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Functional Expression of the Leftward Open Reading Frames of the A Component of Tomato Golden Mosaic Virus in Transgenic Tobacco Plants

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The genome of the geminivirus tomato golden mosaic virus (TGMV) consists of two circular DNA molecules designated as components A and B. We have constructed *Nicotiana benthamiana* plants that are transgenic for the three overlapping open reading frames, AL1, AL2, and AL3, from the left side of TGMV A. In the transgenic plants, the AL open reading frames are under the control of the cauliflower mosaic virus (CaMV) 35S promoter. In TGMV infectivity assays, seven of 10 transgenic lines complemented TGMV A variants with mutations in AL1, AL2, or AL3 when co-inoculated with the B component. The 35S-AL construct was transcribed as a single RNA species in the transgenic plants, indicating that AL1, AL2, and AL3 were expressed from a polycistronic mRNA. This differs from the complex transcription pattern in TGMV-infected plants, which contains five AL transcripts. There was no quantitative correlation between the efficiency of complementation in the infectivity assay and the level of expression of transgenic AL RNA in the leaves of a transgenic line. One line that failed to complement defects in AL1, AL2, and AL3 in infectivity assays contained high levels of transgenic AL RNA and functional AL1 protein. These results provide evidence that chromosomal position can affect the cell- and tissue-specific transcription of the 35S promoter in transgenic plants. Comparison of the complementing plants and wild-type infected plants may provide insight into the TGMV infection process and the use of the CaMV 35S promoter for gene expression in transgenic plants.

INTRODUCTION

Geminiviruses are a group of plant DNA viruses that infect both dicots and monocots, and include tomato golden mosaic virus (TGMV), maize streak virus, and beet curly top virus (for review see Davies, Townsend, and Stanley, 1987; Lazarowitz and Pinder, 1988; Davies and Stanley, 1989). They fall into two subgroups characterized by their insect vector and by the structure of their genome, which exist in either single or two-component configurations. Both genome components of the two component viruses are required for infectivity (Hamilton et al., 1983; Stanley, 1983). The genomes of geminiviruses are small, circular DNAs that are packaged as single strands in virions and occur as single and double strands in plants. Geminivirus DNA is infectious at low efficiency when mechanically inoculated (Hayes et al., 1988b) or at high efficiency when inoculated via *Agrobacterium* (Grimsley et al., 1987; Elmer et al., 1988b; Hayes, Coutts, and Buck, 1988a). Comparison of the nucleotide sequences of several geminivirus genomes has revealed that their gene organization is conserved (Stanley and Gay, 1983; Hamilton et al., 1984; Lazarowitz, 1988), with the open reading frames arranged and transcribed divergently (Morris-Krsinich et al., 1985; Townsend et al., 1985; Hanley-Bowdoin, Elmer, and Rogers, 1988; Sunter, Gardiner, and Bisaro, 1989). The A and B genome components of TGMV contain four and two open reading frames, respectively. The rightward open reading frame (AR1) of TGMV A encodes the viral coat protein (Kallender et al., 1988), which is not required for symptom development in plants (Gardiner et al., 1988). In earlier studies, we assigned possible functions to the other proteins encoded by TGMV by determining the phenotypes conferred by mutations in each open reading frame (Elmer et al., 1988a). These experiments established that AL1, the first leftward open reading frame of the A component, encodes the only viral protein essential for DNA replication. The precise role of AL1 in viral DNA replication remains to be elucidated. These and other studies showed that AL2, BR1, and BL1 mutants are not infectious, and that AL3 mutants display delayed and attenuated symptoms (Brough et al., 1988; Elmer et al., 1988a; Etessami et al., 1988). The phenotypes of the mutants implicate AL2, BR1, and BL1 in systemic movement of the virus and AL3 in symptom development. The results clearly demonstrate that the products of the AL2, AL3, BR1, and BL1 genes

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are central to the TGMV infection process, but nothing is known about the functions of these proteins.

One approach to elucidating the various functions of the proteins encoded by a plant virus is to construct transgenic plants with integrated copies of the viral genes. The transgenic plants expressing the integrated viral genes can be used to study the roles of different viral proteins during infection and generation of symptoms. Using transgenic plants containing integrated multimers of TGMV A and B, we have shown that TGMV functions are divided between the two genome components and confirmed that both DNAs are essential for productive infection (Rogers et al., 1986). Transgenic plants have also been used successfully to identify the single genes for the movement protein of tobacco mosaic virus (Deom, Oliver, and Beachy, 1987), a protein that may be involved in cauliflower mosaic virus symptom production (Baughman, Jacobs, and Howell, 1988), and the replicase of alfalfa mosaic virus (van Dun, van Vloten-Doting, and Bol, 1988). We describe here the construction of transgenic tobacco plants that contain the leftward open reading frames of TGMV A under the control of the cauliflower mosaic virus (CaMV) 35S promoter. We demonstrate that these transgenic plants produce functional AL1, AL2, and AL3 proteins by complementation of TGMV A mutants in infectivity assays. These transgenic plants can be used to identify and characterize the functions of AL1, AL2, and AL3 separate from other viral activities.

