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Regulation of *Tomato golden mosaic virus AL2* and *AL3* gene expression by a conserved upstream open reading frame

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ABSTRACT

A translational regulatory mechanism for *Tomato golden mosaic virus* (TGMV) complementary-sense gene expression has been characterized. TGMV transcribes two mRNAs, AL-1935 and AL-1629 transcripts, both of which contain the AL2 and AL3 open reading frames. However, AL2 is only expressed from AL-1629 whereas AL3 is expressed from both. Three AUG translation initiation codons are located upstream of both the AL2 and AL3 coding regions, within the 5′-untranslated region (UTR) of the AL-1935 transcript. Translation can initiate at the first AUG, specifying the C-terminal 122 amino acids of the AL1 protein (cAL1). Initiation of translation at this AUG is inhibitory for the downstream expression of both AL2 and AL3. This is most likely due to the terminator codon of cAL1 being positioned after the AUG initiation codon for the AL2 ORF. The mechanism by which AL3 is expressed from AL-1935 is currently unknown but a gap between the cAL1 termination codon and the start of AL3 suggests that it may involve reinitiation and/or internal initiation. In contrast, expression of AL3 from AL-1629 most likely occurs via leaky ribosome scanning since the AL3 initiation codon occurs before the terminator codon of AL2. Mutation of the AUG encoding cAL1 in the curtovirus, *Spinach curly top virus*, leads to increased infectivity as measured by a shorter latent period. Together this suggests that geminiviruses use a post-translational regulatory mechanism to regulate the synthesis of viral proteins important for replication and suppression of host defenses.

Introduction

The bipartite genome of *Tomato golden mosaic virus* (TGMV), a begomovirus, consists of two DNA components, A and B, of approximately 2.6 kb (Hamilton et al., 1984). Replication of the single stranded DNA genome occurs in the nuclei of infected plant cells producing double-stranded (ds) DNA replicative form (RF) intermediates (Stenger et al., 1991). Virus-specific mRNAs are transcribed from RF DNA in a bidirectional manner from an approximately 230 nt region, common to both DNA components (Fig. 1A) (Fontes et al., 1994; Hanley-Bowdoin et al., 1990; Petty et al., 1988; Sunter and Bisaro, 1989; Sunter et al., 1989, 1993). Viral RNAs are polyadenylated and initiate downstream of either consensus TATA box or initiator elements indicative of transcription mediated by host RNA polymerase II. It is unknown at this time whether TGMV transcripts are capped at the 5′-end. A single virion-sense RNA is transcribed from TGMV DNA A initiating at nt 319 (AR-319), providing the template for translation of CP (Sunter et al., 1989). Complementary-sense transcription is more complex, producing multiple overlapping RNAs with different 5′-ends but a common 3′-end. The largest transcript initiates at nt 62 (AL-62), encodes the entire left side of TGMV A (Sunter et al., 1989), and is the only transcript that can encode full-length AL1 protein. However, AL-62 also has the potential to code for the AL2, AL3 and AL4 proteins.

Three additional complementary sense transcripts initiate at nt 2548, 2540, and 2515 (Hanley-Bowdoin et al., 1990; Sunter et al., 1989), downstream of the AL1 translation initiation codon. These could potentially serve as mRNAs for the AL2, AL3 and AL4 proteins. Two smaller RNAs, initiating at nt 1935 and 1629 (AL-1935 and AL-1629), specify both AL2 and AL3 ORFs (Fig. 1A). Transcription of functional polycistronic viral mRNAs is supported by data demonstrating that an RNA template similar to AL-62 can be used to translate the AL1, AL2, AL3 and AL4 proteins *in vitro* (Thömmes and Buck, 1994). However, transgenic tobacco plants expressing RNA encoding all four ORFs is capable of complementing mutant TGMV *al1* and *al3* viruses in 100% of cases, but these plants only complemented TGMV *al2* mutant virus at a very low percentage (0% to 25%) (Hanley-Bowdoin et al., 1989). Also, recent results have shown that AL2 is only expressed from AL-1629, whereas AL3 is expressed from both AL-1935 and AL-1629 (Shung et al., 2006). This might suggest post-transcriptional mechanisms that influence expression of viral proteins from polycistronic viral mRNAs.

Viruses utilize the host translation machinery to translate viral proteins and have evolved numerous strategies to efficiently and

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preferentially amplify their genome in the host cell. These strategies include internal ribosome entry (IRES), leaky scanning, and ribosome reinitiation (for review see Dreher and Miller, 2006; Gale et al., 2000). IRES mediated translation is cap-independent and is a common translation strategy among animal and plant positive-sense RNA viruses. *Picornaviridae*, such as poliovirus (PV), human rhinoviruses (HRV), hepatitis A virus (HAV) and foot- and mouth disease virus (FMDV), and *Potyviridae*, such as *Tobacco etch virus, Potato virus Y* and *Plum pox virus*, have a viral protein linked to the 5′-end of the RNA genome (Kneller et al., 2006). A highly significant secondary structure at the 5′-UTR can facilitate ribosome entry independent of the cap structure. In plant viruses, the 5′-UTR region is generally around 150 nt as compared to 600–1200 nt in picornaviruses and is also less structured (Dreher and Miller, 2006; Jang, 2006). PV, HRV, and FMDV also encode eIF4G-cleaving proteases to inhibit cap-dependent translation and allow almost exclusive translation of viral proteins (Jang, 2006). Leaky ribosome scanning permits synthesis of two proteins from a single mRNA when the context surrounding the first AUG codon is unfavorable (Kozak, 1991). Ribosomes that fail to initiate at these sites continue to scan for an appropriate initiation site (Dreher and Miller, 2006). Leaking scanning can be revealed when expression of the downstream ORF is blocked either by optimization of the upstream initiation site, as shown for *Barley stripe mosaic virus* (Zhou and Jackson, 1996) and *Tomato bushy stunt virus* (Scholthof et al., 1999) or by insertion of a new upstream AUG in a strong context, as shown for *Potato virus X* (Verchot et al., 1998). In animal viruses, including adenoviruses, Influenza B viruses and SV40, leaky scanning is also a common mechanism for synthesis of viral proteins (Kozak, 1991).

After translation of the first upstream open reading frame (uORF), ribosomes may resume scanning, and reinitiate at a downstream AUG codon (Kozak, 2001). The ability of eukaryotic ribosomes to reinitiate is limited by the size of the uORF (Kozak, 2001). Whether or not uORFs play a role in leaky scanning and/or in reinitiation (Wang and Rothnagel, 2004) they are sometimes employed to reduce the translation efficiency of a downstream ORF (Kozak, 2001). There is growing evidence that cytokines, proto-oncogenes, and transcription factors use uORFs to limit protein synthesis (Lee et al., 2002; Sarrazin et al., 2000; Stockklausner et al., 2006; Vattem and Wek, 2004; Wiese et al., 2004). Some viruses also use uORFs to regulate their own protein expression, such as hepatitis B (core and polymerase), and human cytomegalovirus (gpUL4) (Alderete et al., 1999; Chen et al., 2005). These proteins are only needed in small quantities and could potentially be harmful to the cell if overproduced.

