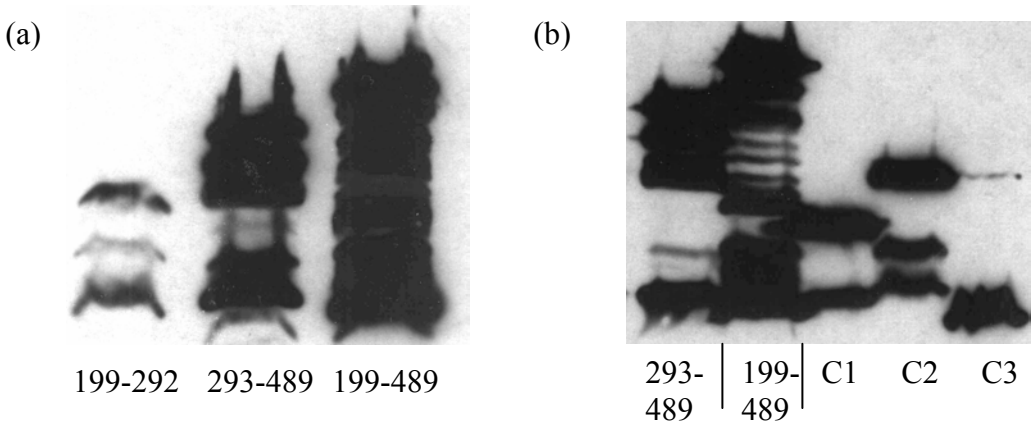

Figure 13. B42AD fused with a series of Nb#62 deletion fragments could accumulate abundantly in yeast. Whole cell extracts were fractionated on a denaturing 12% polyacrylamide gel, transferred to a nitrocellulose membrane and detected by anti-HA tag antibody that binds to the B42AD fusion protein. Proteins were prepared from yeast strains containing B42AD fused with different regions of amino acids of Nb#62: (a) the ankyrin repeats region (amino acids 199-292), the C-terminal region lacking the ankyrin-repeats (293-489), and the Nb#51 encoding region (amino acids 199-489). (b) C1 comprising amino acids 293-356, C2 comprising amino acids 357-460 and C3 comprising amino acids 461-489.