RESULTS

Complementation of TGMV AL Mutants and TGMV B by pMON450 Transgenic Plants

The left side of TGMV A (Figure 1A) contains three overlapping open reading frames—AL1, AL2, and AL3—oriented in the same direction (Hamilton et al., 1984). Previous studies demonstrated that the products encoded by AL1, AL2, and AL3 are all required for full infectivity of TGMV (Elmer et al., 1988a). We obtained transgenic *Nicotiana benthamiana* plants that contain the AL1, AL2, and AL3 open reading frames under the control of the CaMV 35S promoter by *Agrobacterium*-mediated transformation using the binary vector pMON450 (Figure 1B). The transgenic plants were phenotypically normal and fertile. R1 progeny plants were assayed for their capacity to complement mutations in all three AL open reading frames. As shown in Table 1, seven out of 10 transgenic lines complemented mutations in AL1, with 13% to 94% of the kanamycin-resistant progeny plants showing symptoms after agroinoculation of mutant A component DNA (pMON441) and wild-type B component DNA (pMON393). The timing and severity of viral symptoms were indistinguishable from those of transgenic or wild-type plants

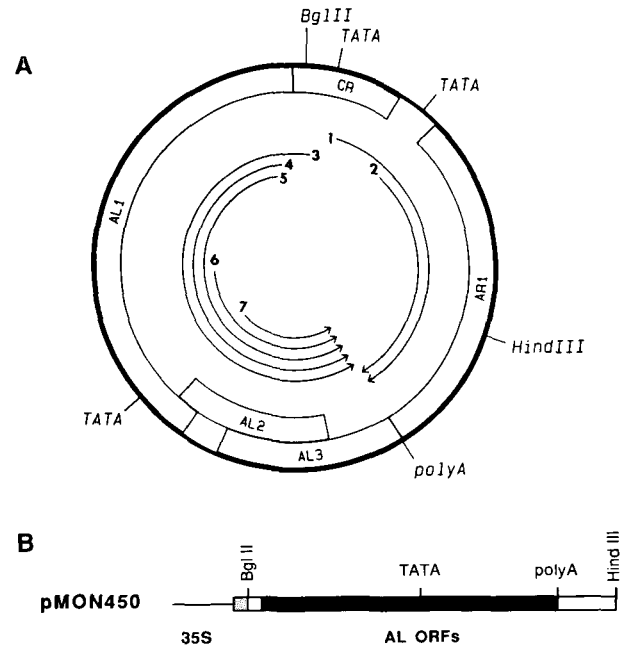

Figure 1. Diagram of the A Genome Component of TGMV and the TGMV A DNA Insert in pMON450.

(A) The 2588-bp, circular DNA of TGMV A is illustrated. TGMV A has four open reading frames, designated by the open boxes: AR1, AL1, AL2, and AL3. The 230-bp sequence that is highly conserved between the A and B genome components of TGMV is indicated by the open box CR (common region). The nucleotide positions of TGMV A DNA are numbered clockwise from the left border of CR (position 0). The RNAs transcribed from TGMV A are shown by the arrows, which indicate the direction of transcription and the range of each RNA. The RNAs are named according to their 5′ ends and direction of transcription: 1, AR137; 2, AR319; 3, AL61; 4, AL2540; 5, AL2515; 6, AL1935; 7, AL1629 (see Figure 4; Hanley-Bowdoin et al., 1988; Sunter et al., 1989). The 3′ ends of the RNAs were inferred from the position of the bidirectional polyadenylation site and the sizes of TGMV A RNAs (Sunter et al., 1989). Potential TATA and polyadenylation signals for transcription of TGMV A are indicated. The BglII and HindIII sites used in the construction of pMON450 are marked.

(B) The BglII-HindIII insert of TGMV A DNA in the binary plant transformation vector pMON450 is shown. The coding sequences for AL1, AL2, and AL3 are indicated by the black box. Other TGMV A DNA sequences are marked by the open boxes. These sequences include 30 bp of the 61-bp 5′ leader of AL1 and the bidirectional polyA site between the 3′ ends of the left and right open reading frames of TGMV A. The AL open reading frames are located downstream of a 327-bp fragment of the CaMV 35S promoter region, which includes 30 bp of transcribed sequences (shaded box).