Whether geminiviruses utilize one or more of these translational regulatory mechanisms has yet to be extensively studied, and some questions remain unanswered. In this paper, we continue and extend our previous studies and examine the role of post-transcriptional regulation in the expression of geminivirus AL2 and AL3. We describe a novel regulatory role for an upstream open reading frame in TGMV and discuss the relevance of this post-transcriptional regulation to the viral life cycle.

Results

The TGMV AL-1935 transcript contains a previously undescribed open reading frame conserved among begomo- and curtoviruses

Our previous data demonstrates that AL2 is translated from AL-1629, but not from AL-1935 (Shung et al., 2006). The AL2 coding region was presumed to be the first ORF in both AL-1935 and AL-1629 (Shung et al., 2006; Sunter and Bisaro, 1989). Upon closer examination of the sequence arrangement of the AL-1935 transcript, we identified a putative ORF that encodes the C-terminal 122 amino acids of the AL1 protein, which we will refer to as cAL1 (Fig. 1B). In addition to this putative ORF, there are two additional AUGs, adjacent to each other, contained within the cAL1 ORF and upstream of AL2 (Fig. 1B). These AUGs could potentially express a short peptide, up to eight amino acids long. In contrast, the AL2 translation initiation codon is the first AUG in the 5′-UTR of AL-1629 (Fig. 1B). To determine whether the AUG codons within AL-1935 are conserved among begomo- and curtoviruses, we analyzed comparable sequences from twenty geminiviruses. These included nine New World (North and South America) and nine Old World (Africa and Asia) begomoviruses, and two curtoviruses, *Beet curly top virus* (BCTV) and *Spinach curly top virus* (SCTV), chosen as representative examples to illustrate conservation. Comparison of viral sequences suggests that the putative cAL1 AUG is highly conserved among the twenty viruses (20/20) (see Fig. 1 in Supplementary data). The second AUG is also highly conserved (19/20), with the third AUG least conserved (2/20).

Translation can initiate at the cAL1 AUG

As the cAL1 AUG is highly conserved in begomo- and curtoviruses, we tested whether the cAL1 AUG can be used to initiate translation. It has been previously shown that the AL-1935 transcript is present at very low levels (Shung et al., 2006; Sunter and Bisaro, 1989) and attempts to use the native viral promoter resulted in expression levels too low to consistently detect differences. Therefore, we generated two DNA constructs in which the cAL1 ORF was linked to the *GUS* reporter gene in a translational fusion, and expressed from the *Cauliflower mosaic virus* (CaMV) 35S promoter. In one construct, 35S[cAL1/GUS], the GUS ORF is fused to the N-terminal 15 amino acids of the cAL1 ORF. In the second construct, 35S[cAL1][GUS], the GUS ORF is in a different reading frame, such that the DNA contains two overlapping ORFs, cAL1 and GUS (Fig. 2A). As can be seen (Fig. 2B), GUS activity detected in extracts from protoplasts transfected with 35S[cAL1/GUS] DNA is eight-fold higher (Student's *t*-test: $P<0.05$) than that detected in extracts from protoplasts transfected with 35S[cAL1][GUS]. GUS activity from the latter construct is approximately eight-fold higher than background levels in protoplasts transfected with DNA lacking the *GUS* gene (data not shown). This data indicates that the AUG codon for TGMV cAL1 can direct expression of a cAL1/GUS fusion protein, and can inhibit expression from a second AUG codon present in a different reading frame.

AUG codons within the 5′ UTR of AL-1935 play an inhibitory role in the expression of TGMV AL2 and AL3

Having demonstrated that the AUG codon for cAL1 can inhibit GUS expression if the GUS coding sequence is in a different reading frame, we tested whether the three AUG codons present within the 5′ UTR of AL-1935 affect AL2 and AL3 expression (Fig. 3). Promoter–reporter constructs were generated containing the 5′-UTR of TGMV AL-1935 linked to GUS in a translational fusion to either the AL2 or AL3 coding regions. For the same reasons outlined above, expression of GUS was directed using the CaMV 35S promoter. Constructs contained either, wild type AUGs (wtAUGs), a single mutation in the AUG for cAL1 (mcAL1), or mutations in all three AUGs (mAUGs) within the 5'-UTR (Fig. 3A). GUS activity detected in extracts from protoplasts transfected with 35S-mcAL1[AL2/GUS] is approximately two-fold higher than activity in extracts from protoplasts transfected with 35S-wtAUGs[AL2/GUS] (Fig. 3B: Student's *t* test, $P<0.05$). No further increase in GUS activity was detected when all three AUGs were mutated (35S-mAUGs[AL2/GUS]). Similar results were observed using promoter–reporter constructs containing an AL3/GUS fusion. Mutation of the AUG for cAL1 (35S-mcAL1[AL3/GUS]) resulted in an approximate five to six-fold increase in GUS activity as compared to wild type (Fig. 3B, Student's *t* test, $P<0.05$). As observed for AL2/GUS constructs no further increase in GUS activity was detected when mutations were introduced into all three AUG codons (35S-mAUGs [AL3/GUS]).

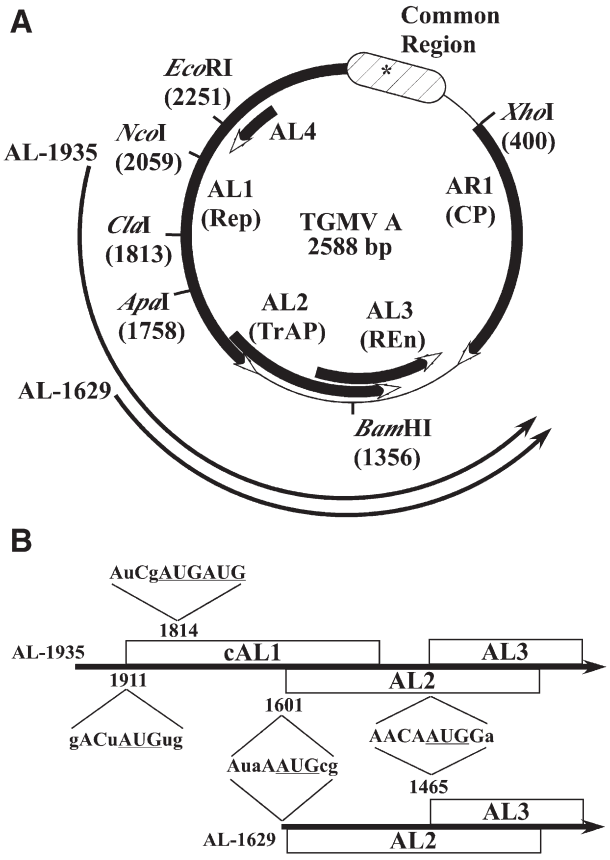

Fig. 1. Organization of complementary sense viral transcripts derived from TGMV DNA A. (A) The circular map illustrates wild type TGMV DNA A with relevant restriction sites and nucleotide coordinates (Hamilton et al., 1984). Open-ended arrows designate coding regions and the two outer arrows indicate the AL-1935 and AL-1629 transcripts and direction of transcription. The origin of plus strand replication is indicated (asterisk) within the ~230 bp common region. (B) The linear maps represent the TGMV AL-1935 and AL-1629 transcripts with the C-terminal region of AL1 (cAL1), AL2 and AL3 ORFs indicated by open boxes. Sequences surrounding AUG codons within the 5′ UTRs of AL-1935 and AL-1629 are shown with nucleotide coordinates (Hamilton et al., 1984). Upper case letters indicate nucleotides identical to the consensus ribosome binding sequence for plants (Joshi et al., 1997), with the AUG start codon underlined.