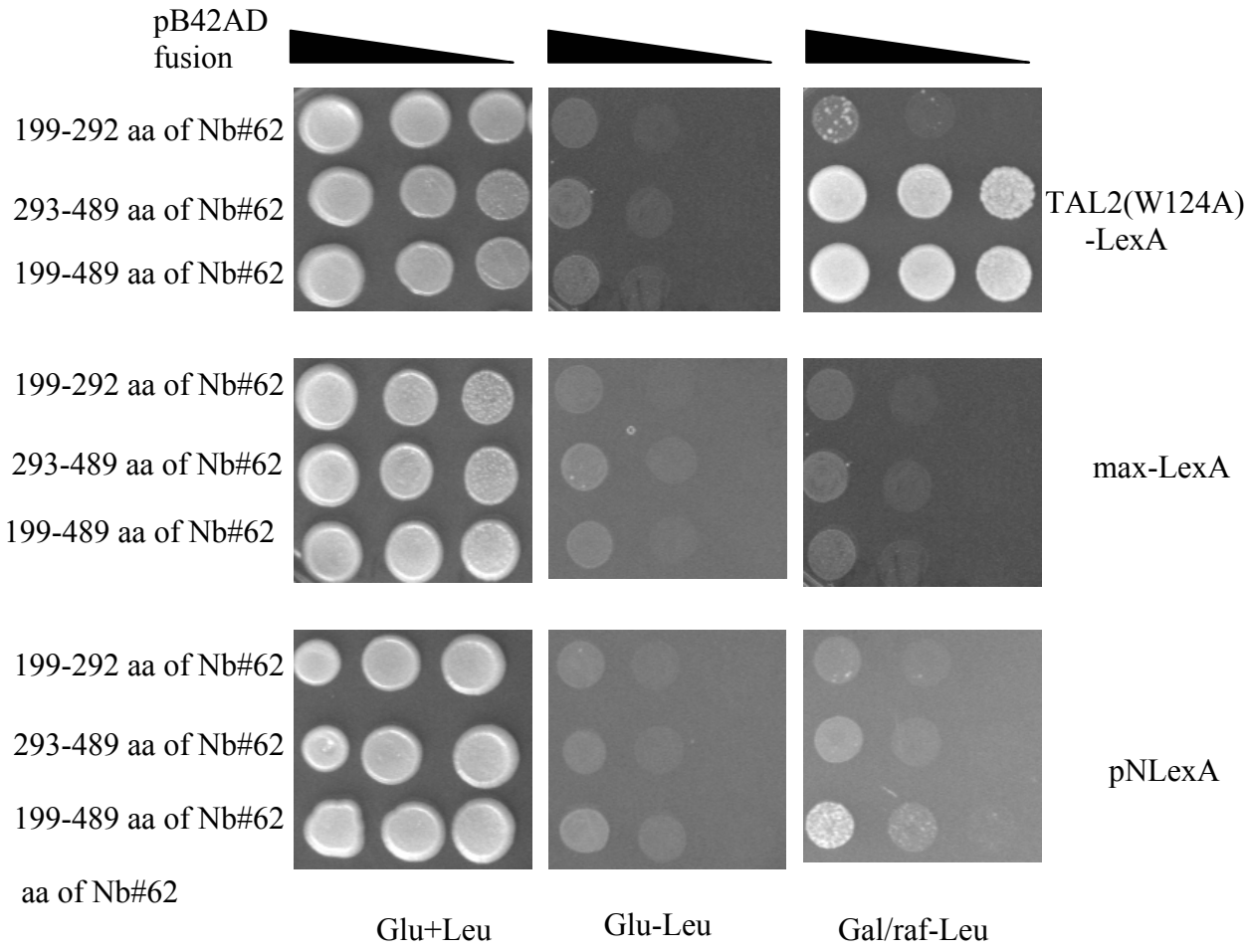

Figure 14. Ankyrin-repeats (Ank) in Nb#62 are not involved in its interaction with TGMV AL2 in the yeast two-hybrid system. Serial dilutions of each yeast strain (10^{-1}, 10^{-2}, 10^{-3}) were spotted onto minimal medium with leucine or without leucine (Glu+Leu, Glu-Leu), or lacking leucine with galactose and raffinose as the carbon source (Gal/raf -Leu). The yeast growth on medium lacking leucine is Gal-dependent. The plates were photographed after 5 days of incubation at 30°C. The ankyrin-repeats alone (amino acids 199-292) exhibited no ability to interact with TGMV AL2. The region of amino acids 293-489 of Nb#62, which does not include the ankyrin repeats, specifically interacted with TGMV AL2. So did the region of amino acids 199-489.

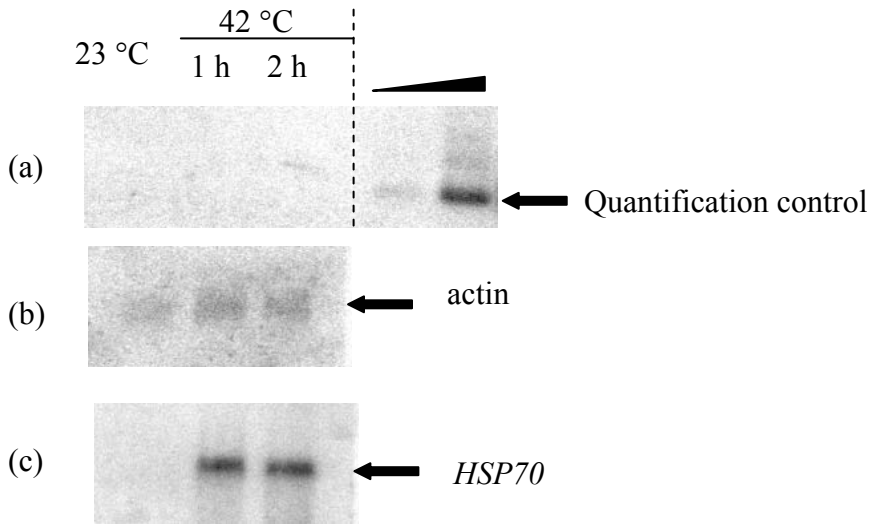

Figure 15. Heat shock did not detectably induce the expression of Nb#62. Total nucleic acids were extracted from control plants maintained at 23 °C, or plants heat-shocked at 42 °C for 1 hour or 2 hours. Equal amounts of poly $(A)^+$ RNA (3.0 μg) were resolved by agarose gel electrophoresis and northern blotted. Replicate blots were hybridized with ^{32}P-labeled probes specific for (a) Nb#62 (corresponding to nucleotides 920-1700 of cDNA Nb#62), (b) *N. benthamiana* β-actin, a constitutively expressed housekeeping gene, and (c) *N. benthamiana HSP70*, a positive control. A dilution series of *in-vitro* transcripts corresponding to the Nb#62-specific probe (1 pg and 10 pg) was used as to quantify the sensitivity of detection of Nb#62 mRNA, and indicated that the detection threshold of the northern hybridization was 1 pg.

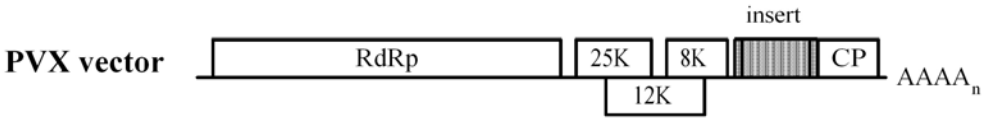

Figure 16. Genomic organization of PVX vectors used in VIGS assays. The PVX open reading frames encode RNA-dependent RNA polymerase (RdRp), movement proteins (25K, 12K and 8K), coat protein (CP), and the insert. The vector constructs were assembled as cDNA and transcribed into RNA for inoculation of plants. $AAAA_n$ represents the 3'-terminal poly (A) tail of the PVX genome.

