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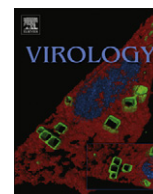
Sequences within the *Spinach curly top virus* virion sense promoter are necessary for vascular-specific expression of virion sense genes

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A B S T R A C T

Sequences necessary for activity of the *Spinach curly top virus* virion sense promoter have been identified within an 84 bp region upstream of two transcription start sites located at nt 252 and 292. RNAs initiating at these sites are expressed at equivalent levels in SCTV-infected *Arabidopsis* and from promoter-reporter constructs. The promoter is capable of directing expression of all three virion sense genes, although not to the same degree. While CP and V3 expression are similar, expression of V2 is elevated. The promoter is active in transient leaf infusion assays in the absence of C2. In *Nicotiana benthamiana* plants the promoter is active in vascular tissue and under no conditions did we detect promoter activity in the mesophyll. This is in contrast to begomoviruses where the virion sense promoter is dependent on AL2, a positional homolog of C2, and the promoter is functional in both vascular and mesophyll tissue.

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Introduction

Plant viruses of the family *Geminiviridae* cause significant disease in economically important plants and can be classified into four genera based on genome organization, type of insect vector and host range (Fauquet et al., 2008). The small genomes of geminiviruses, 2.5–3.0 kb, are replicated in the nuclei of infected cells (Preiss and Jeske, 2003; Stenger et al., 1991) where double-stranded DNA replicative (RF) intermediates are used, in part, as template for viral transcription. The limited coding capacity of geminivirus genomes obligates them to make use of cellular RNA polymerase for viral gene expression, which means that geminiviruses are valuable models for the study of host cell transcription (Hanley-Bowdoin et al., 1999).

Viruses of the *Curtovirus* genus are leafhopper-transmitted, infect dicotyledonous hosts and possess a monopartite circular ssDNA genome of ~3.0 kb. *Spinach curly top virus* (SCTV) is a recently characterized curtovirus (Baliji et al., 2004) with a genome containing seven open reading frames. Three (*V1, V2* and *V3*) are encoded in the virion sense and four (*C1, C2, C3* and *C4*) in the complementary sense (Fig. 1A). The gene *C1* encodes the replication-associated protein Rep, gene *C3* encodes the replication enhancer protein REn and *C4* is a pathogenicity determinant. The gene product of *C2* was shown to interact with

the host kinases AKIN11 and ADK2, which may lead to suppression of host defenses (Baliji et al., 2007). The coat protein (CP) is encoded by the *V1* gene, which in curtoviruses also functions as a movement protein (Briddon et al., 1989; Soto et al., 2005). In *Beet curly top virus* (BCTV), a curtovirus related to SCTV, the V2 gene product is required for accumulation of viral ssDNA and the V3 gene product has a direct role in systemic spread (Hormuzdi and Bisaro, 1993). SCTV has a bidirectional transcription strategy with transcription units diverging from a ~300 bp intergenic region (Fig. 1A). The most abundant RNA maps to the virion sense (1.1-kb transcript) and is comparable in size and location to that observed in BCTV. The virion sense transcription unit comprises two major overlapping transcripts, one initiating upstream of CP, V2, and V3 at nt 252, and the second upstream of CP and V2 at nt 292 (Baliji et al., 2007). TATA box sequences are present ~20–30 nt upstream of both 5′ termini suggesting that these represent authentic transcription start sites for the virion sense transcription unit (Baliji et al., 2007). An additional start site was detected at nt 417, upstream of the CP ORF but downstream of the V2 and V3 ORFs. However, no TATA sequence is present within 30 nt of this putative start site and most likely does not represent an authentic transcription start site (Baliji et al., 2007). This is very different from members of the bipartite begomoviruses, including *Tomato golden mosaic virus* (TGMV) and *Cabbage leaf curl virus* (CaLCuV), where a single virion sense transcript is detected that encodes a single protein, the coat protein (Sunter et al., 1989).

In TGMV and CaLCuV, the *CP* gene is regulated by AL2 (Sunter and Bisaro, 1991, 1992; Lacatus and Sunter, 2008). It has been
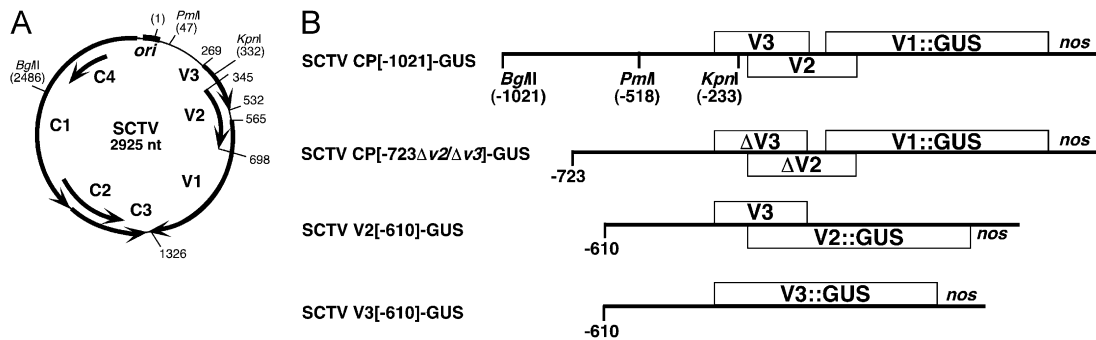
Fig. 1. Genome organization of SCTV: (A) the circular map illustrates the double-stranded replicative form DNA for wild type SCTV with relevant restriction sites, open reading frames (arrows), and the origin of plus strand replication (ori) indicated. Nucleotide coordinates are according to the SCTV sequence as determined by Baliji et al. (2004). (B) The linear maps illustrate derivatives of SCTV DNA containing different 5′ flanking regions of the virion sense genes (V1/CP, V2 and V3) cloned as translational fusions with the *GUS* reporter gene. Clone designations indicate both the amount of 5′ flanking sequence relative to the translation initiation codon for the CP coding region (+1) and the virion sense gene fused to GUS. Position of the virion sense ORFs (wild type and mutant) and the nopaline synthase (nos) 3′ polyadenylation signal are shown. Diagrams are not drawn to size.

previously demonstrated that the *CP* gene in TGMV and CaLCuV is activated by AL2 in mesophyll tissue and de-repressed by AL2 in phloem tissue (Lacatus and Sunter, 2008; Sunter and Bisaro, 1997). This suggests two mechanisms for regulation of the *CP* gene depending on the tissue involved. In BCTV and SCTV a functional homolog of TGMV AL2 is expressed but does not appear to play a role in activating expression of the *CP* gene. This is based on observations that BCTV L2 is unable to activate the TGMV *CP* promoter (Sunter et al., 1994).

The question of how virion sense gene expression is regulated in curtoviruses therefore remains unanswered. In this article we present results that allow us to describe viral sequences that are necessary for virion sense promoter activity. Our results suggest that the SCTV virion sense promoter is active in phloem tissue, in a manner similar to TGMV, where sequences 5′ to the *CP* gene direct promoter activity in the absence of C2. We discuss the possible impact of this on the life cycle of SCTV and the possible relevance to the phloem limitation of some geminiviruses.