Mutation of the cAL1 AUG codon has little effect on viral replication or autorepression

Results demonstrating that an AUG codon in the 5'-UTR of TGMV AL-1935 mRNA can initiate translation of a putative upstream ORF (cAL1) led us to test whether mutation of this AUG affected viral pathogenicity. A mutation of the cAL1 AUG codon was introduced into TGMV DNA A, and the ability of a TGMV *cal1* mutant genome to direct replication was analyzed in protoplasts. Replicating viral DNA was detected in protoplasts transfected with either wild type (Fig. 4A, lane 2) or mutant (Fig. 4A, lane 1) DNAs derived from TGMV. The amount of replicating *cal1* mutant DNA was approximately equal to the corresponding wild type DNA, as estimated by phosphorimager. As cAL1 is also part of the AL1 ORF we also tested whether the mutation affected the ability of AL1 to repress its own promoter (Eagle et al., 1994; Sunter et al., 1993). Protoplasts were co-transfected with DNA containing the *GUS* reporter gene fused to the *AL62* promoter (AL62-GUS) (Sunter et al., 1993), and DNA capable of expressing wild type AL1 (35S-AL1) or DNA containing a full-length AL1 ORF with the cAL1 AUG mutation (35S-m*al1*) from the CaMV 35S promoter. GUS activity detected in extracts isolated from protoplasts co-transfected with AL62-GUS and 35S-AL1 is significantly decreased, approximately 10-fold, as compared to the control (AL62-GUS+pUC19; Fig. 4B), consistent with previous data (Sunter et al., 1993). GUS activity detected in extracts isolated from protoplasts co-transfected with AL62-GUS and 35S-m*al1* was also reduced to the same degree (Fig. 4B). This demonstrates that the cAL1 AUG mutation does not affect the ability of full-length AL1 protein to repress its own promoter. Similar levels of complementary sense viral transcripts were detected in *Nicotiana benthamiana* plants infected with either wild type or *cal1* mutant viruses, confirming that the cAL1 AUG mutation does not impact viral transcription (data not shown).

Mutation of cAL1 affects viral pathogenicity in spinach

To examine the biological relevance of cAL1 to geminivirus infection, we tested whether introduction of the cAL1 AUG mutation influenced the infectivity of TGMV. *N. benthamiana* plants were agroinoculated with wild type TGMV A or mutant *cal1* TGMV A, in the presence of TGMV DNA B. Symptoms detected on *N. benthamiana* plants inoculated with TGMV *cal1* mutant virus were indistinguishable from those induced by wild type virus (data not shown). The same percentage of plants inoculated exhibited symptoms and symptoms appeared at the same time post-inoculation (Table 1) using an *Agrobacterium* culture at two different doses (OD_{600}=0.5 and 0.1). This suggests that the mutation in the TGMV cAL1 ORF has little effect on infectivity in *N. benthamiana* plants. As TGMV infectivity is limited to solanaceous hosts, we used a second virus, SCTV, to extend our observations to non-solanaceous hosts. As observed for TGMV, introduction of the *cal1* mutation had no apparent effect on infectivity of SCTV in *N. benthamiana* or in *Arabidopsis* (Table 1) and no difference in mean latent period or ID_{50} (Sunter et al., 2001) was observed (data not shown). In contrast, spinach plants proved more susceptible to infection with SCTV *cal1* mutant virus than wild-type virus as judged

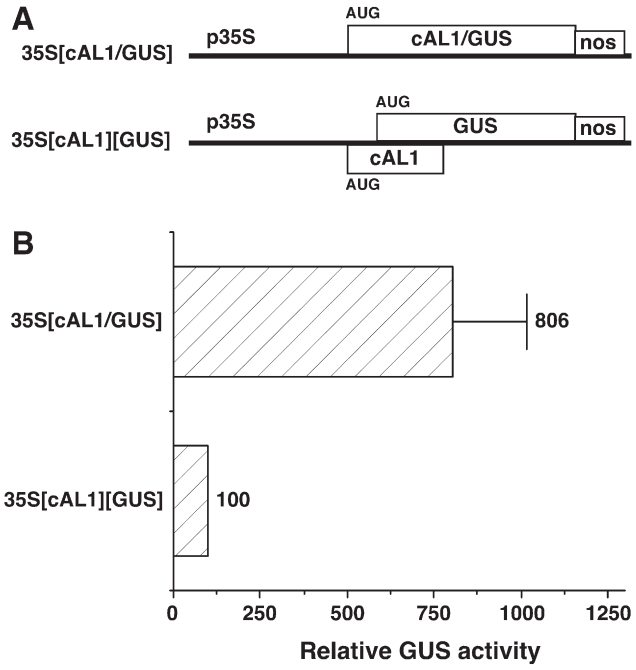

Fig. 2. The cAL1 AUG can initiate translation in *N. benthamiana* protoplasts. (A) The linear maps represent promoter–reporter constructs containing the GUS coding sequence fused either in-frame (35S[cAL1/GUS]), or out-of-frame (35S[cAL1][GUS]) with the TGMV cAL1 ORF. The CaMV 35S promoter and *nos* termination signal are indicated. (B) The ability of promoter–reporter constructs to direct GUS expression was determined by comparing GUS activity to background (pTGA26; ~6% activity). *N. benthamiana* protoplasts (5×10^5 cells) were transfected with 10 μg of a promoter–reporter construct and significant differences in GUS activity (Student's *t*-test: $P<0.05$) measured in extracts isolated three days post-transfection. Columns represent relative GUS activity as compared to 35S[cAL1][GUS], which was arbitrarily assigned a value 100%. Error bars represent the standard error of the mean from three independent experiments.