Table 1. Complementation of AL1 Mutant TGMV by pMON450 Transgenic Tobacco Plants

Line	Copy Number[a]	Relative[b] AL1 RNA	Complementation Efficiency[c] of Kan^r R1 Plants
9982	1	6	94% (16/17)
9969	>1	5	94% (16/17)
9972	1	3	89% (16/18)
9978	1	5	78% (14/18)
9676	>1	88	67% (12/18)
9970	1	5	61% (11/18)
9971	>1	7	13% (2/15)
9674	>1	23	0% (0/48)
9968	>1	6	0% (0/17)
9966	1	0	0% (0/12)

[a] The copy number of independent T-DNA insertions was estimated from the frequency of kanamycin-resistant R1 progeny.
[b] The S1 hybridization signal for 35S-AL RNA in uninfected, kanamycin-resistant R1 plants was normalized relative to AL61 RNA in wild-type infected plants.
[c] Kanamycin-resistant R1 plants were inoculated with mixed cultures of *Agrobacterium* containing pMON441 and pMON393. Complementation frequency was scored as infected/inoculated.

inoculated with wild-type TGMV DNAs (data not shown). The symptoms were not due to reversion of the TGMV A mutations because crude virus isolated from these plants did not produce symptoms on wild-type plants and could only be propagated on plants transformed with pMON450 (data not shown). Table 2 shows that similar complementation frequencies were observed for three AL1 mutants (pMON421, pMON441, pMON446) and one AL3 mutant (pMON438) when co-inoculated with the B component on four transgenic lines. Complementation of an AL2 mutant (pMON422) was much less efficient. After agroinoculation with TGMV B DNA (pMON393) alone, R1 progeny of two of the four lines developed symptoms at a frequency similar to that of AL2 complementation (Table 2), demonstrating that AL1, AL2, and AL3 can be expressed simultaneously in the same plant.

The complementing plants are able to support replication and systemic movement of TGMV DNA. We isolated total DNA from systemically infected leaves and analyzed it by DNA gel blot hybridization using TGMV A- and B-specific probes. As seen in Figure 2, double- and single-stranded forms of the A and B components were detected in symptomatic tissue from plants co-inoculated with an AL1 (pMON441, lane 2), an AL2 (pMON422, lane 3), or an AL3 mutant (pMON438, lane 4), and wild-type B component DNA (pMON393). TGMV B DNA was also detected in symptomatic tissue from plants agroinoculated only with the B component (pMON393, lane 5). (The single-stranded forms of A and B component DNAs were detected in lanes 4 and 5 upon long exposure of the autoradiogram.) The amount of TGMV DNA, in particular the A component, was lower in complementing plants inoculated with TGMV A mutants than in transgenic plants infected with wild-type TGMV DNA (cf. lane 1 with lanes 2 to 5). The level of TGMV B DNA and the ratio of single- to double-stranded TGMV DNA were reduced in plants inoculated with an AL3 mutant (lane 4) or B (lane 5) alone.

A Single AL RNA Species Is Produced by the pMON450 Transgenic Plants

The different complementation efficiencies observed for the AL1 and AL3 mutants and the AL2 mutant may reflect different expression patterns for the AL open reading frames in infected and transgenic plants. We examined this possibility at the level of transcription by characterizing and comparing the AL RNAs found in healthy transgenic and infected wild-type plants.

We mapped the 5′ ends of the AL transcripts in TGMV-infected plants by S1 nuclease protection and primer extension. The three single-stranded TGMV A S1 probes, which span the AL open reading frames and were used to protect RNA from systemically infected *N. benthamiana*

Table 2. Complementation of AL1, AL2, AL3, and TGMV B by pMON450 Transgenic Tobacco Lines

Inoculated Constructs	Genotype	Complementation Efficiency of Kan^r R1 Plants[a]			
		9676	9972	9978	9982
pMON337/393	Wild-type A + B	91% (10/11)	100% (12/12)	100% (6/6)	100% (6/6)
pMON421/393	AL1 mutant + B	60% (12/20)	92% (11/12)	83% (10/12)	92% (11/12)
pMON441/393	AL1 mutant + B	67% (12/18)	87% (26/30)	87% (26/30)	96% (27/28)
pMON446/393	AL1 mutant + B	90% (9/10)	100% (12/12)	100% (12/12)	100% (12/12)
pMON422/393	AL2 mutant + B	0% (0/18)	0% (0/6)	25% (3/12)	17% (1/6)
pMON438/393	AL3 mutant + B	71% (12/17)	100% (12/12)	100% (12/12)	100% (12/12)
pMON393	B	0% (0/12)	0% (0/6)	17% (1/6)	33% (2/6)

[a] Kanamycin-resistant R1 plants of each pMON450 line were inoculated with mixed cultures of *Agrobacterium* containing the listed constructs. Complementation frequency was scored as infected/inoculated.