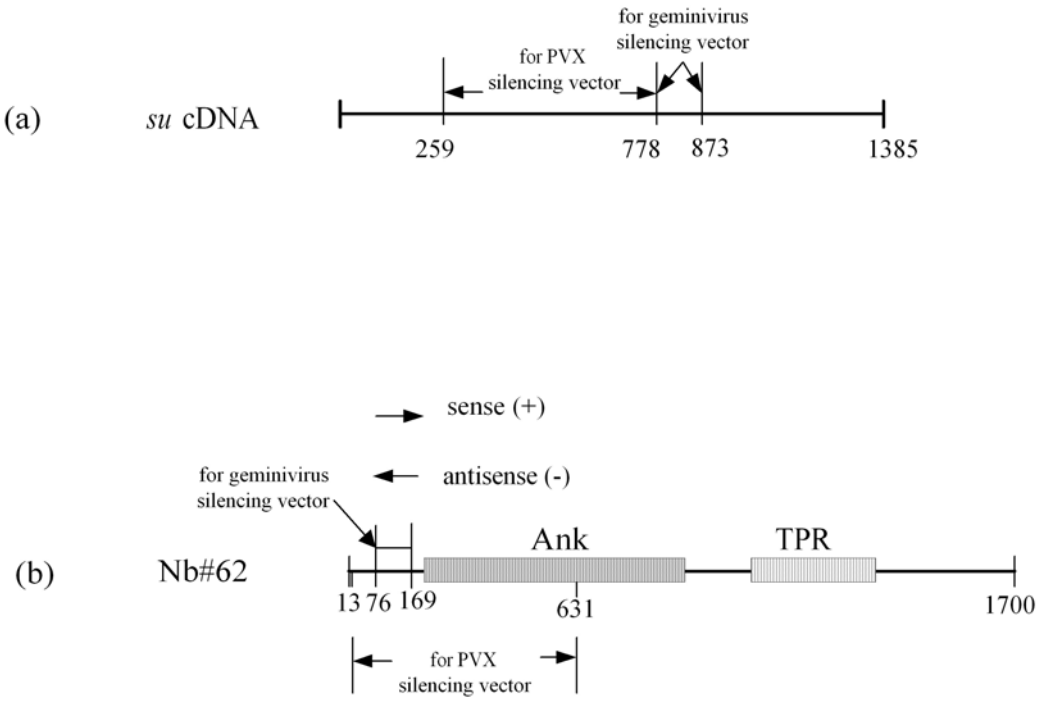

Figure 17. The locations of fragments used in gene silencing experiments.

(a) In the *su* cDNA (1385-bp), the 519-bp fragment (nucleotides 259-778) was used to make PVX::su. The 95-bp fragment (nucleotides 778-873) was used to make TGMV/B::su.

(b) In cDNA Nb#62 (1700-bp), the 618-bp fragment (nucleotides 13-631) was used to make PVX::62. The 95-bp fragment (nucleotides 76-169) in sense (+) or antisense (-) orientation was used to make TGMV/B::62(+) or TGMV/B::62(-).