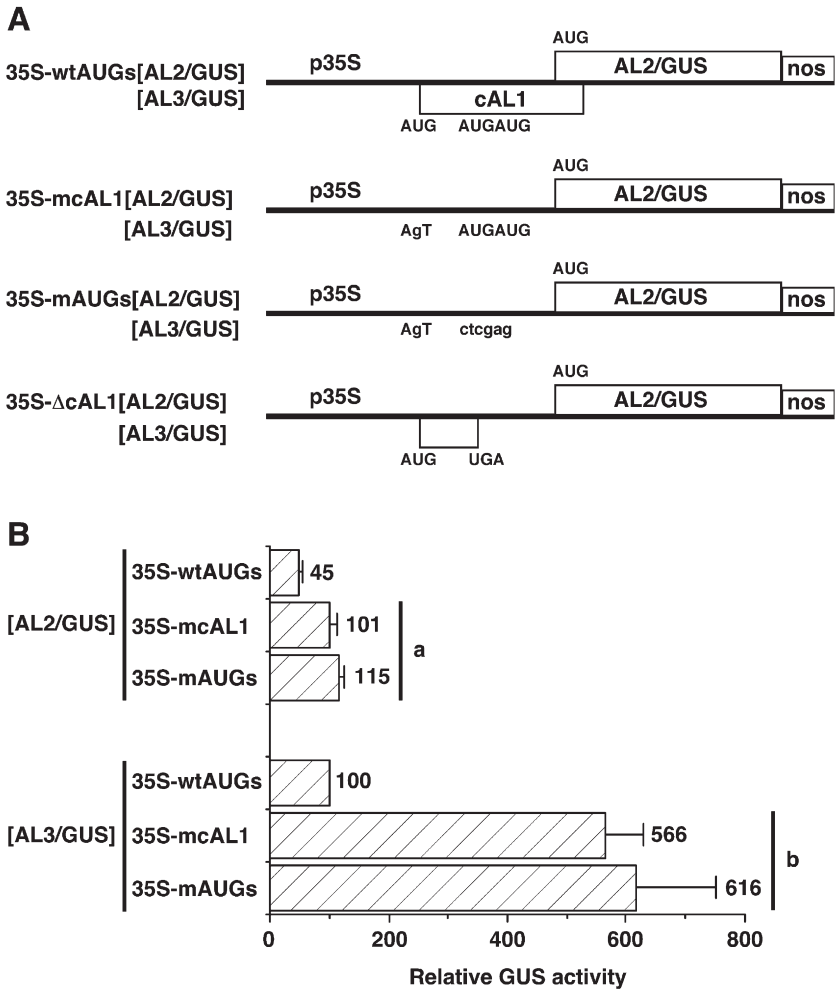

Fig. 3. AUG codons within the 5′-UTR of AL-1935 affect TGMV AL2 and AL3 expression. (A) The linear maps represent promoter–reporter DNAs where GUS is combined with the AL2 or AL3 coding sequence in a translational fusion. Constructs contain wild type 5′-UTR sequences (35S-wtAUGs), or mutations in a single (35S-mcAL1) or multiple (35S-mAUGs) AUG codons within the 5′-UTR. The CaMV 35S promoter, *nos* termination signal and any sequence alterations are shown. (B) The ability of promoter–reporter DNAs to direct GUS expression was determined by comparing GUS activity to background (pTGA26; ~6% activity). *N. benthamiana* protoplasts (5×10^5 cells) were transfected with 10 μg of a promoter–reporter construct and significant differences in GUS activity (Student's *t*-test: $P<0.05$) measured in extracts isolated three days post-transfection. Columns represent relative GUS activity as compared to wild type (35S-wtAUGs), which was arbitrarily assigned a value 100%. Error bars represent the standard error of the mean from three independent experiments. Significant differences in GUS activity were measured by Student's *t*-test.

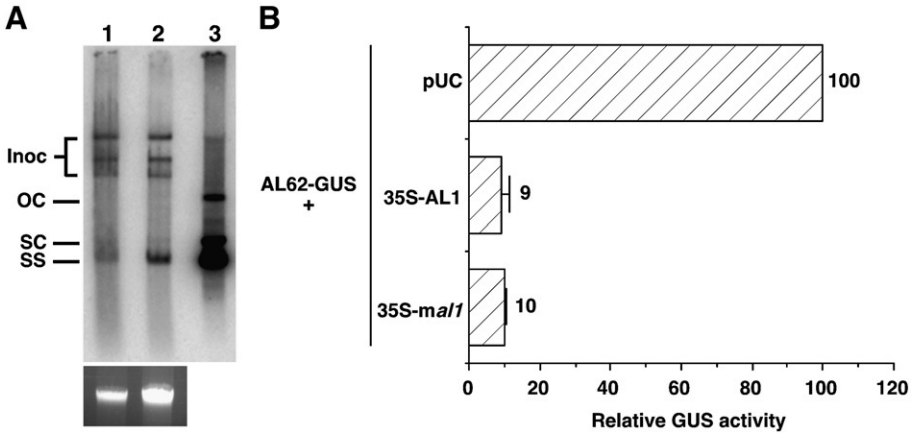

Fig. 4. TGMV cAL1 ORF does not affect AL1 function. (A) The top panel represents an autoradiogram of DNA samples hybridized to a TGMV-specific probe. One representative example of three independent experiments is illustrated. Lane 1, DNA (3 μg) isolated from protoplasts transfected with pGS99 (TGMV *cal1* mutant); Lane 2, DNA (3 μg) isolated from protoplasts transfected with pTGA26 (wild type TGMV); Lane 3, DNA (20 ng) isolated from TGMV-infected plants. The open circular (OC), supercoiled (SC) and single-stranded (SS) DNA forms of TGMV are indicated on the left side of the panel. The lower panel represents an ethidium bromide-stained gel of DNA samples from protoplasts (lanes 1 and 2 only), as a loading control. No genomic DNA was present in the sample isolated from TGMV-infected plants. (B) The ability of promoter–reporter constructs to direct GUS expression was determined by comparing GUS activity to background (pTGA26; ~6% activity). *N. benthamiana* protoplasts (5×10^5 cells) were co-transfected with 10 μg of AL62-GUS (*GUS* reporter gene fused to the *AL-62* promoter), and either pUC, 35S-AL1 (wild type AL1) or 35S-m*al1* (full-length AL1 ORF with the cAL1 AUG mutation) DNA. GUS activity was measured in extracts isolated three days post-transfection. Columns represent percent GUS activity as compared to Al62-GUS + pUC, which was arbitrarily assigned a value 100%. Error bars represent the standard error of the mean from two independent experiments.

Table 1
Infectivity of TGMV and SCTV *cal1* mutant viruses on *N. benthamiana* and *Arabidopsis*

Inoculum[a]	*N. benthamiana*[b]		*Arabidopsis*[b]	
	OD_{600}=0.5	OD_{600}=0.1	OD_{600}=0.5	OD_{600}=0.1
Wild type TGMV	10/12 (7 days)	18/18 (8 days)	ND	ND
TGMV *cal1* mutant	12/18 (8 days)	16/17 (8 days)	ND	ND
Wild type SCTV	15/18 (7 days)	16/18 (7 days)	11/12 (8 days)	8/18 (9 days)
SCTV *cal1* mutant	14/16 (7 days)	16/18 (6 days)	18/18 (8 days)	9/18 (11 days)

[a] Inoculum (as OD determined by spectrophotometer at 600 nm) consisted of a mixture of *Agrobacterium* containing Ti plasmids harboring 1.5 copies of wild type or mutant TGMV A with wild type TGMV B, or wild type or mutant SCTV.
[b] Values represent the numbers of plants exhibiting symptoms typical of a TGMV infection compared to the total number of plants inoculated. The numbers in parentheses indicate the number of days post-inoculation for symptoms to first appear.

by a reduction in the time to first appearance of symptoms (mean latent period). Under the conditions of the test *cal1* mutant virus showed an intrinsic mean latent period of 16.3 days (Table 2). In contrast, the latent period for wild type SCTV was 18.2 days. Thus, plants inoculated with *cal1* mutant SCTV exhibited symptoms approximately two days earlier than plants inoculated with wild type SCTV. Analysis of the data by one-way analysis of variance (ANOVA) and Dunnett's test confirmed that the reductions were statistically significant.