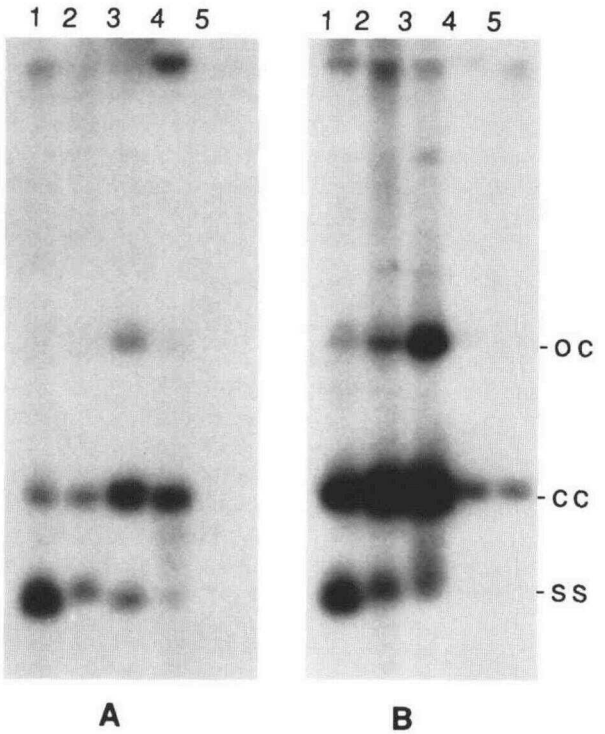

Figure 2. Replication of TGMV DNA in pMON450 Transgenic Plants Agroinoculated with TGMV A Variants and TGMV B.

DNA was isolated from systemically infected leaves of *N. benthamiana* plants agroinoculated with TGMV A and/or TGMV B. Total DNAs from transgenic pMON450 R1 plants (line 9982) inoculated with mixed cultures of *Agrobacterium* carrying pMON337 (wild-type TGMV A, lane 1), pMON441 (AL1 mutant, lane 2), pMON422 (AL2 mutant, lane 3), or pMON438 (AL3 mutant, lane 4), and pMON393 (wild-type TGMV B) were electrophoresed in duplicate. In lane 5, DNA from symptomatic leaf tissue of line 9982 R1 plants agroinoculated with pMON393 alone was resolved. Fivefold more DNA was electrophoresed in lanes 2 to 5 than lane 1. The DNAs were transferred and hybridized with [^{32}P]dCTP-labeled DNA probes.

(A) The blot was hybridized with a 2.3-kb TGMV A-specific probe.

(B) The blot was hybridized with a 1.5-kb TGMV B-specific probe. Neither probe included common region sequences. TGMV DNA forms are indicated: oc, double-stranded open circles; cc, double-stranded closed circles; ss, single-stranded circles.

leaves, are illustrated in Figure 3D. A 746-base SalI-XhoI probe 5′ end-labeled at the SalI site (probe A) protected three RNAs (Figure 3A, lane 2). A single RNA was detected using a 631-base NheI-RsaI probe 5′ end-labeled at the NheI site (probe B; Figure 3B, lane 2). Similarly, a single predominant RNA was detected using a 703-base BamHI-NcoI probe 5′ end-labeled at the BamHI site (probe C; Figure 3C, lane 2). Many larger protected fragments were reproducibly observed with probe C and may reflect minor

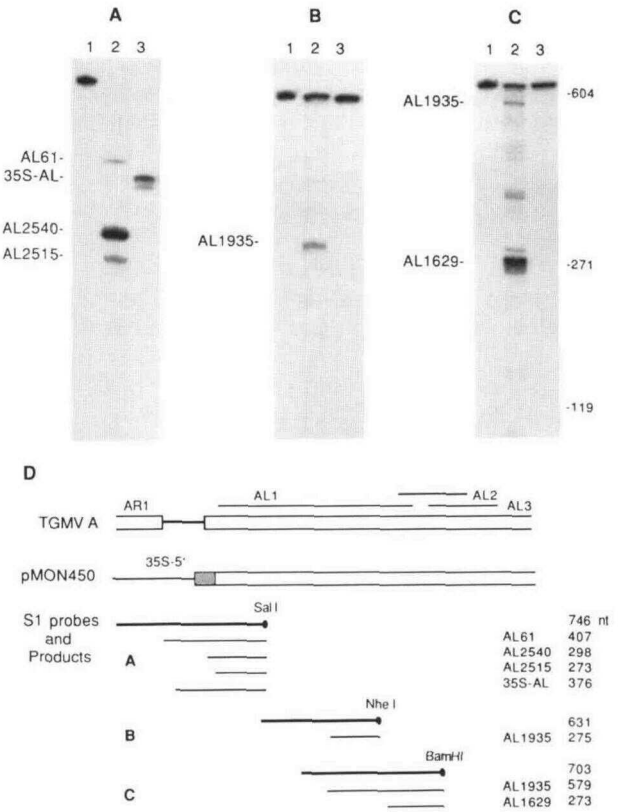

Figure 3. Analysis of AL RNAs from TGMV-Infected and pMON450 Transgenic Plants.