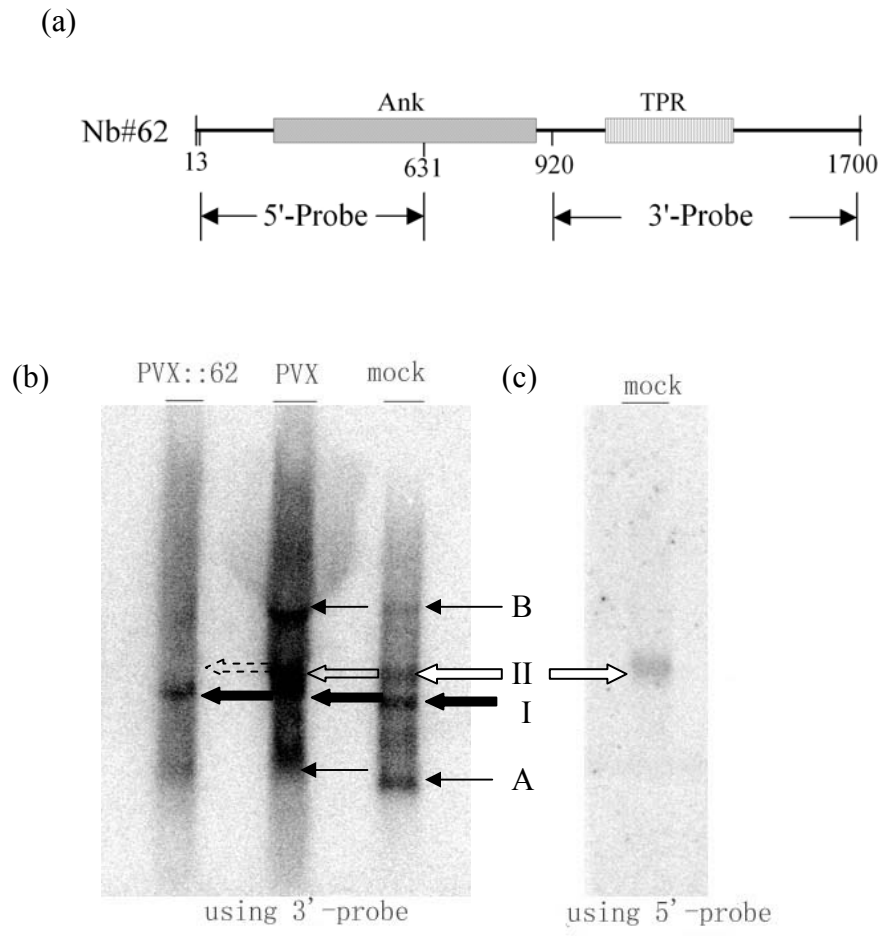

Figure 18. Nb#62-specific transcripts were silenced in *N. benthamiana* by a PVX-based VIGS vector in a sequence-specific manner. Nucleic acids were extracted at 21 dpi from systemically infected leaves of plants that had been mock inoculated with buffer only, or inoculated with the PVX vector or PVX::62. Equal amounts of poly $(A)^+$ RNA (4.5 μg) were resolved by agarose gel electrophoresis and northern blotted. The filter was hybridized with ^{32}P-labeled probes to detect the presence of Nb#62 mRNA. (a) The locations of fragments from cDNA Nb#62 used for making probes. The 5'-probe corresponds to nucleotides 13-631 and the 3'-probe corresponds to nucleotides 920-1700. (b) RNA species detected by 3'-probe. (c) RNA species detected by 5'-probe. Arrows

indicate the positions of the Nb#62 transcripts. The white solid two-way arrows indicate the position of band II, corresponding to a longer transcript, which hybridized to both 5'- and 3'-probes. This transcript was eliminated in RNA samples from PVX::62 inoculated plants (indicated by dashed arrow). The black solid arrows indicate the positions of the faster-migrating band I, corresponding to a shorter transcript, which only hybridized to the 3'-probe. This transcript was detected in RNA samples from mock inoculated plants, and PVX vector as well as PVX::62 inoculated plants. The black arrows indicate the positions of band A and B. These bands were probably the electrophoresis artifacts and could be resolved by a denaturing gel.

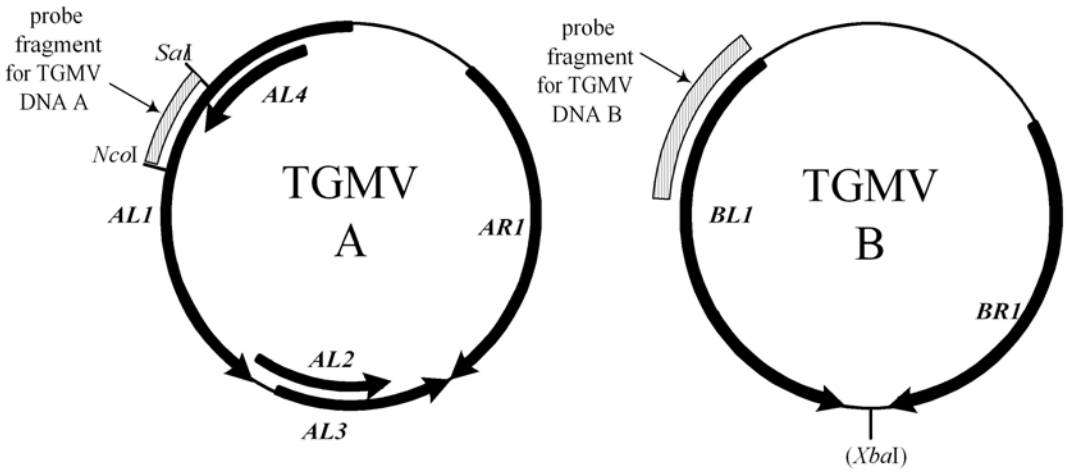

Figure 19. Schematic illustration of TGMV DNA components. The locations of restriction sites for cloning or isolation of probe fragments in Southern hybridization are shown. The *Xba*I is shown parenthetically in the figure to indicate that it was artificially introduced for cloning. Shaded areas indicate the locations of probe fragments for TGMV DNA A or TGMV DNA B.