Attempts to further characterize the phenotype by assessing the ID_{50} for mutant and wild type virus (Sunter et al., 2001) proved difficult as less than 50% of plants became infected at the doses used, even an OD_{600} of 0.5. The use of inoculum doses above an OD_{600} of 0.5 resulted in significant numbers of plants becoming necrotic (Baliji et al., 2004), making it difficult to differentiate symptoms from damage due to the inoculation procedure. Despite this, it can be seen that *cal1* mutant SCTV infected 30–36% of spinach plants, at all inoculum doses. In contrast, wild type SCTV infected only 17–30% of plants inoculated. It was concluded from these experiments that mutation of the cAL1 AUG results in increased infectivity, as measured by a decrease in mean latent period and an apparent decrease in the amount of virus inoculum required to infect the plants as compared to wild type virus.

Mutation of cal1 has no effect on virus load

Although spinach plants display a reduced latent period following challenge with *cal1* mutant SCTV there was no evidence of increased disease severity. As this is not a quantitative assessment of virulence we investigated whether there were differences in SCTV accumulation in mutant and wild type infected plants. DNA extracts were obtained from comparable systemically infected leaves of individual, randomly selected plants approximately 14 days after the mean latent period for each virus. The presence of SCTV viral DNA was detected by DNA gel blot hybridization using an SCTV-specific probe (Baliji et al., 2004). Quantitation was performed by phosphorimager analysis and the data were normalized using an internal 18S rDNA control. The accumulation of SCTV genomic ssDNA and dsDNA replicative forms was variable for both wild type and mutant viruses, as expected (see Fig. 2 in Supplementary data). However, no statistical difference in total viral DNA accumulation was detected between wild type and mutant viruses (Student's *t*-test), and well within the limits of normal variation. Some variability in viral nucleic acid levels is to be expected in a small and random sample of infected plants, but the data suggest that increased infectivity of the *cal1* mutant virus is not a consequence of increased virus loads.

Table 2
Mean latent period and infectivity for SCTV inoculation of spinach plants

Inoculum dose (OD_{600})[a]	Mean latent period[b]	Infectivity[c]	
	0.5	0.1	0.02
Wild type SCTV	18.2±0.7 (10/34)	6/36 (17%)	7/36 (19%)
SCTV *cal1* mutant	16.3±0.7 (12/35)	13/36 (36%)	11/36 (31%)

[a] Inoculum dose consisted of a mixture of *Agrobacterium* of differing concentrations (as OD determined by spectrophotometer at 600) containing Ti plasmids harboring 1.5 copies of wild type or mutant SCTV.
[b] Values represent the mean latent period of plants exhibiting symptoms typical of an SCTV infection, calculated using data from plants inoculated with an inoculum dose of OD_{600}=0.5. The significance of mean latent period differences observed (mean latent period±SE) was confirmed by one-way ANOVA ($P<0.05$) followed by Dunnett's test. Numbers in parentheses indicate infectivity (number of plants infected/number plants inoculated).
[c] Values represent the numbers of plants exhibiting symptoms typical of a TGMV infection compared to the total number of plants inoculated. The percentage of plants infected is given in parentheses.

AL3 expression from AL-1629 via context-dependent leaky scanning

In contrast to AL-1935, both AL2 and AL3 can be expressed from the bicistronic AL-1629 mRNA (Shung et al., 2006). Examination of the short (20 nt) 5′-UTR of AL-1629 reveals that the AL2 translation initiation codon is the first AUG. Based on the unfavorable ribosome binding sequence (RBS) (Joshi et al., 1997; Kozak, 1984, 1999) surrounding this AUG, we hypothesized that AL3 is translated from AL-1629 via leaky ribosome scanning. DNA constructs were generated containing 210 nt upstream of the transcription initiation site for TGMV AL-1629 linked to GUS in a translational fusion to the AL2 or AL3 coding region. This fragment of the TGMV genome contains the promoter for expression of the AL-1629 transcript (Shung et al., 2006; Tu and Sunter, 2007). DNAs contained either the wild type AL2 RBS (AL1629-wtAL2[AL2/GUS]) or mutant sequences more closely matching the optimal RBS (AL1629-optimal AL2[AL2/GUS]). As shown in Fig. 5, AL2/GUS activity detected in extracts from protoplasts transfected with DNA containing an optimal RBS is approximately two-fold higher than that detected in extracts from protoplasts transfected with DNA containing the wild type AL2 RBS. The opposite result was observed in an AL3/GUS background. GUS activity detected in extracts from protoplasts transfected with DNA containing an optimal RBS for AL2 (AL1629-optimal AL2[AL3/GUS]) is approximately seven-fold lower than that detected in extracts from protoplasts transfected with DNA containing the wild type AL2 RBS (AL1629-wtAL2[AL3/GUS]). AL2/GUS activity from DNA containing a wild type AL2 RBS is approximately three- to four-fold lower than AL3/GUS expression in the same background, consistent with previous observations (Shung et al.,

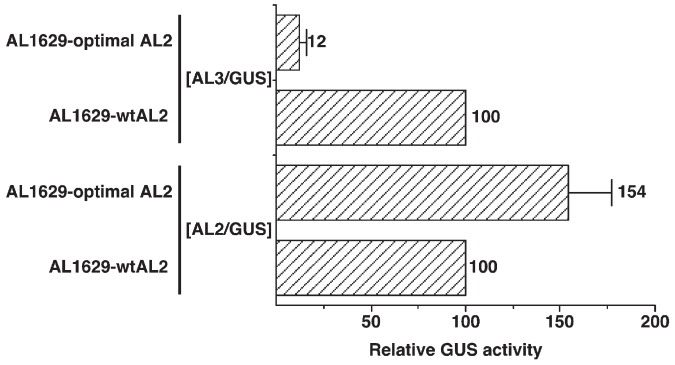

Fig. 5. Sequence context of the AL2 translation initiation codon is important for AL2 and AL3 expression. The ability of promoter–reporter constructs to direct GUS expression was determined by comparing GUS activity to background (pTGA26; ~1% activity). *N. benthamiana* protoplasts (5×10^5 cells) were transfected with 10 μg of a promoter–reporter construct and significant differences in GUS activity (Student's *t*-test: $P<0.05$) measured in extracts isolated three days post-transfection. Columns represent relative GUS activity as compared with AL1629-wtAL2[AL3/GUS], which was arbitrarily assigned a value 100%. Error bars represent the standard error of the mean from three to four independent experiments.

2006). This data is consistent with TGMV AL3 being translated from AL-1629 via leaky ribosome scanning.