Results

Identification of sequences necessary for activity of the SCTV virion sense promoter

Virion sense transcription in SCTV diverges from a ~300 bp intergenic region (Fig. 1A), which likely contains sequences important for transcription. To determine whether sequences within the intergenic region of SCTV contain promoter activity we constructed a series of truncated promoters beginning with SCTVCP[−1021]–GUS and including −518 and −233 (Fig. 1B). The truncated promoters were linked to the GUS reporter at the AUG codon for the *CP* (*V1*) gene, where +1 refers to the translation initiation codon for the CP coding region. In these experiments, as the AUG codons for the V2 and V3 ORFs were still present, it was essential to use a translational fusion to the AUG of CP in order to distinguish sequences required for *CP* gene expression due to the fact that CP, V2 and V3 overlap in different open reading frames (Fig. 1). Constructs were transformed into *Agrobacterium* and cultures used to infuse leaves of *Nicotiana benthamiana*. Extracts were prepared 3 days post-infusion and promoter activity measured by fluorometric GUS assay as described previously (Sunter and Bisaro, 1991). Constructs with deletion end points at −1021 and −518 exhibited significant GUS activity of up to 400-fold greater than background activity from the pMON521 vector control (Fig. 2A). Activity from DNA with a

deletion end point at −518 was approximately eight-fold lower than that of the full-length promoter (Fig. 2A). GUS activity above background was not detected in extracts from leaves infused with *Agrobacterium* cultures containing DNA with a deletion end point at −233 (Fig. 2A). This suggests that sequences necessary for SCTV virion sense promoter activity lie between −518 and −233. However, it is also apparent that sequences between −1021 and −518 function to stimulate activity of the SCTV virion sense promoter.

To further define sequences necessary for transcription from the virion sense promoter, additional deletions were made using PCR and included truncations with 5′ end points located both upstream and downstream of −518. As can be seen (Fig. 2B), promoter truncations with 5′ end points at −723 and −610 retained GUS activity ranging between 5154 and 5653 pm MU/min/mg protein. This is approximately four-fold lower than that of the full-length promoter (Fig. 2B) and approximately two-fold higher than that observed for the promoter truncation with a 5′ end point at −518 (Fig. 2A). A promoter truncation with a 5′ end point at −430 showed a 17-fold decrease in GUS activity (1282 pm MU/min/mg protein) relative to activity at −1021, which relates to a four-fold decrease relative to −610 (Fig. 2B). However, no significant GUS activity was detected from a promoter deletion with a 5′ end-point at −346 (Fig. 2B). In a separate experiment, significant GUS activity was detected in extracts from leaves infused with *Agrobacterium* cultures containing DNA with a deletion end point at −476, at a level that was statistically indistinguishable from activity in extracts from plants with DNA containing a deletion end point at −518 (Fig. 2C). However, no activity was detectable in extracts from plants containing DNA with a deletion end point at −392 (Fig. 2C), which was similar to extracts from plants containing DNA with a deletion end point at −346 (Fig. 2B).

Together, these results allow us to conclude that sequences necessary for activation of the SCTV virion sense promoter lie within 431 bp of the translation start site for the *CP* gene (+1) and that an element(s) required for promoter activity most likely lies between −430 and −392. Core promoter sequences are located within 79–157 bp upstream of the major virion sense transcription start sites at nt 252 and 292, which in turn are located 313 and 273 bp respectively, upstream of the translation start site for the *CP* gene. In addition, we can conclude that the promoter is active in the absence of the viral C2 gene product, in contrast to TGMV and CaLCuV where AL2 is required to activate the promoter in transient assays (Sunter and Bisaro, 1997; Lacatus and Sunter, 2008).

Impact of V2 and V3 on expression of the CP::GUS promoter-reporter

The experiments described above identify sequences within 431 bp of the translation start site for the *CP* gene that confer
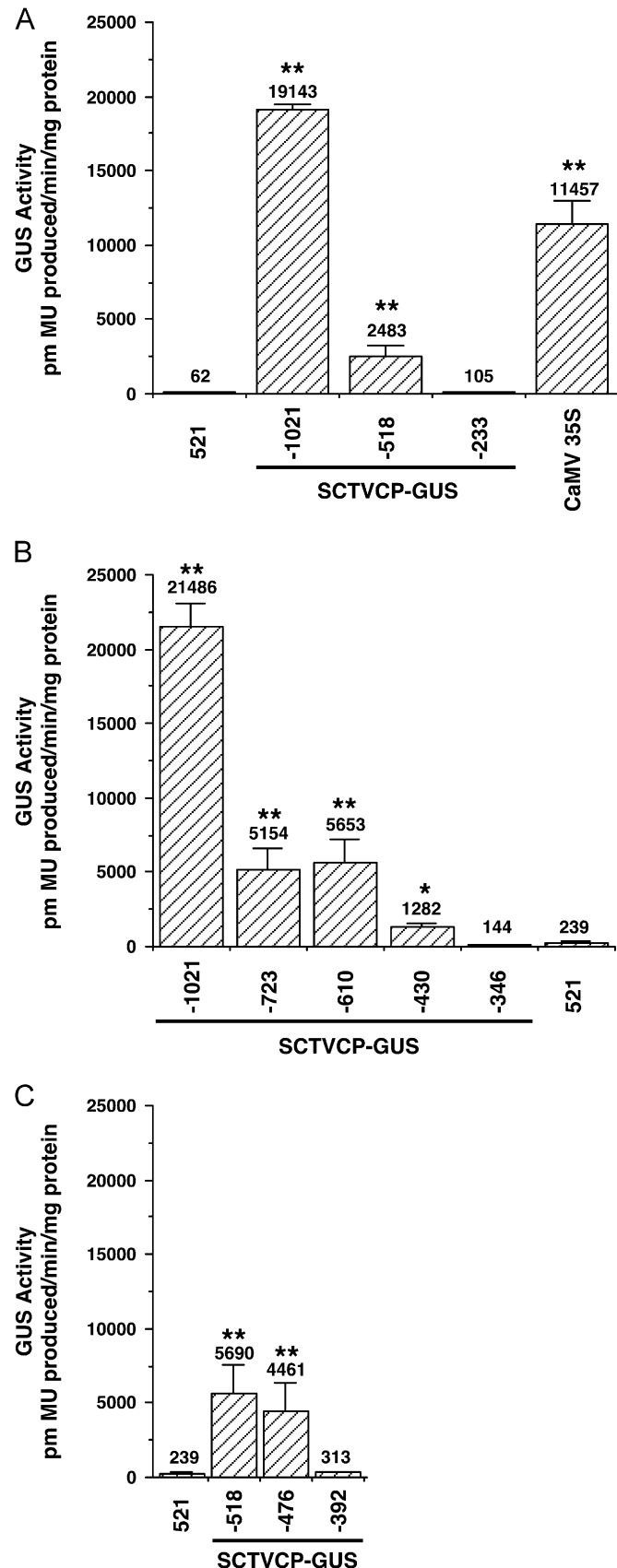
promoter activity. However, the V3 and V2 coding regions are also contained within this region, and the V2 ORF overlaps the CP ORF (Fig. 1A). We therefore investigated whether these coding regions affect expression of CP::GUS from the virion sense promoter. For these experiments a promoter truncation with a 5′ end point at −723 was constructed (SCTVCP[−723 Δv2/Δv3]-GUS) that contained mutations within the ATG codons for V2 and V3 (Fig. 1B). The truncated promoter was again linked to the GUS reporter in a translational fusion with the *CP* gene. Activity from this promoter construct was compared to a promoter truncation with a 5′ end point at −723 (SCTVCP[−723]-GUS) containing wild type sequences for V2 and V3. As shown (Fig. 3A), GUS activity, significantly higher than background, was detected in extracts from leaves containing either SCTVCP[−723 Δv2/Δv3]-GUS or SCTVCP[−723]-GUS. For the two constructs tested, mean expression was higher than that detected for CP::GUS, but the difference was not significant as determined by ANOVA (Fig. 3A). This would seem to indicate that the presence of sequences representing functional ATG codons for the V2 and V3 ORFs have little or no impact on expression of CP from the virion sense promoter located between −430 and −392 upstream of the CP start codon.