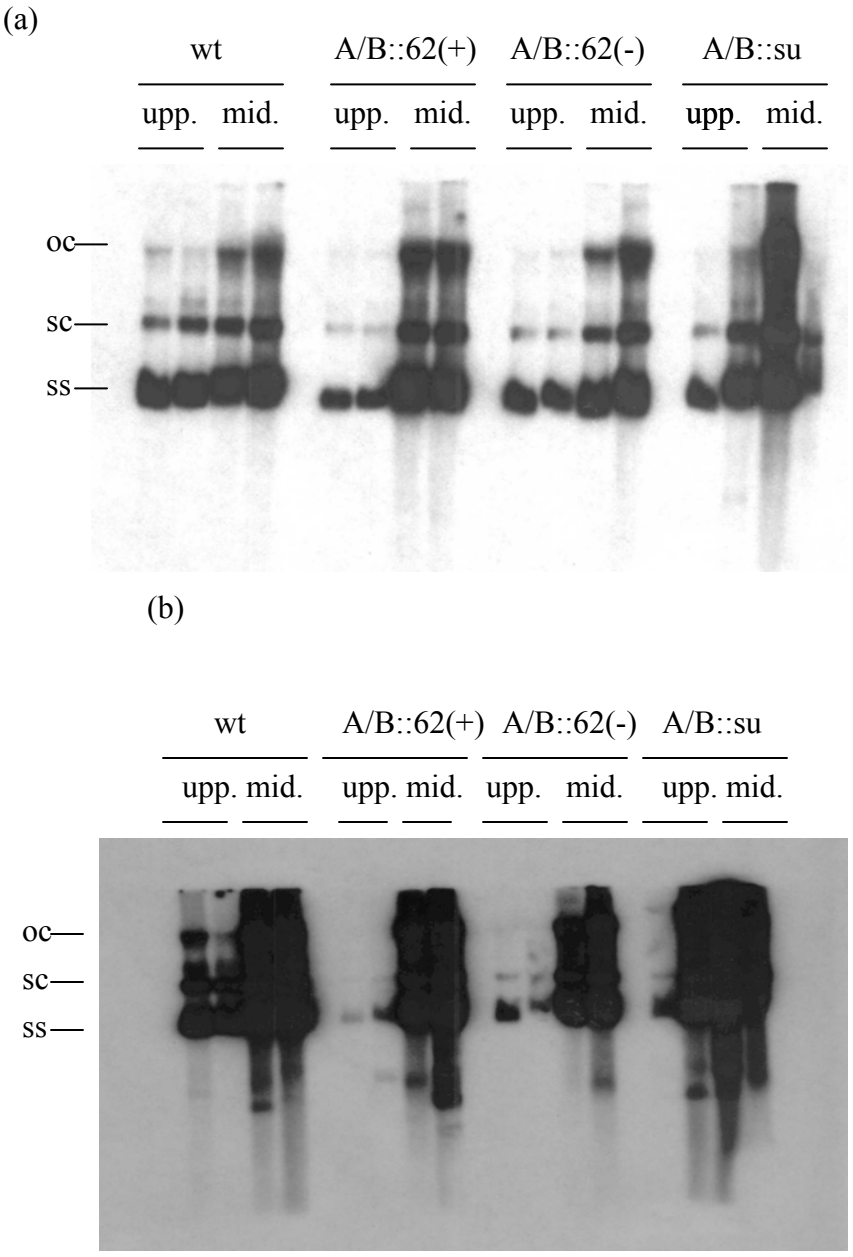

Figure 20. Viral DNA accumulation was reduced in *N. benthamiana* plants inoculated with TGMV A/B::62 compared to wild-type TGMV or TGMVA/B::su. Total nucleic acids were extracted at 21 dpi from pairs of plants which had been inoculated with plasmids containing TGMV-based DNA B silencing vectors

TGMV/B::62 (+), TGMV/B::62(-), TGMV/B::su, or wild-type TGMV B, together with the plasmid containing wild-type TGMV A. Equal amounts of total nucleic acids (2.5 μg) from leaves with strongest symptoms in the middle of the plant (mid.), and from upper leaves showing various degrees of recovery (upp.) were resolved by agarose gel electrophoresis and Southern blotted. (a) Accumulation of TGMV DNA A. (b) Accumulation of TGMV DNA B. The probe for TGMV DNA A was a 182-bp *Nco*I-*Sal*I fragment from the *AL1* gene of TGMV (Figure 19). The probe for TGMV DNA B was a 345-bp PCR product from the 5'-flanking region of the TGMV *BL1* gene (Figure 19). The positions of viral open-circular (oc) and supercoiled dsDNA forms and single stranded DNA (ss) are indicated on the left. Comparisons can be made between gene silencing vectors and wild-type controls within each panel, but the panels cannot be compared directly because the probes have different specificities and the exposure time is different.

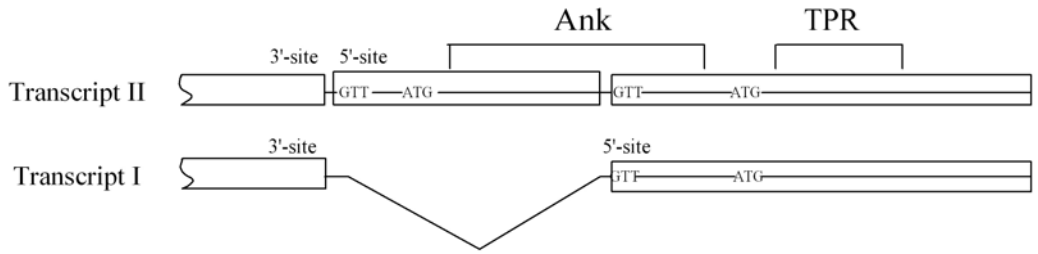

Figure 21. Alternative splicing hypothesis that transcripts I and II might be two alternatively spliced transcripts from a common pre-mRNA. The hypothetical, identical 5'-end sequences, which were truncated during cloning procedures, are shown as curved boxes. Two identical 5'-end splice sites (GTT) in different downstream locations compete for the same upstream 3'-splice site. If the 3'-splice site joins to the closest GTT, transcript II is formed. In some cases, the more distant 5'-splice site is chosen and thus transcript I is formed. The locations of ankyrin-repeats (Ank) and the tetratricopeptide region (TPR) encoded by Nb#62 are also shown.