Discussion

Complementary-sense transcription in geminiviruses is complicated, with multiple overlapping RNAs with unique 5′-ends and a common 3′-end (Sunter et al., 1989). The largest transcript initiates at nt 62 (AL-62), and is the only transcript that can encode full-length AL1 protein, but can also potentially encode the AL2, AL3 and AL4 ORFs. Previous studies using *in vitro* translation assays (Thömmes and Buck, 1994) and transgenic plants (Hanley-Bowdoin et al., 1989) suggest that the AL1, AL2 and AL3 proteins can be translated from a polycistronic viral RNA. Our previous work has demonstrated that the TGMV transcript initiating at nt 1935 (AL-1935) can express AL3, whereas the TGMV transcript initiating at nt 1629 (AL-1629) can express both AL2 and AL3 (Shung et al., 2006). This raised questions of how geminiviruses differentially regulate expression of AL2 from AL-1935 and AL-1629. Examination of the genetic map for AL-1935 reveals three AUG start codons upstream of the AL2 ORF (Fig. 1B), the first of which is highly conserved among begomoviruses and curtoviruses (Supplementary data). There is growing evidence that the first AUG rule for translation is not always observed (Kozak, 1987), and uORFs within the 5′-UTR limit or repress expression of many cellular and viral genes. This has been observed for thrombopoietin (Cazzola and Skoda, 2000), Bcl-2 (Harigai et al., 1996), Huntingtin proteins (Lee et al., 2002), basic region leucine zipper (bZIP) transcription factors (Wiese et al., 2004), the Fli-1 proto-oncogene (Sarrazin et al., 2000), Hepatitis B virus (HBV) core and polymerase (Chen et al., 2005) and Human cytomegalovirus gpUL4 early (Alderete et al., 1999). Our data suggests that TGMV may use a similar strategy to regulate the synthesis of viral proteins important for replication and suppression of host defenses. In TGMV, the first AUG within the 5'-UTR of AL-1935 encodes a putative protein consisting of the C-terminal 122 amino acids of AL1 (cAL1). We have shown that translation can initiate at the cAL1 AUG (Fig. 2) and that mutations in AUG codons within the 5'-UTR of AL-1935 lead to an increase in expression of both AL2 and AL3 (Fig. 3). These mutations do not increase transcription in infected plants, which therefore suggests a post-transcriptional regulatory mechanism. One interpretation of this data is that initiation of translation at one, or more, of these AUG codons reduces the number of ribosomes capable of initiating translation of the AL2 and AL3 coding regions, thereby repressing downstream AL2, and reducing AL3, expression. Based on the leaky scanning model for translation, the presence of an upstream AUG would only pose a problem if it occurs in a favorable context and is not followed by a terminator codon before the start of the major open reading frame (Kozak, 2002). For the TGMV cAL1 ORF, the sequence context surrounding the cAL1 AUG is not favorable for initiation of translation (Fig. 1B), but a terminator codon is positioned after the AUG initiation codon for the AL2 ORF. Thus translation of the cAL1 ORF could potentially inhibit translation of the AL2 coding region. Even though we detect AL2 expression in the presence of the wild type cAL1 AUG using promoter–reporter assays this may not be enough for expression of biologically relevant levels of AL2. This interpretation is supported by results demonstrating that transgenic *N. benthamiana* plants expressing the AL-1935 transcript do not complement TGMV *al2* mutant virus, but do complement TGMV *al3* mutant virus (Shung et al., 2006). The mechanism by which AL3 is expressed from this bicistronic viral mRNA could involve reinitiation and/or internal initiation (Kozak, 2001, 2002), although this is currently unknown and under investigation.

Although the TGMV AL-1935 transcript appears to initiate translation of the cAL1 ORF there is no comparable transcript in SCTV. Three complementary sense transcripts that initiate within the C1 ORF have however, been identified in SCTV-infected plants (Baliji et al., 2007). Within these transcripts there are two major ORFs, the first encoding C4 and the second encoding the C-terminal 212 amino acids of C1, within which is the cAL1 ORF. The reason for different coding capacities of TGMV and SCTV transcripts is unknown but may reflect host differences. Alternatively, AL-1935 and AL1629 may be expressed in different tissues reflecting the ability of TGMV to invade mesophyll tissue in contrast to curtoviruses that are phloem-limited (Stenger et al., 1990), and express a single transcript equivalent to AL1629 (Baliji et al., 2007).

Evidence that the regulatory effect of the cAL1 AUG on AL2 and AL3 expression has a biologically relevant function was provided by infectivity studies. Although translation can initiate at the cAL1 AUG (Fig. 2), mutation of the cAL1 AUG has little effect on replication, autorepression or complementary-sense transcription. Interestingly, mutation of the cAL1 AUG in either TGMV or SCTV has little if any effect on pathogenicity in *N. benthamiana* or *Arabidopsis*. However, in SCTV the mutation does influence infectivity in spinach, the original host for isolation of SCTV (Baliji et al., 2004), where *cal1* mutant virus was more infectious than wild type virus. This is characterized by an approximate 10% reduction in mean latent period following virus inoculation (Table 2). Although the shorter latent period could indicate an increase in virulence, there was no obvious enhancement of disease symptoms, and mutant virus did not accumulate to greater levels than wild type virus (Fig. 2, Supplementary data).

The phenotype observed in plants infected with *cal1* mutant SCTV is strikingly similar to the enhanced susceptibility phenotype (ES) observed in transgenic plants expressing a transgene capable of expressing the TGMV AL2 protein (Sunter et al., 2001). The ES phenotype is attributable to the inactivation of an SNF1-related protein kinase (SnRK1) by geminivirus AL2 and L2 proteins and provides evidence that SnRK1 is a component of innate antiviral defenses (Hao et al., 2003). AL2 also interacts with adenosine kinase (Wang et al., 2003) resulting in suppression of RNA silencing, an activated host defense response (Wang et al., 2005). Targeting of SnRK1 and ADK by geminiviruses is therefore postulated to be a counter-defensive measure. Increases in viral infectivity observed upon mutation of the cAL1 ORF would be consistent with higher levels of AL2, which could more efficiently inactivate both basal and activated defense responses. Levels of AL3 would also be postulated to increase, which could elevate viral replication. However, we do not see increases in replication of *cal1* mutant DNA in protoplasts (Fig. 4A) or in infected plants (Fig. 2, Supplementary data). Therefore we conclude that increased AL3 expression is not likely to be responsible for the increased infectivity observed. It may seem counter-intuitive to down-regulate genes involved in suppression of host defenses and viral replication. However, if these proteins are only needed in small quantities they could potentially be harmful to the cell if over-produced and thus detrimental to virus survival. This has been observed for a high percentage of eukaryotic genes controlling cell growth, including oncogenes and growth factors (Kozak, 1991). The presence of uORFs within the 5'-UTR can lead to inefficient translation of these proteins under normal conditions and mutation of the uORF can lead to overexpresssion in cancer cells. For example, the Her-2 uORF is translated and strongly inhibits the expression of the downstream coding region and in certain breast cancers the Her-2 receptor is overexpressed (Mehta et al., 2006). The oncogenic potential of these tumor cells appears to be a consequence of post-transcriptional mechanisms that repress the function of the uORF leading to overexpression of Her-2 (Mehta et al., 2006).