(A), **(B)**, and **(C)** Total RNA (20 μg) isolated from systemically infected leaves of wild-type *N. benthamiana* plants agroinoculated with pMON337 and pMON393 was assayed by S1 nuclease protection in lane 2 using probes A, B, and C shown in **(D)**. Total leaf RNA (20 μg) from healthy pMON450 transgenic R1 plants (line 9982) was protected from S1 nuclease digestion in lane 3 by probes A, B, and C. Undigested probes A, B, and C were electrophoresed in lane 1. The protected products corresponding to viral RNAs AL61, AL2540, AL2515, AL1935, and AL1629 are indicated. The protected product resulting from transgenic AL RNA is designated as 35S-AL. The numbers on the left correspond to the sizes and positions of [^{32}P]φX174-HaeIII DNA markers.

(D) The single-stranded S1 probes that were isolated from TGMV A DNA and used to analyze infected and transgenic AL RNAs and their reaction products are compared with TGMV A and pMON450. The AL1, AL2, AL3, and AR1 coding sequences in TGMV A and pMON450 are illustrated by the open boxes and the lines above TGMV A. The TGMV A common region is shown by the heavy line. The CaMV 35S promoter region of pMON450 is indicated by the shaded box and the fine line. The letter designating each probe corresponds to the panel in which it was used. The position of the ^{32}P-labeled 5′-end of each probe is marked (●). The sizes in nucleotides (nt) of the probes and reaction products are listed.

transcripts or artifacts due to RNAs that initiate upstream. Upstream RNAs also resulted in full-length protection of probes B and C (Figures 3B and 3C, lane 2). The S1-protected products may reflect transcription start sites, 3′ splice sites of processed RNAs, or AT-rich regions that are susceptible to nuclease digestion. To distinguish between these possibilities, 21-bp synthetic oligonucleotides corresponding to the 5′ ends of each S1 probe were used in primer extension assays of AL RNAs, and the primer extension products were compared to the S1-protected products (data not shown). The same products were detected with S1 probes A and C and their respective primers, verifying that the reaction products correspond to the 5′ ends of AL RNAs. In contrast, three primer extension products were observed with the primer corresponding to S1 probe B, two that were larger than the S1 product and one that was the same size. One possible explanation is that the AL RNA detected with S1 probe B and its primer is a processed species, and that the band seen in Figure 3B (lane 2) reflects a 3′ splice site. This possibility will be explored further by sequence analysis of AL cDNA clones. The 5′ ends (AL61, AL2540, AL2515, AL1935, and AL1629) of the AL RNAs in Figure 4 were determined by comparison of the primer extension and S1 products with dideoxy sequencing ladders generated using the corresponding primer (data not shown). Multiple RNAs corresponding to the leftward open reading frames of TGMV A have also been characterized by Sunter et al. (1989).

In contrast to the complex AL transcription pattern in infected wild-type tissue, a single AL RNA species was detected in healthy transgenic plants (Figure 3A, lane 3). The S1 assay product (35S-AL) in transgenic plants corresponds to the break in homology at the junction between wild-type TGMV A sequences and the 35S promoter. The lower band of the doublet varied in intensity between experiments and was probably due to S1 nuclease trimming of the larger band. The 5′ end of the transgenic AL RNA is located within the 35S 5′ sequences and results from 35S promoter activity. The AL2540, AL2515, AL1935, and AL1629 TGMV transcripts were not detected in healthy transgenic plants even though the 5′ ends of these RNAs are present in pMON450 DNA (Figure 1A). The same protection patterns were seen for all the pMON450 lines. These results show that the AL open reading frames are not transcribed or processed in the transgenic plants in the same manner as in infected plants. The existence of a single 35S-AL RNA suggests that the AL1, AL2, and AL3 polypeptides are translated from a tricistronic mRNA in the transgenic plants. In infected plants, the AL proteins are probably translated from individual or bi-cistronic mRNAs, which may have different translational efficiencies than the 35S-AL mRNA.