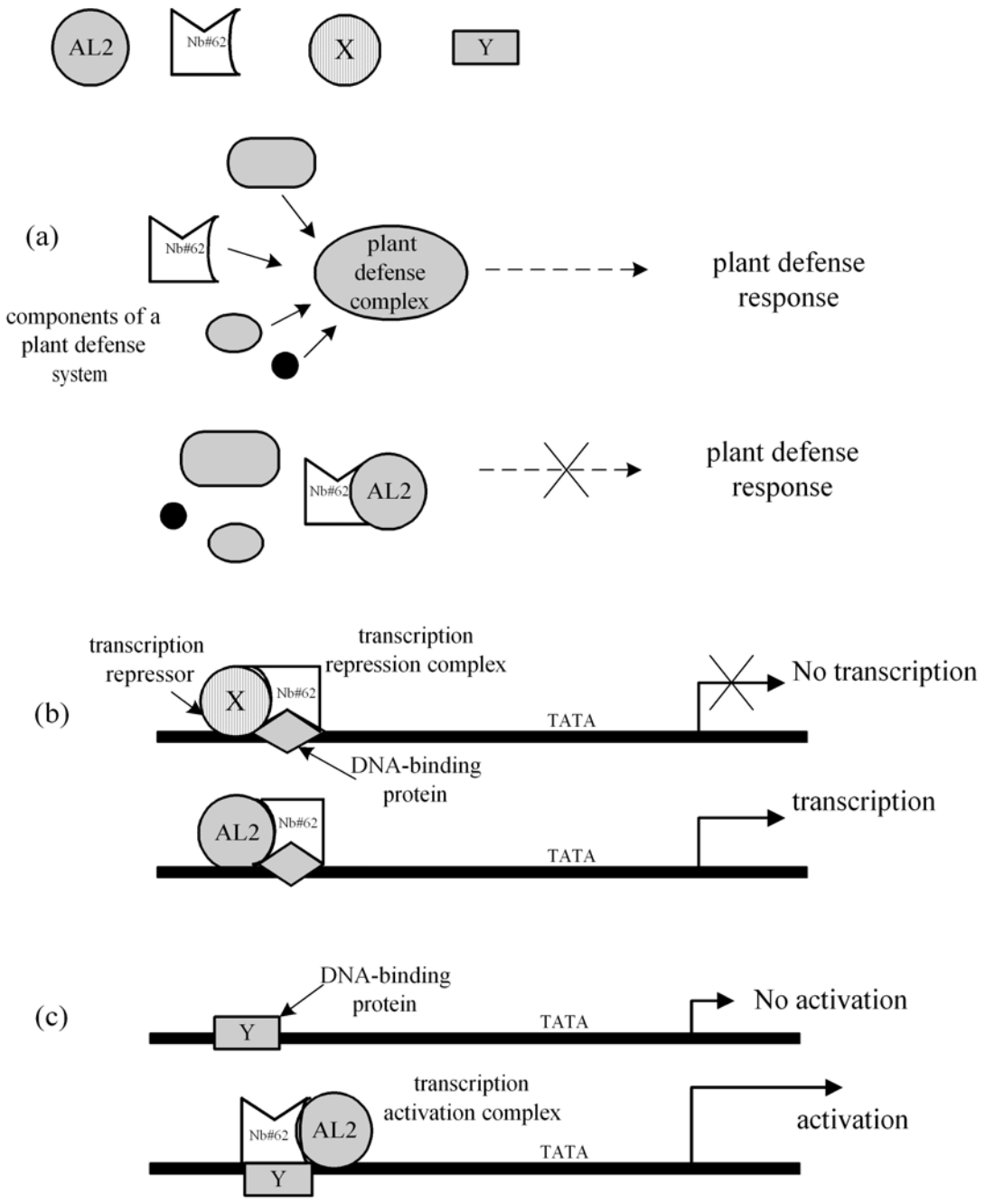

Figure 22. Possible models for roles of interactions between Nb#62 and TGMV AL2 protein. (a) TGMV AL2 protein could compete with components of a plant defense system for binding to Nb#62 and consequently interfere with the plant defense responses. (b) Nb#62 could be an adaptor protein in a cellular transcription repression complex.

TGMV AL2 protein could interact with Nb#62, to block binding of a cellular transcription repressor X, and so de-repress the promoter. (c) Nb#62 could act as an adaptor protein to form a complex between a promoter-specific DNA-binding protein Y and TGMV AL2 protein, leading to activation of the promoter by AL2.

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APPENDICES

APPENDIX A: Evaluation of bean golden mosaic virus DNA B as a vector to trigger virus-induced gene silencing in *Nicotiana benthamiana*

Virus-induced gene silencing (VIGS) has become a new tool for plant genomics research. Homology-dependent gene silencing triggered by viruses carrying a fragment of a target gene could mimic 'knockout' phenotypes to study gene function in the host plant. Tomato golden mosaic virus (TGMV) has been developed as an effective VIGS vector for silencing endogenous genes in *N. benthamiana* plants (Kjemtrup *et al*., 1998; Peele *et al.,* 2001). However, using TGMV as a VIGS vector has limitations in determining the phenotype of silencing, especially when the VIGS effect will be directed not only against the target gene, but also against viral gene expression. Also the symptoms caused by TGMV infection in *N. benthamiana* plants could mask the phenotype caused by the silencing of the target gene.