The virion sense promoter is capable of directing expression of SCTV V2 and V3

In an attempt to determine whether the sequences within 431 bp of the translation start site for the *CP* gene can also drive expression of V2 and/or V3, we generated promoter truncations that were linked to the GUS reporter in a translational fusion with either the *V2* (SCTVV2[−610]-GUS) or *V3* (SCTVV3[−610]-GUS) genes (Fig. 1B). Both constructs contained a 5′ end point equivalent to the CP −610 promoter, which was previously shown to yield GUS activity approximately four-fold lower than that of the full-length promoter (Fig. 2B). GUS activity, significantly higher than background, was detected in extracts containing SCTVV2[−610]-GUS or SCTVV3[−610]-GUS (Fig. 3B). Activity from the V2::GUS fusion was significantly higher than that from CP::GUS, whereas activity from the V3::GUS fusion was equivalent to that from the CP::GUS construct (Fig. 3B). This indicates that CP, V2 and V3 can all be expressed from the sequences located between −430 and −392 upstream of the CP start codon. The results also suggest that expression of V2 is higher than either CP or V3.

Identification of minimal sequences required for activation of a heterologous promoter core
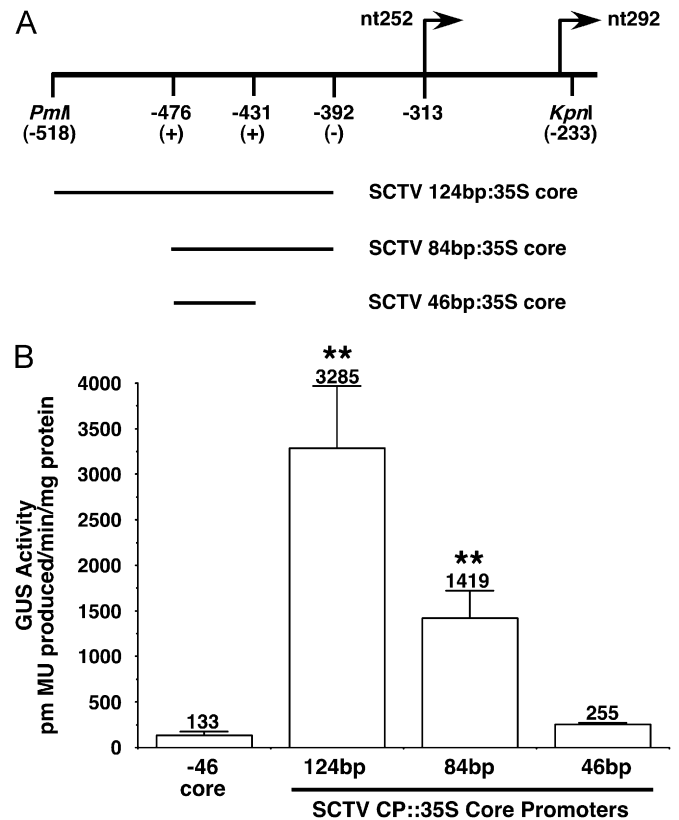
To test the ability of SCTV sequences to activate expression of a core promoter, putative promoter fragments were placed upstream of the heterologous *Cauliflower mosaic virus* (CaMV) 35S promoter core (−46 to +8; Benfey and Chua, 1990). Chimeric promoters (SCTV:35S core) linked to the *GUS* reporter gene as transcriptional fusions were analyzed in leaf infusion assays as

Fig. 2. Activity of SCTV virion sense promoter-reporter constructs. *N. benthamiana* leaves were infused with *Agrobacterium* cultures containing *CP* promoter-reporter constructs and activity determined by measuring GUS expression in extracts isolated 3 days post-transfection. Columns represent mean GUS activity in pm MU produced per min per mg protein and error bars represent the standard error of the mean. Values for each promoter-reporter construct were generated from three independent experiments, except for SCTVCP[−1021]-GUS and the pMON521 vector control in panel B where two independent measurements were taken. Error bars represent the standard error of the mean. Significant differences observed between samples were confirmed by one-way ANOVA. Double asterisks indicate means that are significantly different ($P < 0.05$) from the control mean (pMON521 vector control), as determined by Dunnett's test. The single asterisk in panel B indicates that activity from SCTVCP[−430]-GUS was not significantly different from pMON521 vector control using Dunnett's test, but was significantly different using a Student's *t*-test ($P < 0.05$).
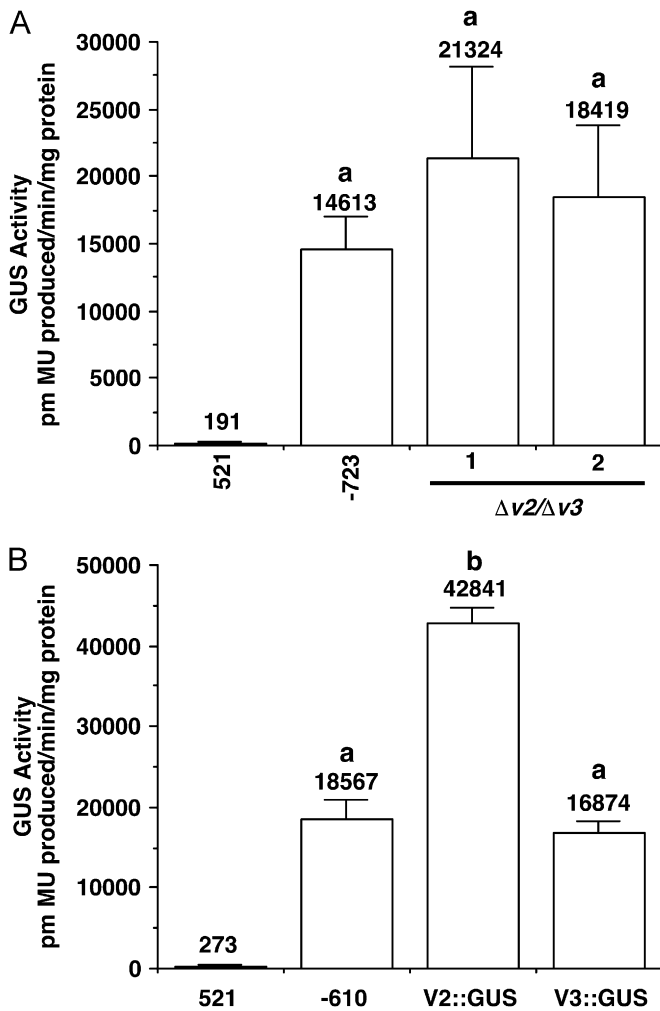
Fig. 3. V2 and V3 can be expressed from the virion sense promoter. *Agrobacterium* cultures containing promoter-reporter constructs were infused into *N. benthamiana* leaves, and promoter activity determined by measuring GUS expression in extracts isolated 3 days post-infusion. Columns represent mean GUS activity for each promoter-reporter construct from three independent experiments, and error bars represent the standard error of the mean. Significant differences observed between samples were confirmed by one-way ANOVA. Lower case letters indicate means that are significantly different from the control mean (pMON521 vector control), as determined by Dunnett's test ($P < 0.05$): (A) experiments were conducted using a promoter truncation with a 5′ end point at −723 (SCTVCP[−723 $\Delta v2/\Delta v3$]-GUS) containing mutations within the ATG codons for V2 and V3 (two independent constructs). The truncated promoter was linked to the GUS reporter in a translational fusion with the *CP* gene and compared to SCTVCP[−723]-GUS containing wild type sequences for V2 and V3. (B) Promoter truncations were linked to the GUS reporter in a translational fusion with either the *V2* (SCTVV2[−610]-GUS) or *V3* (SCTVV3[−610]-GUS) genes. Both constructs contained a 5′ end point equivalent to the CP −610 promoter, and were compared to the CP-GUS promoter-reporter (SCTVCP[−610]-GUS).
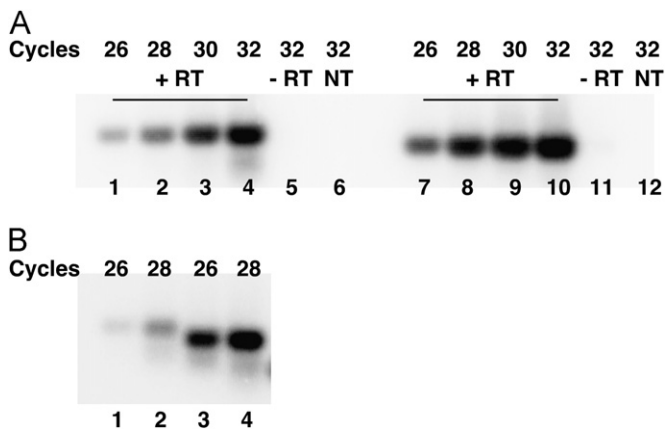