					+1 ●
AL61	AATTCTAATA	TATAAGAGCT	ACCTTACTAC	CAATTCATAT	**AA**CTTTTGGA
AL2540	TTTCAAATAA	ATGCCAAAAA	TTATTTTCTT	ACATATCCTC	**A**GTGCTCCTT
AL2515	TTCTTACATA	TCCTCAGTGC	TCCTTGTCCA	AAGAAGAATC	**A**CTTTCTCAA
AL1935	CGCTGCGCGG	CCGGAGAGAC	CTATTAGTAT	TATCATCGAG	**G**GCGATAGTC
AL1629	GGTGCTAGGC	TATAAAGTTT	TCCTCGACAA	AGAGGAAAAC	**A**CTCCACTAA

Figure 4. DNA Sequences at the 5′ ends of AL RNAs.

The 5′ ends of AL RNAs were mapped by co-electrophoresis of S1 protection and primer extension products of total TGMV-infected leaf RNA and dideoxy DNA sequencing ladders primed by the matching 21-nucleotide synthetic oligonucleotide. The 5′ ends of the two primers used in the analysis corresponded to the 5′ ends of the S1 probes A and C in Figure 3D. The 5′ ends of the three transcripts detected using S1 probe A (Figure 3A, lane 2) were mapped to positions 61, 2540, and 2515 on the TGMV A genome. The 5′ end of the major transcript detected using S1 probe C (Figure 3C, lane 2) was mapped to position 1629. The 5′ end of the RNA detected with the S1 probe B (Figure 3B, lane 2) was mapped to position 1935 by co-electrophoresis of the S1 product with a dideoxy sequencing ladder generated from its matching 5′ primer. The 5′ ends of the AL RNAs are shown in bold letters. Potential TATA sequences (AL61 and AL1629) and a putative 3′ splice consensus sequence (AL1935) are underlined.

Tissue-Specific Expression of 35S-AL1,2,3 Gene in Transgenic Plants

There is no quantitative correlation between the capacity of different pMON450 *N. benthamiana* lines to complement AL1 mutations and the steady-state levels of total leaf 35S-AL RNA expressed by the lines (Table 1). This is most clearly demonstrated with line 9674, which over-expressed 35S-AL RNA 23-fold relative to AL61 RNA, the only AL RNA that can encode a full-length AL1 protein in infected plants. Line 9674 failed to complement mutations in AL1 (Table 1), AL2, and AL3 (data not shown) in infectivity assays. Two possible explanations for the failure of line 9674 to complement AL mutations are that line 9674 does not express functional AL proteins or does not express them in plant tissues capable of propagating TGMV. We have used a transient assay for TGMV DNA replication to examine these possibilities for AL1. Leaf discs from wild-type, 9676, and 9674 plants were co-cultured with *Agrobacterium* containing pMON393 with one and one-half tandem copies of TGMV B DNA in the T-DNA. In Figure 5, total DNA isolated from the leaf discs 5 days after inoculation was analyzed by DNA gel blot hybridization using a TGMV B probe. Double- and single-stranded forms of TGMV B DNA were detected in 9676 (lane 3) and 9674 (lane 4) leaf discs, whereas no free episomal B DNA was detected in wild-type leaf discs (lane 2). These results demonstrated that leaf discs from line 9674 express functional AL1 and can supply it in *trans* for the replication of TGMV B DNA in leaf discs. Protein gel blot analysis has also shown that full-length AL1 protein is present in 9674 plants at levels comparable with those detected in plants

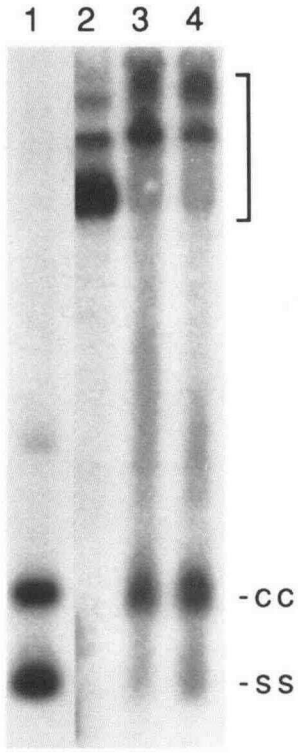

Figure 5. Transient TGMV B DNA Replication by Complementing and Noncomplementing Transgenic pMON450 Lines.

DNA was isolated from *N. benthamiana* leaf discs cultured with *Agrobacterium* carrying pMON337 (TGMV A) and/or pMON393 (TGMV B). Total DNA from wild-type leaf discs agroinoculated with pMON337 and pMON393 was electrophoresed in lane 1. Total DNAs from wild-type (lane 2), pMON450 line 9676 (lane 3), and pMON450 line 9674 (lane 4) leaf discs agroinoculated with pMON393 alone were also resolved. The DNAs were transferred and hybridized with a 1.5-kb TGMV B-specific probe labeled with [^{32}P]dCTP. TGMV DNA forms are indicated: cc, double-stranded closed circles; ss, single-stranded circles. The signal marked by the bracket reflects hybridization to *Agrobacterium* vector DNA containing TGMV B sequences and trapping of TGMV DNA by high molecular weight plant chromosomal DNA.

of the complementing lines 9676 and 9982 (data not shown). These results suggest that AL1 is expressed in the appropriate tissues for TGMV infection in 9676 and 9982 plants but not in 9674 plants, thereby providing evidence that chromosomal position may affect the tissue-specific activity of the 35S promoter in transgenic plants.