In contrast to AL-1935, both AL2 and AL3 can be expressed from the bicistronic AL-1629 mRNA transcribed by TGMV. The AL2 translation initiation codon is the first AUG in the 5'-UTR of AL-1629 but has an unfavorable ribosome binding sequence (RBS) according to the model for leaky ribosome scanning (Joshi et al., 1997; Kozak, 1999). The second AUG is the initiation codon for AL3, which has an optimal RBS, and our data suggests that AL3 is translated from AL-1629 via

leaky scanning. Changing the sequence surrounding the AL2 AUG to more closely match an optimal RBS results in increased AL2 and decreased AL3 expression (Fig. 5) as predicted. This is interpreted as an increase in ribosome binding at the AUG for AL2 leading to increased translation of this protein. This would effectively reduce the number of ribosomes that scan past the first AUG and initiate at the AUG for AL3.

It is currently unclear why geminiviruses would express AL3 from multiple transcripts, AL-62, AL-1935 and AL-1629. One possible explanation is that large amounts of AL3 are required for its multiple functions in replication (AL1/AL3 and AL3/AL3 oligomers), and interaction with host cell cycle components (pRB, and PCNA) (Settlage et al., 2001, 2005). AL3 could be also required at several stages of the viral life-cycle requiring expression from different RNAs transcribed at different times. However, down-regulation of AL3 expression from AL-1935 may indicate that excessive levels of AL3 are detrimental to the virus. Alternatively, it may be more critical to regulate the level of AL2 protein, which is involved in inactivating defense responses and regulation of late gene expression. Therefore TGMV may utilize the AUG from the C-terminal region of AL1 to down-regulate AL2 expression from AL-1935, ensuring AL2 expression only occurs from AL-1629. This is supported by data that shows AL2 is not expressed from AL-1935 (Shung et al., 2006). A second consequence of this would be to also down-regulate AL3 from AL-1935, which could be a reason the virus expresses AL3 from multiple transcripts. Nonetheless, independent regulation of these two genes would appear to be crucial to ensure successful completion of the viral life cycle. It is currently unknown whether the cAL1 ORF produces a functional protein although cAL1 protein could not be detected in extracts isolated from plants infected by TGMV using an antibody raised against the full length AL1 protein (data not shown). However, we are currently investigating whether cAL1 plays any additional role in viral pathogenesis.

Materials and methods

DNA constructs

Cloned DNA capable of generating a replicating TGMV A genome component (pTGA26), DNA containing the *GUS* reporter gene in place of the AL1 ORF (AL62-GUS; pTGA60) and DNA containing the AL1 ORF under control of the CaMV 35S promoter (35S-AL1; pTGA73) have been described previously (Sunter et al., 1990, 1993). Promoter–reporter constructs containing varying 5′ flanking sequences of AL2 (AL2[−1779]GUS, AL2[−1391]GUS, AL2[−654]GUS and AL2[−244]GUS) or AL3 (AL3[−790]GUS and AL3[−380]GUS) fused to the *GUS* reporter gene have been described (Shung et al., 2006). Cloned DNA capable of expressing the C-terminal 5 amino acids of AL1 (cAL1) was generated by PCR using AL2 [−1391]GUS as template. A 1060 bp fragment of TGMV DNA A (nt 399 to 1896), containing the common region, was generated (Primers TGMV-1 and TGMV-2) (Table 3). After restriction with NcoI and PmlI (within primer), the resulting 168 bp fragment was used to replace a 703 bp NcoI–BamHI fragment of AL2[−654]GUS (Shung et al., 2006). The BamHI site of AL2[−654]GUS was treated with either Klenow or Mungbean nuclease to generate DNA containing an in-frame (pGS73), or out-of-frame (pGS71) translational fusion between GUS and the N-terminal 5 amino acids of the cAL1 coding region. Both pGS71 and pGS73 were then used as template for PCR using primers that anneal at the cAL1 ATG and the 3′-end of GUS (cAL1 5′ and GUS 3′) (Table 3). The resulting 1860 bp fragments were treated with T4 polynucleotide kinase (T4 PNK) and EcoRI, and cloned into pMON530 digested with BglII (end filled with Klenow) and EcoRI, generating 35S[cAL1][GUS] and 35S[cAL1/GUS], respectively.

DNA capable of expressing an RNA equivalent to TGMV AL-1935, from the CaMV 35S promoter, was generated by PCR using AL2[−1391] GUS or AL3[−790]GUS as template. DNA fragments of 2490 bp were amplified (Primers cAL1 ATG and GUS 3′) (Table 3) and restricted with SmaI and EcoRI. The resulting DNA fragments were ligated into pMON530 restricted as above, generating 35S-wtAUGs[AL2/GUS] and 35S-wtAUGs[AL3/GUS]. Both DNAs contain three wild type ATG codons upstream of the AL2 ORF (Fig. 5A). A mutation in the ATG translation initiation codon of cAL1, in AL2/GUS and AL3/GUS backgrounds, was generated by PCR (Primers cAL1*atg*⁻F and GUS 3′) (Table 3) using AL2[−1392]GUS or AL3[−790]GUS as template. Amplified DNA (2490 bp) was treated with T4 PNK followed by restriction with EcoRI. DNA fragments were ligated into pMON530, digested with BglII (end filled with Klenow) and EcoRI to generate 35S-mcAL1[AL2/GUS], and 35S-mcAL1[AL3/GUS].

Cloned DNAs containing mutations in the three ATG codons within the 5'-UTR of AL-1935, upstream of the AL2 ORF in AL2/GUS, were generated as follows. First, a 2280 bp DNA fragment was amplified using AL2[−1392]GUS as template (Primers mATG-1 and GUS 3′) (Table 3), treated with T4 PNK and restricted with EcoRI. The DNA was ligated into pUC119 digested with BamHI (end filled with Klenow) and EcoRI to generate pGS94. Second, a 100 bp fragment was amplified from 35S-mcAL1[AL2/GUS], which contains the cAL1 ATG mutation, using primers cAL1*atg*⁻F and mATG-2 (Table 3). Following treatment with T4 PNK and XhoI, the DNA was inserted into pGS94 treated with HindIII (end filled with Klenow), and XhoI to generate pGS96. The BamHI site in pGS96 was end-filled with Klenow and religated, resulting in a +1 frame shift to generate pGS98. Using pGS96 or pGS98 as template, 2490 bp DNA fragments containing mutations in all three ATG codons were amplified (Primers cAL1*atg*⁻F and GUS 3′), treated with T4 PNK followed by restriction with EcoRI. The resulting DNA fragments were cloned into pMON530 restricted with BglII (end filled with Klenow) and EcoRI to generate 35S-mAUGs[AL2/GUS] and 35S-mAUGs[AL3/GUS], respectively.