Fig. 4. Activation of SCTV:35S core promoters: (A) linear representation of the SCTV virion sense promoter between −518 and −233. Relevant promoter-reporter deletion end points, SCTV:35S core promoter fragments and the two major transcription initiation sites (arrows) are indicated. (B) GUS activity in extracts isolated 3 days post-transfection from *N. benthamiana* leaves infused with *Agrobacterium* cultures containing SCTV:35S core promoters is shown. Columns represent mean GUS activity in pm MU produced per min per mg protein and error bars represent the standard error of the mean. Values for each promoter-reporter construct were generated from two independent experiments. Asterisks indicate means that are significantly different (Student's *t*-test; $P < 0.05$) from the control mean (35S minimal: −46). SCTV:35S core promoters tested included 124 bp (−518 to −392), 84 bp (−476 to −392) and 46 bp (−476 to −431) fragments within the virion sense promoter.

Differential expression of SCTV virion sense transcripts

We next measured RNA levels to determine whether a single or multiple transcripts, initiating at nt 252 and nt 292 (Baliji et al., 2007), are produced. Total RNA was isolated from SCTV-infected Arabidopsis, or from leaves infused with *Agrobacterium* containing a promoter-reporter construct with a deletion end-point at −723 (SCTVCP[−723]-GUS). We used a semi-quantitative RT-PCR approach with nested primer pairs, to estimate the relative steady-state RNA levels of SCTV RNAs initiating at nt 252 and nt 292, which overlap extensively (Fig. 1). Total RNA was reverse transcribed into cDNA and PCR performed using a common primer that anneals either within the GUS sequence for the promoter-reporter construct, or within the V1 ORF for SCTV-infected Arabidopsis, in conjunction with a primer that would specifically amplify a fragment from RNA transcribed from initiation at either nt 252 alone, or from both nt 252 and nt 292. Subsequent hybridization to a probe that would anneal to sequences contained within both RNAs allowed us to obtain a relative ratio of complementary sense RNAs within any given sample as described previously (Shung and Sunter, 2007). Products of the predicted size for RNA initiating at nt 252 and nt 292 were detected in RNA isolated from both infected Arabidopsis and leaves infused with the promoter-reporter construct (Fig. 5).

above. Significant GUS activity (Student's *t*-test: $P < 0.05$), up to 25-fold greater than basal levels from the 35S core promoter was detected with fragments from −518 to −392 and −476 to −392 (Fig. 4). However, no activity was detectable from the 35S core promoter with a fragment from −476 to −431. The most direct interpretation of these data is that sequences between −431 and −392 are absolutely required for activity. This is consistent with the deletion analysis where a complete loss of activity is observed when sequences between −431 and −392 are deleted (Fig. 2). The results identify a 84 bp fragment of viral DNA (−476 to −392) capable of activating expression of the 35S core promoter in *N. benthamiana* protoplasts.

Fig. 5. Expression of SCTV virion sense RNAs: (A) each panel illustrates RT-PCR products derived from RNA isolated from SCTV-infected Arabidopsis using the following primer pairs: V3mutF+CPRT1 (lanes 1–6) or V2mutF+CPRT1 (lanes 7–12). PCR was conducted at varying cycles as indicated. Lanes 5 and 11 contain samples from RT reactions in which the reverse transcriptase was omitted (−RT). Lanes 6 and 12 contain PCR reactions in which the template was omitted (NT). (B) The panel illustrates RT-PCR products derived from RNA isolated from *N. benthamiana* leaves infused with *Agrobacterium* containing a promoter-reporter construct with a deletion end-point at −723 using the following primer pairs: V3mutF+GUSRT1 (lanes 1 and 2) or V2mutF+GUSRT1 (lanes 3 and 4). PCR was conducted at varying cycles as indicated.

A product generated using primers V2mutF and either CPRT1 or GUSRT1 would amplify a product from RNA initiating at nt 252 and nt 292. This signal is therefore comprised of cDNA product derived from both RNAs. Primers V3mutF and either CPRT1 or GUSRT1 would generate a product derived from RNA initiating at nt 252 alone. By subtracting the amount of signal detected for RNA initiating at nt 252, we can estimate that the residual signal is a consequence of amplification from RNA initiating at nt 292. The results show that in RNA extracted from SCTV-infected Arabidopsis, steady state SCTV virion sense RNAs comprise approximately 45% initiating at nt 252 and 55% initiating at nt 292 (Fig. 5A, Table S1 in Supplementary Data). In RNA extracted from *N. benthamiana* leaves infused with the promoter-reporter construct, RNAs comprised approximately 32% initiating at nt 252 and 68% initiating at nt 292 (Fig. 5B, Table S1 in Supplementary Data). Thus, expression of virion sense SCTV RNAs initiating at nt 252 and nt 292 appear to be approximately equivalent, although initiation at nt 252 is slightly lower. No product was detectable in reactions lacking reverse transcriptase or in PCR reactions lacking template (Fig. 5A).

The SCTV virion sense promoter appears to be active in the phloem but not in the mesophyll.

In TGMV and CaLCuV, regulation of the *CP* promoter is AL2-dependent, with the promoter being activated by AL2 in mesophyll and de-repressed by AL2 in phloem (Lacatus and Sunter, 2008; Sunter and Bisaro, 1997). In contrast, the SCTV virion sense promoter appears to be active in the absence of C2 (Fig. 2). To determine whether the SCTV virion sense promoter is regulated in a tissue-specific manner, transgenic *N. benthamiana* plants expressing SCTV *CP* promoter-reporter constructs containing 5′ deletion end points at, either −1021, −723, −610, −518 or −233 were generated. The presence of each transgene was confirmed by PCR (data not shown). Histochemical staining was performed on leaves of both T0 (primary) and T1 transgenic *N. benthamiana* plants expressing each of the truncated *CP* promoter-reporter constructs. Leaves from transgenic plants containing constructs with 1021 bp, 723 bp, 610 bp (Table 1) or 518 bp (Fig. 6A) upstream of the CP ORF exhibited promoter activity in

Table 1
SCTV virion sense promoter activity in transgenic *N. benthamiana* plants.

Construct	C2-Independent expression		Activation	
	Vascular	Mesophyll	TGMV	SCTV
SCTVCP[−1021]-GUS	+	−	nd	nd
SCTVCP[−723]-GUS	+	−	nd	nd
SCTVCP[−610]-GUS	+	−	nd	nd
SCTVCP[−518]-GUS	+	−	−	−
SCTVCP[−233]-GUS	−	−	nd	nd
SCTVCP[−723 Δv2/Δv3]-GUS	+	−	nd	nd
SCTVV2[−610]-GUS	+	−	nd	nd
SCTVV3[−610]-GUS	+	−	nd	nd

vascular tissue. No expression was observed in mesophyll or other cell types. As these constructs do not contain an intact *C2* gene, vascular expression of the virion sense promoter is therefore independent of C2. This is similar to TGMV and CaLCV where *CP* promoter activity in vascular tissue is AL2-independent (Lacatus and Sunter, 2008; Sunter and Bisaro, 1997). In contrast, transgenic plants containing a promoter construct with 233 bp upstream of the CP ORF showed no staining in either tissue (Fig. 6B), consistent with results observed in transient GUS assays demonstrating no significant activity (Fig. 2A). As GUS is fused to the CP ORF, these results would appear to indicate that sequences necessary for SCTV CP expression in vascular tissue are positioned within 518 bp of the CP coding region, most likely within a 285 bp fragment between −518 and −233.