DISCUSSION

We have constructed transgenic tobacco plants that contain the leftward open reading frames of TGMV A under the control of the CaMV 35S promoter. We identified transgenic plants that express functional AL proteins by complementation of TGMV A mutants. In the complementing plants, the severity and timing of TGMV symptoms were similar to those of plants infected with wild-type viral DNA. Upon closer examination, we have found that there are several important differences between the complementing plants and wild-type infected plants. These differences may provide insight into the TGMV infection process and the use of the CaMV 35S promoter for gene expression in transgenic plants.

Transcription of the leftward open reading frames of TGMV A in infected tissue is complex, resulting in at least five AL RNAs. AL61 is the only RNA that can encode full-length AL1. None of the other AL RNAs includes the initiator methionine codon for AL1. [A 41-kD polypeptide has been detected in infected tissue, verifying the AL1 translation start site (L. Hanley-Bowdoin, J.S. Elmer, and S.G. Rogers, manuscript submitted).] The entire AL2 and AL3 open reading frames are present on all five AL RNAs. Four of the AL RNAs are derived from different transcription start sites located at TGMV A positions 61, 2540, 2515, and 1635 (Figure 4). Only positions 61 and 1635 are preceded by putative TATA boxes. The leftward open reading frames of TGMV B (L. Hanley-Bowdoin, unpublished results; G. Sunter and D. Bisaro, personal communication) and the single genome component of *Digitaria* streak virus also show complex transcription patterns (Accotto, Donson, and Mullineaux, 1989). The fifth AL transcript, AL1935, may be a spliced RNA with its 3′ splice site at position 1935 on the TGMV A component (Figure 4). It is interesting that the nucleotide sequence surrounding position 1935 conforms to the consensus sequence for eucaryotic 3′ splice sites (Brown, 1986). Further experiments are in progress to determine whether AL1935 is a spliced RNA and to identify its putative 5′ exon. Spliced RNA derived from the leftward transcription unit has been found in leaves infected with the single component geminiviruses, *Digitaria* streak virus (Accotto et al., 1989), and wheat dwarf virus (Schalk et al., 1989). The widespread occurrence of multiple transcription start sites and spliced RNAs suggests that transcription initiation and RNA processing may be involved in controlling gene expression of the leftward open reading frames of geminiviruses.

Comparison of the AL RNAs in TGMV-infected and healthy transgenic tissues revealed that the expression patterns are very different in the two tissues. In contrast to the complex AL RNA profile seen in infected tissue, a single AL RNA resulting from 35S promoter activity is found in transgenic tissue. None of the TGMV AL RNAs whose transcription start sites are present in the integrated TGMV A DNA was detected. Despite the sensitivity of the single-stranded S1 assay, the TGMV AL RNAs could be present in transgenic tissue at low levels. The absence or very low levels of the TGMV AL RNAs may reflect their weak promoters and the low copy number of the integrated

DNA relative to freely replicating TGMV A DNA in infected tissue (L. Hanley-Bowdoin, unpublished results). Clearly, the upstream 35S enhancer did not significantly increase the activities of the AL promoters in the transgenic plants. Strong transcription from the 35S promoter may also interfere with the transcription of the weak TGMV AL promoters. An alternative and interesting explanation is that the expression of the TGMV AL RNAs may be temporally regulated during infection by earlier events in the infection process that do not occur in healthy transgenic plants. Temporal regulation of transcription and RNA processing is well documented for other eucaryotic viruses (Khoury and May, 1977; Nevins et al., 1979; Nevins and Wilson, 1981; Hyde-DeRuyscher and Carmichael, 1988).