Cloned DNA containing a stronger ribosome binding site (RBS) surrounding the AL2 ATG translation initiation codon was generated by PCR. A 2180 bp DNA fragment was amplified using AL2[−244]GUS as template (primers AL2RBS⁺F and GUS 3′) (Table 3). Following treatment with T4 PNK and EcoRI, DNA was ligated into pUC119 restricted with BamHI (end filled with Klenow) and EcoRI to generate pGS603. A 230 bp fragment was then generated by PCR (Primers TGMV-3 and AL2RBS⁺R) using pTGA26 as template, treated with T4 PNK and ligated into pGS603 restricted with HpaI to generate DNA containing an optimal RBS for AL2 (pGS604), in an AL2/GUS

Table 3
Sequences of primers used for PCR and cloning reactions

Primer	Sequence[a]	Nucleotide coordinates[b]
TGMV-1:	5′-gcgaagcttctcgagGAGAATAATTAGCAGAGCG GGAAACCC-3′	374–399
TGMV-2:	5′-gcgcacgtgGTGAACGAGCCCAC-3′	1896–1909
cAL1 5′:	5′-gcgcccgggATGTGGGCTCGTTCACTAGG-3′	1892–1911
cAL1*atg*⁻F:	5′-gcgact***AGT***TGGGCTCGTTCA-3′	1897–1911
cAL1*atg*⁻R:	5′-gcg***ACTAGT***CTTTCCCGTCCG-3′	1915–1926
GUS 3′:	5′-gcggaattcTCATTGTTTGCCTCCCTCGTGCG-3′	N/A
mATG-1:	5′-gcgctcg***AG***TCACACCGCAAT-3′	1797–1811
mATG-2:	5′-gcg***CTCGAG***CGATGACGTTAT-3′.	1815–1826
AL2RBS⁺F:	5′-gcgGT***TAAC***AATGG***C***AAATTCGTCTTCCTC-3′	1582–1608
AL2RBS⁺R:	5′-GAAAGTCCAGTTCTTTAGTGG-3′	1606–1626
TGMV-3:	5′-CAACCTTGTTTGAGT-3′	1830–1844
AL1 5′:	5′-gcgggatccGATGCCATCGCATCCAAAACG-3′	14–2582
AL1 3′:	5′-cgcaagcttAGCTGCTCTGTGAAGAG-3′	1545–1561
SCTV-1:	5′-***GATATC***GGGCCAGGTCTTTGGG-3′	2145–2160
SCTV-2:	5′-gcgctcgagAGATCTTCCATCGATCTGAA-3′	2486–2505
SCTV-3:	5′-TGTTTTTCCTGTTCTAGAAT-3′	2165–2184
SCTV-4:	5′-gcgGAATTCAACCTTCGCAAAAA-3′	1837–1856

[a] Restriction sites within the primer are underlined. Non-TGMV sequences are in lower case letters and TGMV sequences are shown in upper case letters. Mutated sequences are shown in bold italics.

[b] Nucleotide coordinates are given according to the TGMV sequence (Hamilton et al., 1984).

background. Using BamHI, a +1 frame shift was generated as described above, to yield DNA containing an optimal RBS for AL2 in an AL3/GUS background (pGS605). A 1737 bp SnaBI to EcoRI DNA fragment from AL2[−654]GUS containing part of the GUS sequence and the *nos* 3′ end was used to replace the 1400 bp SnaBI to EcoRI fragment of pGS604 and pGS605 to generate mAL1629[AL2/GUS] and mAL1629[AL3/GUS] respectively.

The cAL1 mutation was introduced into the TGMV as follows. A 700 bp fragment was amplified by PCR with primers cAL1*atg*⁻F and AL1 3′ (Table 3) using pTGA26 as template. The DNA was restricted with SpeI and ApaI and the resulting 150 bp DNA fragment ligated into pGEM5Zf(+) to generate pGS95. A 870 bp fragment was amplified using AL2[−1779]GUS as template (Primers AL1 5′ and cAL1*atg*⁻R) and restricted with SpeI and PstI. The resulting 250 bp fragment was ligated into pGS95 restricted with SpeI and PstI to generate pGS97. A 200 bp fragment containing the mutation in the AUG of cAL1 was isolated from pGS97, digested with EagI and ApaI and used to replace the corresponding wild type DNA fragment from pTGA26. The resulting DNA (pGS99) contains 1.5 copies of TGMV DNA A with a single copy of the mutant cAL1 ORF (Met to Ser change). pGS99 was restricted with PvuII and the approximately 4 kbp DNA fragment cloned into the binary plasmid vector pMON521 to generate TGMV *cal1*⁻. The AL1 coding region containing the cAL1 mutation was amplified by PCR using pGS99 as template, and the resulting DNA fragment used to replace the wild type AL1 coding region of pTGA73. The resulting DNA (pGS609) is capable of expressing the AL1 mutant protein from the CaMV 35S promoter (35S-*mal*1). The SCTV cAL1 mutant was generated as follows. Using pSCTV-WT (Baliji et al., 2004) as template, a 2600 bp DNA fragment containing the LIR was amplified (primers SCTV-1 and SCTV-2) (Table 3). Following restriction with EcoRV and XhoI the DNA fragment was cloned into similarly cleaved pMON521 generating pGS618. A second DNA fragment (2600 bp) containing the LIR was amplified by PCR (Primers SCTV-3 and SCTV-4) (Table 3) using pSCTV-WT as template. Following restriction with EcoRI the DNA fragment was cloned into pMON521 restricted with EcoRV and EcoRI. The resulting DNA construct (SCTV *cal1*⁻) contains 1.75 copies of the SCTV genome with two LIR sequences and a single copy of the cAL1 mutation. The introduction of a restriction site to generate the mutation results in two amino acid substitutions (Met–Trp to Tyr–Arg).

All DNA constructs were mobilized into *Agrobacterium tumefaciens* strain GV3111SE by triparental mating (Rogers et al., 1987) and used for agro-inoculation. *Agrobacterium* cultures containing tandemly repeated copies of the wild type TGMV A and B genome components, and wild type SCTV have been described previously (Baliji et al., 2004; Elmer et al., 1988).

Protoplast transfection and analysis

Protoplasts were isolated from an *N. benthamiana* suspension culture cell line (Sunter and Bisaro, 2003), transfected with various DNAs, and fluorometric GUS assays performed using equivalent amounts of protein as described (Sunter and Bisaro, 1991). Differences in GUS activity were assessed by Student's *t* test. For analysis of viral replication, DNA was isolated from protoplasts and analyzed by hybridization to a ^{32}P-labeled probe specific for TGMV DNA A, as described previously (Baliji et al., 2007).

Plant inoculation

Healthy *N. benthamiana*, *Arabidopsis* or spinach (*Spinacea oleracea*, var. viroflay) were inoculated with *Agrobacterium* cultures containing either tandemly repeated copies of wild type or mutant SCTV or TGMV DNA, using a standard dose ($O.D._{600}$ = 1.0) as described previously (Baliji et al. 2004). Inoculated plants were scored for the appearance of symptoms typical of an SCTV or TGMV infection on systemically infected tissue on a daily basis. Latent periods and ID_{50} measurements were made as described previously (Sunter et al., 2001). DNA was isolated from infected, and healthy mock-inoculated plants and analyzed for viral DNA accumulation as described previously (Baliji et al., 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.10.020.

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