To further evaluate the virion sense promoter we also generated transgenic *N. benthamiana* plants expressing SCTV promoter truncations that were linked to the GUS reporter in a translational fusion with either the *V2* (SCTVV2[−610]-GUS) or *V3* (SCTVV3[−610]-GUS) genes (Fig. 1B). Both constructs contained a 5′ end point equivalent to the CP −610 promoter. Histochemical staining on leaves of both T0 (primary) and T1 transgenic *N. benthamiana* plants expressing each of the truncated *CP* promoter-reporter constructs indicated promoter activity in vascular tissue (Table 1). Again, no expression was observed in mesophyll or other cell types. As GUS is fused to the V2 or V3 ORFs in these constructs, these results would appear to indicate that the same sequences necessary for SCTV CP expression in vascular tissue are also necessary for expression of V2 and V3 in the same tissue. Mutation of the V2 or V3 ATG start codons in the context of a CP-GUS fusion (SCTVCP[−723 Δv2/Δv3]-GUS) also had no impact on the staining pattern (Table 1) confirming the results observed for transient GUS assays (Fig. 3A).
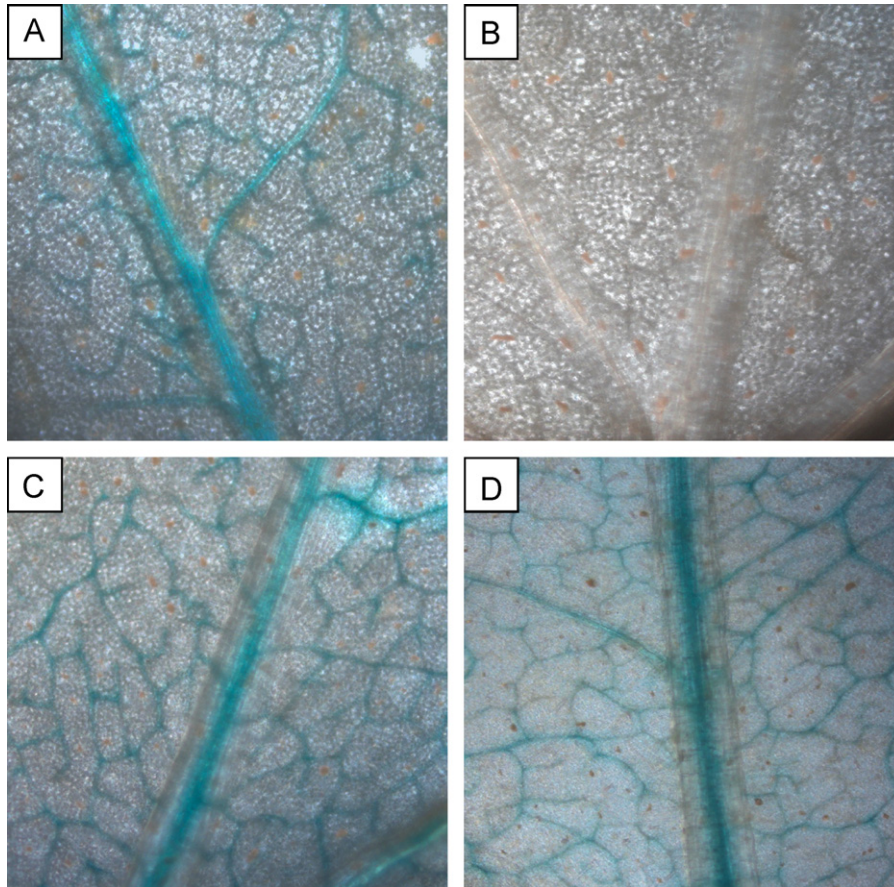
SCTV C2 and TGMV AL2 are unable to activate the SCTV CP promoter in mesophyll tissue

As the *CP* promoter of TGMV and CaLCuV can be activated by AL2 in mesophyll tissue (Lacatus and Sunter, 2008; Sunter and Bisaro, 1997), we tested whether SCTV can activate transcription of the SCTV *CP* promoter in mesophyll. Leaves from transgenic plants (T1) expressing the 518 bp *CP* promoter-reporter construct were infected with wild-type SCTV (Fig. 6C). However, the staining pattern was indistinguishable from non-infected samples (Fig. 6A). Thus SCTV appears unable to activate the SCTV *CP* promoter in mesophyll tissue. However, as SCTV is a curtovirus and it has been previously shown that the related BCTV is phloem-limited, it is unlikely to infect mesophyll tissue. Thus it is possible that the inability of C2 to activate the *CP* promoter is a consequence of SCTV being limited to the phloem. To test whether the SCTV *CP* promoter can be activated by the begomovirus homolog AL2, we infected transgenic plants expressing the

Fig. 6. Activity of SCTV virion sense promoter-reporter constructs in transgenic *N. benthamiana* plants. Tissue samples stained with X-Gluc were examined under a light microscope: (A) leaves from transgenic plants containing the SCTVCP[−518]-GUS transgene exhibited staining limited to the vascular region. (B) No staining is detectable in leaves from transgenic plants containing the SCTVCP[−233]-GUS transgene. Systemically infected leaves from plants containing the SCTVCP[−518]-GUS transgene exhibiting symptoms typical of SCTV (C) or TGMV (D) exhibit staining limited to the vascular region.
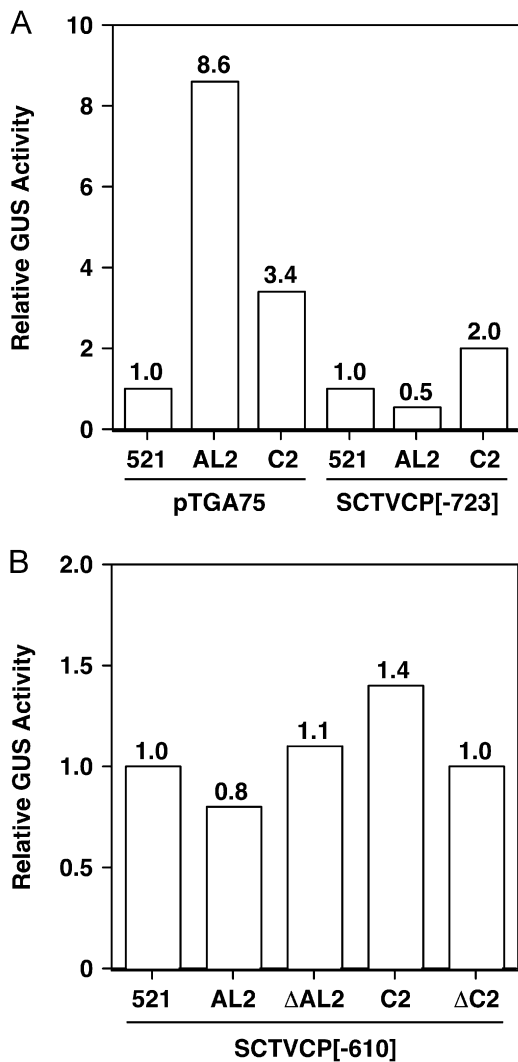
518 bp *CP* promoter-reporter construct with wild type TGMV. Again, the staining pattern (Fig. 6D) was indistinguishable from non-infected samples (Fig. 6A). This suggests that the SCTV *CP* promoter cannot be activated in the mesophyll by AL2.