Bean golden mosaic virus (BGMV) is also a member of the genus *Begomovirus*. Several features of BGMV make it an attractive candidate for a VIGS vector in *N. benthamiana* plants. First, unlike TGMV, which establishes a severe systemic infection in *N. benthamiana* plants, wild-type BGMV doesn't cause symptoms in infected leaves. Therefore, any observed phenotype would be caused by the silencing of the target gene. Second, BGMV does not interfere with TGMV infection. Therefore, it should be possible to elicit silencing of the target gene by BGMV and then superimpose TGMV infection to elucidate any effect of the target gene silencing on TGMV infection. Finally, BGMV is closely related to TGMV genetically, so the methodology for construction of TGMV DNA B as a VIGS vector might also be applicable to BGMV DNA B.

To test the feasibility of using the BGMV B component as a silencing vector, a 95-bp fragment of the tobacco *Sulfur* cDNA (*su*) was cloned into a BGMV B vector at an *Xba*I site downstream from the *BR1* gene (Figure A1) in either the sense or antisense orientation. The resulting plasmids containing BGMV B::su (+), BGMVB::su (-) or the wild-type BGMV DNA B were bombarded into *N. benthamiana* plants, together with the wild-type BGMV DNA A. Mock inoculation by bombardment with gold particles alone was also included.

Silencing of the *Sulfur* gene would cause the typical phenotype of white leaves (Kjemtrup *et al*., 1998). However, none of the 12 plants inoculated with test constructs showed any visible signs of silencing. At 21 days post-inoculation (dpi), total nucleic acids were extracted from both the inoculated and upper leaves. Nucleic acid samples were analyzed by PCR to detect the presence of viral DNA. As shown in Table A1, over a series of three experiments, BGMV DNA A was readily detected from all plants (6/6) inoculated with either BGMV A/B::su(+) or BGMV A/B::su(-), in both the inoculated leaves and upper leaves. However, BGMV DNA B was detected only once, in the inoculated leaves of one plant infected with BGMV A/B::su(+). It could not be detected in the upper leaves of any plants inoculated with BGMV A/B::su(+) or BGMV A/B::su(-). In contrast, both DNA A and B were detected in the inoculated and systemically infected leaves of all plants (6/6) inoculated with wild-type BGMV.

The absence of detectable DNA B in upper leaves of all the plants inoculated with test constructs showed that BGMV B::su DNA was unable to move systemically. In contrast, BGMV A was able to move systemically in the co-inoculated plant. Previous studies have shown that BGMV A cannot move systemically in the absence of movement

protein expression from DNA B. However, replication or movement of co-inoculated DNA B is not required (Petty *et al*., 1995). This suggests that movement protein expression from BGMV B::su constructs was unimpaired, but either DNA replication or systemic movement might be affected *in cis*. The suggestion that *BR1* expression may be occurring normally in plants inoculated with BGMV B::su would also be consistent with the lack of any apparent silencing of the *Sulfur* gene in these experiments. It's not clear why insertion of a 95-bp DNA fragment into the *Xba*I site of BGMV B would affect DNA replication, since the *cis*-acting sequence known to be important are all located in the common region (Fontes *et al*., 1994; see Figure A1). On the other hand, it has been shown for TGMV that there is a limit to the size of insertions that can be made without interfering with the ability of the DNA to move from cell to cell (Peele *et al*., 2001). Although the existence of a similar size limit on the movement of BGMV DNA B is likely, this has not been investigated previously.

In either case, silencing of the *Sulfur* gene would not be achieved, since studies on TGMV-based VIGS have shown that the initiation and maintenance of the endogenous gene silencing require viral replication and movement (Peele *et al*., 2001).

To distinguish between these explanations for the failure of BGMV B::su to accumulate in upper leaves, lack of replication versus lack of movement, protoplast inoculations could be performed (Fontes *et al*., 1994). This would allow DNA replication to be assayed independently from movement, or movement protein gene expression.

In conclusion, the results suggested that BGMV DNA B could not effectively trigger gene silencing in *N. benthamiana* plants.

Table A1. PCR analysis of BGMV DNA components in *N. benthamiana* plants inoculated with BGMV-based silencing constructs

Inoculum	Silencing of *su* gene[b]	BGMV DNA A detected[c]		BGMV DNA B detected[c]	
		inoc.	upper	inoc.	upper
Wild-type BGMV A/B	Not applicable	3/3	3/3	3/3	3/3
BGMV A/B::su (+)	0/6	3/3	3/3	1/3	0/3
BGMV A/B::su (-)	0/6	3/3	3/3	0/3	0/3

a. Inoculum included 5 μg of a plasmid containing BGMV B::su (+), BGMV B::su(-) or the wild type BGMV B, together with 5 μg of a plasmid containing the wild type BGMV DNA A. They were inoculated into *N. benthamiana* plants by microprojectile bombardment.

b. Number of plants in which silencing of *su* was triggered /number of the plants inoculated; determined by visual inspection for loss of chlorophyll which is characteristic for silencing of the tobacco *Sulfur* gene.

c. Number of plants in which viral DNA was detected by PCR/number of the plants analyzed. Samples tested by PCR were obtained from both directly inoculated leaves (inoc.) and upper leaves (upper), to which viral DNA must move systemically.