To directly test the ability of SCTV C2 to activate the virion sense promoter we took two approaches. First, leaves of non-transgenic *N. benthamiana* leaves were infused with Agrobacterium cultures containing a TGMV (pTGA75) or SCTV (SCTVCP[−723]) promoter-reporter construct along with constructs capable of expressing either SCTV C2 (Baliji et al., 2010) or TGMV AL2 (Sunter et al., 1994) from the CaMV 35S promoter. Extracts were prepared 3 days post-infusion and promoter activity measured by fluorometric GUS assay as described previously. As can be seen (Fig. 7A), the presence of SCTV C2 or TGMV AL2 had little impact on expression of the SCTV *CP* promoter (SCTVCP[−723]). In contrast, the presence of TGMV AL2 resulted in an approximate nine-fold increase in TGMV *CP* promoter activity (pTGA75). SCTV C2 induced a small increase in activity of the TGMV *CP* promoter, which is most likely a consequence of the anti-silencing function of the curtovirus C2 protein (Baliji et al., 2007; Raja et al., 2008). In a second approach, we utilized transgenic plants expressing an SCTV *CP* promoter-reporter construct containing a 5′ deletion end point at −610 (Fig. 7B). In this experiment leaves of the transgenic plants were infused with Agrobacterium containing constructs capable of expressing either SCTV C2 or TGMV AL2 from the CaMV 35S promoter. In both cases little or no change was detected in activity of the SCTV −610 promoter (Fig. 7B). A second set of test constructs were used that could only express the first 100 amino acids of SCTV C2 or TGMV

AL2 from the CaMV 35S promoter. These constructs contain the region of AL2 and C2 that is responsible for inhibition of the host RNA silencing pathway, and were used to eliminate any potential effects of silencing suppression by AL2 or C2. However, as can be seen, there was not additional activity from the SCTV −610 promoter (Fig. 7B). Together these results would indicate that regulation of the SCTV virion sense promoter, at least with respect to mesophyll expression appears to be fundamentally different to that observed for begomoviruses like TGMV and CaLCuV.

Discussion

The coding capacity of the virion sense DNA in bipartite begomoviruses is relatively simple. For example, TGMV and CaLCuV encode a single ORF for expression of CP (Abouzid et al., 1992; Hamilton et al., 1984). The transcription unit for expression of the TGMV *CP* gene comprises a single virion sense RNA spanning the CP ORF (Sunter et al., 1989). Expression of the *CP* gene in CaLCuV and TGMV is dependent on sequences contained within the 230 bp intergenic region and is mediated by the AL2 gene product (Lacatus and Sunter, 2008; Sunter and Bisaro, 1992, 2003). In contrast, the coding capacity for the virion sense DNA of curtoviruses, including BCTV and SCTV, is more complex. In these monopartite viruses there are three viral sense ORFs that overlap extensively (Baliji et al., 2004; Stanley et al., 1986). Analysis of transcripts produced during an infection with SCTV revealed the presence of two putative transcription start sites, one initiating upstream of the CP, V2 and V3 ORFs and a

Fig. 7. SCTV C2 cannot activate the SCTV or TGMV virion sense promoters: (A) N. benthamiana leaves were infused with Agrobacterium cultures containing a TGMV (pTGA75) or SCTV (SCTVCP[−723]) promoter-reporter construct and constructs capable of expressing either TGMV AL2 or SCTV C2. (B) Transgenic N. benthamiana plants containing the (SCTVCP[−610]) promoter-reporter construct were infused with Agrobacterium cultures containing either TGMV AL2, SCTV C2 or constructs consisting of the first 100 amino acids of each protein (ΔAL2 and ΔC2). Promoter activity was determined by measuring GUS expression in extracts isolated 3 days post-infusion, with columns representing the fold change in GUS expression relative to the promoter-reporter construct infused with a vector control (pMON521 vector control).

second initiating upstream of the CP and V2 ORFs (Baliji et al., 2007). A third minor site, lacking a corresponding TATA sequence was detected upstream of CP (Baliji et al., 2007). However, little is known about the sequences regulating transcription of these viral RNAs in curtoviruses. Our studies demonstrate that the SCTV virion sense promoter exhibits C2-independent regulation in leaf infusion assays (Fig. 2). These results are in stark contrast to the CP gene in bipartite begomoviruses where promoter activity is dependent on the AL2 gene product in transient assays using leaf infusions or protoplasts (Lacatus and Sunter, 2008; Sunter and Bisaro, 1992, 2003). Virion sense promoter activity is clearly dependent on sequences located within 431 bp of the CP coding region, as deletion of sequences from −430 to −392 cause a complete loss of activity (Fig. 2). This was confirmed when SCTV sequences between −431 and −392 were shown to be absolutely required for activation of the heterologous CaMV 35S core

promoter (Fig. 4). However, there are sequences upstream of this region that appear to modulate activity of the virion sense promoter. Specifically, deletion of sequences between −1021 and −610, −610 to −518, and −476 to −430 lead to a four- two-, and three-fold decrease in activity respectively (Fig. 2). This suggests that the promoter could be modular, with several elements contributing to full promoter activity. It is of course also possible that additional sequences within the V1 coding region itself could play a role in modulating promoter activity, given that this was replaced by the GUS reporter gene sequence in the constructs used in the analysis.

Analysis of sequences between −476 and −392 using the PLACE database (Prestridge, 1991; Higo et al., 1999) has revealed a number of putative transcription factor binding sites (Table S2: Supplementary Data). Within the region that appears to be absolutely required for activity (−430 to −392) there are few putative binding sites, although most of those predicted were implicated in dehydration and/or low temperature responses (CBFHV; DRECRTCOREAT; LTRECOREATCOR15). How these particular sites play a role in the viral life cycle is currently unknown. Of the putative transcription factor binding sites identified within the region that appears to modulate promoter activity (−476 and −430) putative binding sites for WRKY transcription factors and E2F are potentially relevant to a virus infection. WRKY transcription factors are implicated in both positive and negative regulation of plant defense, responses to abiotic stress and growth and development. These factors globally regulate host defenses at various levels, including direct modulation of downstream target genes, activation or repression of additional transcription factors, and by regulating WRKY genes through feed-forward and feedback regulation (Pandey and Somssich, 2009). How this degree of regulation is achieved is not clearly understood. These proteins bind the W box sequence (TTGACT/C), although adjacent DNA sequences and the arrangement of these elements appear to play a role in binding preference (Agarwal et al., 2010). Thus, the interaction between WRKY and other proteins could lead to the formation of distinct complexes, resulting in different transcriptional outputs. It will be interesting to determine which, if any of these binding sites, contribute to transcriptional activity of the SCTV virion sense genes and whether the virus utilizes members of the WRKY transcription factor family to regulate expression.

As promoter-reporter constructs in which GUS is joined either to CP, V2 or V3 as a translational fusion exhibit activity, it suggests that a single sequence is involved in the expression of all three virion sense genes. Analysis of transcripts produced in SCTV-infected Arabidopsis plants indicates the presence of virion sense RNAs initiating at nt 252 (VS252) and 292 (VS292) in roughly equal amounts, although there did seem to be a slightly lower level of RNA initiating at nt 252 (Fig. 5A). This was also observed in RNA isolated from N. benthamiana leaves infused with Agrobacterium containing a promoter-reporter construct with a deletion end-point at −723 (Fig. 5B), suggesting that the promoter constructs are utilizing the authentic viral transcription initiation sites. In neither case could we detect any evidence for an RNA initiating at nt 417 (data not shown), which represents a minor site detected by primer extension in infected plants, and no TATA box sequence is located within 30 nt of this site (Baliji et al., 2007).

To interpret how this could affect expression of the three proteins we can assess the potential coding capacity of the putative transcripts initiating at nt 252 and 292 (Fig. 8). Both can potentially code for CP and V2 (VS292), and the second has the capacity to code for CP, V2 and V3 (VS252). It is therefore likely that the RNAs express more than one protein, and analysis of sequence surrounding the AUG codon for CP, V2 and V3 may give some insight. In higher plants, authentic translation start
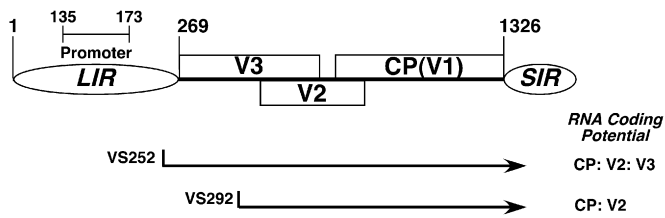
Fig. 8. Coding capacity of SCTV virion sense transcripts. The linear diagram illustrates part of the SCTV genome encoding virion sense transcripts (Baliji et al., 2007). Positions of the large (LIR) and small (SIR) intergenic regions, ORFs, and the virion sense promoter are shown. Arrows designate transcripts and direction of transcription with the start site and potential coding capacity of each mRNA indicated.

codons are included in a consensus sequence for initiation (A(A/C)aAUGGC) (Joshi et al., 1997), which has an overall similarity with the consensus sequence found in vertebrate sequences (GCCRCCAUGG) (Joshi et al., 1997; Kozak, 1991). Sequence surrounding the AUG for V2 (AAaAUGGg) indicates a strong RBS, with three of the conserved nucleotides including the most important located at −3 and +4. In the model for leaky ribosome scanning (Joshi et al., 1997; Kozak, 1999) V2 is the first ORF encountered in VS292 RNA, and we predict expression at a high level. In contrast, CP expression from VS292 would be expected to be lower as few ribosomes are predicted to scan by the AUG for V2. However, as the sequence context for CP is also unfavorable (uauAUGag) we would also predict fewer ribosomes initiating translation. With respect to VS292 we can make similar predictions. First some ribosomes are expected to scan through the AUG codon for V3, which is in an unfavorable context (uaaAUGat). Second, we predict high V2 expression and third, lower expression of CP for the reasons outlined above. It is also possible that CP expression from VS292 would be higher than from VS252 given that there would have to be leaky scanning through two ORFs in the latter RNA. Based on this we can predict that V3 would be mostly likely expressed from VS252, V2 from both VS252 and VS292, and CP from VS292. Our data is consistent with this interpretation as experiments where V2 is fused to GUS expression from the virion sense promoter appears to be significantly higher than either CP or V3 (Fig. 3B). Expression of V3 and CP are approximately equal, although V3 is somewhat lower which would reflect expression from a single RNA. Another line of evidence for this conclusion is provided by experiments in which the ATG codons for V2 and V3 are mutated. Mutation of the ATG codons for V2 and V3 results in 30–40% increase in CP::GUS activity. Thus, the presence of the V3 and V2 ORFs appears to have some impact on expression of the downstream CP. This would presumably be mainly due to removal of the V2 ATG, which lies in a favorable sequence context. Our data suggests that the virus may upregulate expression of V2 relative to V3 and CP. We do not currently understand why this appears to be the case, but may in some way reflect the role of the V2 gene product in the accumulation of viral ssDNA, and CP and V3 in movement (Hormuzdi and Bisaro, 1993).

Previous analysis of the CaLCuV and TGMV CP promoters showed that the CP promoter was active in different cell types through distinct viral sequence elements (Lacatus and Sunter, 2008; Sunter and Bisaro, 1997). In mesophyll tissue the promoter is activated in the presence of AL2, but in phloem the promoter is active unless a sequence located between 1.2 and 1.5 kb upstream of the CP transcription start site is present. AL2 then acts to derepress the CP promoter (Sunter and Bisaro, 1997). This led us to examine the tissue specificity of the SCTV virion sense promoter in N. benthamiana. Sequences necessary for vascular

expression are located within 518 bp of the CP coding region, but the promoter is highly active in the absence of C2. This is similar to bipartite begomoviruses where AL2-independent expression is observed in the phloem. However, unlike begomoviruses all attempts to activate expression in mesophyll tissue were unsuccessful. This may reflect observations that TGMV and CaLCuV are not restricted to the phloem (Morra and Petty, 2000; Qin and Petty, 2001) in contrast to curtoviruses, which are phloem-limited (Stenger et al., 1990). There can be many reasons for restriction of viruses to phloem tissue, but our data indicates that the SCTV virion sense promoter does not appear to be active in tissue other than phloem. Thus, even if viral DNA and/or virions were able to move out of the vascular tissue, the virion sense promoter would not be active and so CP, V2 and V3 would not be made.

One interpretation of our results is that curtoviruses do not temporally regulate virion sense gene expression, unlike begomoviruses, and that CP is constitutively expressed. However, we do not believe this to be the case. In begomoviruses, AL2 activates the CP promoter in mesophyll tissue, which is not the case for SCTV. There is, however, a second mechanism for controlling CP gene expression in begomoviruses, which involves derepression in the phloem (Lacatus and Sunter, 2008; Sunter and Bisaro, 1997). Thus, it is possible that SCTV virion sense expression is regulated in a similar manner, given the phloem restriction of the virus, and this is currently under investigation.

Materials and methods

Cloning of truncated promoter-reporter constructs

DNAs containing various lengths of the SCTV virion sense promoter were cloned as translational fusions between SCTV CP and the β-glucuronidase (GUS) gene followed by the nopaline synthase (NOS) terminator sequence. A PCR fragment was generated (Primers SCTV CP −1021+SCTV CP Rev) containing 1021 bp of sequence upstream of the translation start site for the CP gene, and used to replace the 873 bp HindIII to BamHI fragment of pBI221 that contains the CaMV 35S promoter. The resulting DNA, SCTVCP[−1021]-GUS (provided by Dr. Gabriela Lacatus), was restricted with PmlI and EcoRI or KpnI and EcoRI to generate 2618 bp and 2333 bp fragments respectively. These fragments were cloned into the vector pMON521 cut with HindIII, Klenow treated, and restricted with EcoRI to generate (SCTVCP[−518]-GUS) and (SCTVCP[−233]-GUS) which contained 518 bp and 233 bp of sequence upstream of the translation start site for the CP gene. Additional deletion end-points were generated using a PCR-based cloning strategy, using (SCTVCP[−1021]-GUS) as template. Primers used in this process along with the nucleotide co-ordinates are listed in Table 2. Each of the primers was paired with the GUS3Xba primer in PCR reactions. The PCR products were purified and digested with HindIII and SnaBI. The DNA was then used to replace the HindIII-SnaBI fragment from (SCTVCP[−518]-GUS) to generate (SCTVCP[−723]-GUS), (SCTVCP[−610]-GUS), (SCTVCP[−476]-GUS), (SCTVCP [−430]-GUS), (SCTVCP[−392]-GUS) and (SCTVCP[−346]-GUS).

Promoter-reporter fragments were cloned as translational fusions between SCTV V3 or V2 and GUS. Using PCR, 325 bp (SCTVCP −1021+SCTVV3) and 401 bp (SCTVCP −1021+SCTVV2) fragments were amplified (Table 2) using SCTVCP[−1021]GUS as template. Fragments were restricted with HindIII and BamHI and used to replace the 873 bp HindIII to BamHI fragment of pBI221, generating (SCTVV2[−610]-GUS) or V3 (SCTVV3[−610]-GUS).