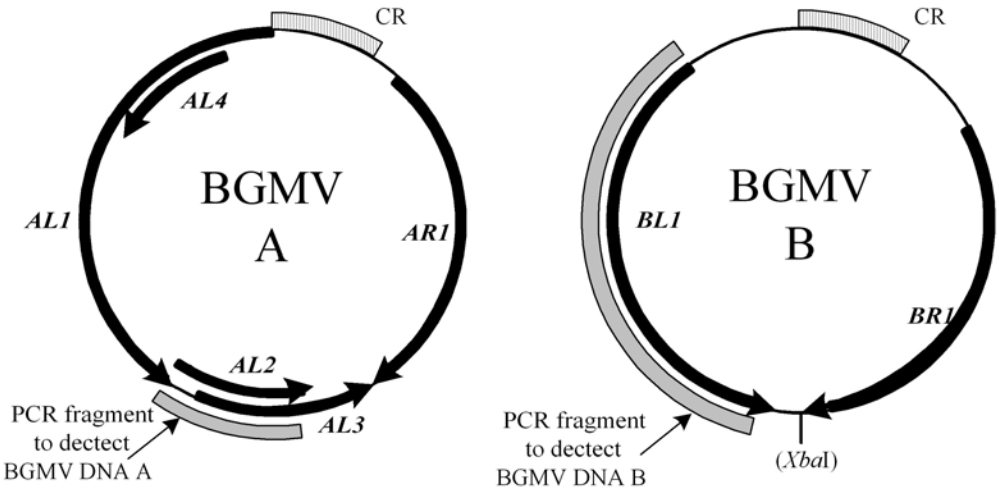

Figure A1. Illustration of BGMV genome components. The common region (CR) that is nearly identical in both DNA components is indicated by hatched boxes. The *Xba*I site shown in the figure was used for cloning of silencing fragments. It is shown parenthetically to indicate that it was artificially introduced for cloning. The locations of the PCR fragments used to detect viral DNA are indicated by gray boxes.

APPENDIX B: The nucleotide sequence data of cDNAs Nb#51 and Nb#62

cDNA Nb#51

ACGAGGGTTAATGCTCAAACAGAAGAAGATATCTGTCCTTTGGTATCAGCTGTTGCAGCCAAT
TCATTGCCTTGCGTGGAACTGTTAGTAAAGGCAGGAGCTGATGTCAATGTTAGGGCTGGTGAA
GCAACCCCTTTGCTCATTGCTGCTCATAATGGTAGTGCAGAAATTGTTAAGTGCTTACTACAA
GCTGGAGCTGATCCTAATGCCACTGATGAGGATGGTAACAAGCCAATACATGTTGCAGCTACA
AGGGGTTGTCGGCCGTCTGTTGAAGCTTTGTTTGCAGTCACACCGCGGATTCAGTGTGTTCCA
GAATGGAGTGTGGATGGACTCATTAAGTTTATGCAATCTGAGCCTATCCTATCTGAAACTAGG
ATAAACCAGGAAAGAACAGAAGCTGGGAGGGAACCAAGTAAGAGAGAAAGTATTATTCCAA
AGAAAGATTTACCCGAGGTGACTCCTGAAGCAAAGAAGAAAGCTGCAGATGCGAAGGTGAG
GGGAGATGAGGCATTTAAGAGGAAGGATTACGCTACGGCTGTGGATGCTTATACACAGGCAA
TCGATTTTGATCCAACTGATGGCATTCTGTTTTCGAATAGAAGTCTTTGTTGGATCCGCTTGGG
GCAAGCTGAACATGCTTTAAGTGATGCCAAGGCTTGCAGACAACTTAGACCAGATTGGGCAA
AAGCTTGTTTTCGGGAAGGGGCAGCTCTACGGCTATTGCAGAGATTTGAAGACGCAGCCAATG
CTTTCTATGAGGGCGTACAGATCGACCCTGAAAATATGGAGCTCGCAACTGCTTTCAGGGAAG
CTGTTGAGGCTGGTAGAGAATTTCATGGCAAGAATAAGAAAAATTCTCAGTCATCCAGGAGTT
AGTGGTGTTAAGAAGTGCATAATGACAGGACTTCTGCTGGAGCAAGTAGATAATTTTATCGGA
CATGCCCTTTTTTGGTAGTTTCAGAATGTATCATGTGATGTTTTGCCCTCTAGTGATTTGTTCTG
TTGCATCTTCAGAAATTTATACTCACAATATAATTTCTTTTTTCGGTTAAGGGTCAAATATATC
CATGTACTATTCAAAATAAGCCATATTTATCCTCCGTTTGAATTCTTTAAAAAAAAAAAAAAA

cDNA Nb#62

ACGAGGGTTCTACATAAACCCCAGAAGCTTCTGCCGAAGTCCAAAGTTTTCTCAGTTATGGCT
CCTGATGCTGCTGATGCTCTTGCAGTTAGAGAAAAGGTTATGAAGTTACTGAATGCTGCTTGT
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