A construct containing mutations in the ATG codons for V2 and V3 were generated using a multi-step PCR-based approach. A 438 bp fragment containing the SCTV virion sense promoter from

Table 2
List of primers used for cloning.

Primer name	Primer sequence[a]	Nucleotide co-ordinates[b]
SCTV CP Rev	5′–CGCGGATCCTATAAAGCACACTTCCTTGC-3′	546–565
SCTV CP − 1021	5′–GCGAAGCTTGCACGCGGAGGTCAAC-3′	2491–2470
SCTVCP − 723	5′–GCGAAGCTTCTTCTTTAGAAATGGAAC-3′	2767–2790
SCTVCP − 610	5′–GCGAAGCTTCTTTGGGAGCTCTTAAATACCATC-3′	2880–2903
SCTVCP − 476	5′–GCGAAGCTTGAAACTTCCTGAAGAAG-3′	89–105
SCTVCP − 430	5′–GCGAAGCTTGTCAAAAAGTAGCCGACATAG-3′	135–155
SCTVCP − 392	5′–GCGAAGCTTAAGTAGATGACTGTGGTCC -3′	173–191
SCTVCP − 346	5′–GCGAAGCTTGAATAATGTCGGTTTATATACG-3′	219–240
SCTV5 MIN1	5′–GCGGGATCCCACGTGGAAAGCATATGATGTCG-3′	46–69
SCTV5 MIN2	5′–GCGGGATCCGGAAACTTCCTGAAGAAGTTTTCTTTC-3′	87–114
SCTV3 MIN1	5′–GCGTACGTATAACTTTATTTCAAAACTATGTCGG-3′	146–171
SCTV3 MIN2	5′–GCGTACGTAAAGTCAAAAAAATGAGCGGGAAG-3′	110–133
GUS3 XBA	5′–GCGTCTAGATCATTGTTTGCCTCCCTGCTG-3′	NA
CPRT1	5′–CAAGCATGTCGTCATACATC-3′	717–736
SCTVV3	5′–GCGGGATCCACAGACCATCATTTATAAGTAC-3′	259–280
SCTVV2	5′–GCGGGATCCGAAAGGTCCCATAAAAATTGG-3′	336–356
V3mutF	5′–GCGGTCGACGTCTGTCTACCAGACTGG-3′	269–292
V3mutR	5′–GCGGTCGACTTATAAGTACATATACATG-3′	250–275
V2mutF	5′–GCGTCTAGACCTTTCAGAGTGGATCAATTTCC-3′	345–373
V2mutR	5′–GCGTCTAGAAAAAAATTGGTACCCGATTG-3′	326–350
GUSRT1	5′–CTTCGCGCTGATACCAGACG-3′	NA

[a] Sequences underlined indicate sequences added to each primer to provide a unique restriction site.
[b] Nucleotide coordinates are given according to the sequence of SCTV (Baliji et al., 2004).

−723 to the V3 ORF was amplified using SCTV CP-723 and V3mutR primers (Table 2) with SCTV DNA as template. Following restriction with HindIII and SalI the fragment was cloned into similarly digested pUC119 (pV2/V3-1). A 76 bp fragment was then amplified using V3mutF and V2mutR primers (Table 2) with SCTV DNA as template, digested with SalI and XbaI and cloned into similarly restricted pV2/V3-1 (pV2/V3-2). The SalI restriction site in this construct changes the sequence at the start of V3 from ATGATG to GTCGAC. In the third step, a 220 bp fragment was amplified using V2mutF and SCTV CP Rev primers (Table 2) with SCTV DNA as template. After restriction with XbaI and BamHI, DNA was cloned into pV2/V3-2 restricted with XbaI and BamHI (pV2/V3-3). The XbaI restriction site in this construct changes the sequence at the start of V2 from ATGGGA to TCTAGA. The promoter region containing the V2 and V3 mutations was then removed by restriction with HindIII and BamHI and used to replace the 1021 bp HindIII to BamHI fragment of SCTVCP[−1021]-GUS.

Fragments of SCTV were amplified by PCR using primers SCTV5Min1+SCTV3MIN2, SCTV5MIN1+SCTV3MIN1, or SCTV5 MIN2+SCTV3MIN2 (Table 2) to yield 124 bp (−518 to −392), 84 bp (−476 to −392) and 46 bp (−476 to −431) fragments within the virion sense promoter. SCTV:35S Core promoters were generated by cloning each fragment upstream of the CaMV 35S core promoter fragment from −46 to +8 (Benfey and Chua, 1990).

Leaf infusions and fluorometric GUS analysis

Promoter::reporter constructs were mobilized into *Agrobacterium tumefaciens* GV3111SE containing the disarmed Ti plasmid pTiB6S3SE as described (Rogers et al., 1986). The presence of each promoter-reporter construct was confirmed by PCR using primers specific for each promoter fragment (Table 2). Leaves of *N. benthamiana* plants were infused with *Agrobacterium* containing truncated promoter::reporter constructs essentially as described (Johansen and Carrington, 2001; Wang et al., 2005). Disks were cut from the infusion sites of nine leaves, from three different plants, and extracts isolated 3 days post-inoculation. GUS activity was compared by fluorometric assay using equivalent amounts of protein as described previously (Shung et al., 2006).

Generation of transgenic plants

N. benthamiana plants transgenic for promoter-reporter constructs were generated as described (Rogers et al., 1986). The presence of each transgene in three primary transformants was confirmed by PCR using GUS3XBA and SCTVCP-476 primers (Table 2). Three to five plants from each T1 line were selected for subsequent analysis.

Inoculation N. benthamiana plants

N. benthamiana plants transgenic for each promoter-reporter constructs were agro-inoculated with *Agrobacterium* containing tandemly repeated copies of SCTV or TGMV genomes using a standard dose (optical density at 600 nm=1.0) as described previously (Baliji et al., 2004; Sunter et al., 2001).

Histochemical staining

Leaf disks from SCTV or TGMV-infected, or non-infected transgenic *N. benthamiana* plants were histochemically stained for GUS activity 10–20 days post-inoculation essentially as described (Jefferson et al., 1987; Sunter and Bisaro, 1997). Disks were surface sterilized, infiltrated with X-Gluc substrate and incubated at 37 °C overnight. Stained samples were fixed with formaldehyde, cleared by a series of washes containing increasing ethanol concentration, and stored in 100% ethanol. Tissue samples were photographed using a light microscope (Axioskop, Carl Zeiss) under a 10 × objective.

RNA isolation and analysis

Total RNA was isolated from *N. benthamiana* leaves using Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase I (RQ1 DNaseI, Promega, Madison, WI) at 37 °C for 1 h. Following phenol/chloroform extraction and ethanol precipitation, RNA was resuspended in H_2O and stored at −80 °C.

Semi-quantitative RT-PCR

Up to 250 ng total RNA was used for detection of SCTV viral RNAs in two-step reverse transcription (RT)-PCR reactions. First,

cDNA was generated using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo (dT) as the primer. This was followed by PCR using 5′ Prime MasterMix (Qiagen, Valencia, CA) according to the manufacturers instructions. The primers used for amplification along with the nucleotide co-ordinates are listed in Table 2. The optimum number of cycles required for a near linear relationship was determined as described previously (Shung and Sunter, 2007). As there is extensive overlap of the virion sense viral RNAs, 150 pm of each primer was used. Specific cDNA products were detected by hybridization to ^{32}P-labeled probes specific for SCTV and quantified by phosphorimager analysis (Molecular Imager FX, Bio-Rad, Hercules, CA). The ratio of mRNAs in each sample was determined by direct comparison of the levels of cDNA generated at a given cycle within the linear range of amplification as described (Shung and Sunter, 2007).

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.05.007.

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