Some lines of tobacco transformed with pMON450 complement mutations in all three TGMV AL open reading frames and can support the replication and systemic movement of TGMV B alone. However, the transgenic lines complement TGMV AL1 and AL3 mutants much more efficiently than they complement AL2 mutants (Table 2). The efficiency of TGMV B complementation may be limited by AL2. The lower complementation efficiency of AL2 may reflect less efficient expression of AL2 than AL1 and AL3 in the transgenic plants. The single AL RNA in the transgenic plants most likely functions as a polycistronic message for all three AL proteins. Complementation of TGMV B suggested that AL1, AL2, and AL3 are translated from the polycistronic RNA in the same transgenic plant. Polycistronic mRNAs have been characterized in other eucaryotes (Kozak, 1986; Sample et al., 1986; Chang et al., 1989). In infected tissue, each of the AL polypeptides may be translated from a different mRNA. AL1 is translated from the first methionine codon in AL61. All five of the AL RNAs in infected tissue contain full AL2 and AL3 open reading frames, and could be translated to produce these proteins. There is one important difference between AL2 and AL3 translation in infected tissue. AL2 translation could initiate at the first methionine codon in AL1629, whereas AL3 translation must always initiate at an internal methionine. The sequence surrounding the AL3 translation start site shows strong homology to the consensus sequence flanking plant translation start sites (Joshi, 1987), and may be optimized for translation initiation from an internal methionine codon. These observations have important implications for the translation of the polycistronic message found in transgenic tissue. AL1, by virtue of being the first open reading frame, and AL3, which may be optimized for internal translation initiation, may both be translated efficiently from the polycistronic RNA, whereas AL2 may be translated poorly. The AL1 and AL2 open reading frames overlap, and upstream AL1 translation may interfere with the initiation of AL2 translation (Hughes et al., 1984; Liu, Simonsen, and Levinson, 1984; Peabody, Subramani, and Berg, 1986), further reducing the efficiency of AL2 expression in the transgenic plants. The AL1 and AL3 open reading frames do not overlap, and AL1 translation would not interfere with the initiation of AL3 translation. The relative translational efficiencies of the AL proteins will be determined when antibodies to all three polypeptides are available. In the present experiments, we have not eliminated the possibility that more AL2 protein is required than AL1 and AL3, or that very little AL3 is required for productive TGMV infection. Antibodies will also allow us to address these possibilities.

Earlier studies have established that there are large variations in the expression of integrated genes by transgenic plants (Jones, Dunsmuir, and Bedbrook, 1985; Nagy et al., 1985; Odell, Nagy, and Chua, 1987). We observed a broad range of transgenic AL RNA expression among the different pMON450 lines (Table 1). The capacities of the different lines to complement TGMV mutants also varied widely. However, there was no quantitative correlation between the level of 35S-AL RNA and complementation efficiency. This lack of correlation is most likely due to a combination of two factors, i.e., a nonuniform expression pattern of the integrated 35S-AL gene and a tissue-specific requirement for TGMV propagation. Recent studies have shown that the CaMV 35S promoter can be divided into discrete domains that can confer different tissue-specific expression patterns (Benfey and Chua, 1989; Benfey, Ren, and Chua, 1989; Fang et al., 1989). These results suggest that the 35S promoter is analogous to other complex eucaryotic promoters composed of several *cis* elements that can show tissue specificity (Zenke et al., 1986; Ondek, Shepard, and Herr, 1987; Nomiyama et al., 1987; Schirm, Jiricny, and Schaffner, 1987). In the case of the 35S promoter, each independent *cis* element may be affected differently by chromosomal position in transgenic plants, and independent transgenic lines may have different tissue expression patterns dependent on the site of insertion. This hypothesis is supported by the variable patterns of β-glucuronidase activity observed in plants transformed with 35S-β-glucuronidase constructs (Jefferson, Kavanagh, and Bevan, 1987). Similarly, the 35S-AL expression pattern may not be uniform across all the pMON450 lines. The transgenic lines that complement AL mutations most efficiently may express high levels of AL proteins in TGMV target tissues, whereas those lines that fail to complement TGMV A mutations may not express the 35S-AL gene in these tissues. Geminiviruses are associated with the phloem (Rushing et al., 1987), and it is likely that complementing lines express the 35S-AL gene in phloem companion cells. The RNA analysis of the pMON450 transgenic lines was performed using leaf RNA, and the level of 35S-AL leaf RNA may not reflect the level in phloem. We have demonstrated that cells in leaf discs from line 9674 can support TGMV B replication and, therefore, produce functional AL1 protein. However, line 9674 fails to complement TGMV AL1 mutants in infectivity assays and must not be expressing AL1 in the appropriate tissues of the plant for systemic infection. Future in situ hybridization studies on the distribution of AL RNA in line

9674 as compared with a complementing transgenic line will address this question directly. Hammer et al. (1987) have shown, using transgenic mice, that chromosomal position can independently affect the activities of α-fetoprotein promoter enhancer elements in a tissue-specific manner, analogous to the tissue-specific effect of position on 35S promoter activity that we observed in the pMON450 transgenic plants.

No functions have been assigned to the proteins encoded by AL2 and AL3, and the precise role of AL1 in viral DNA replication remains to be determined. We have demonstrated that functional AL1, AL2, and AL3 can be produced from a single chromosomal gene in transgenic plants. These plants show normal morphology and fertility, demonstrating that AL1, AL2, and AL3 are not by themselves determinants of disease or pathogenesis. The transgenic plants can now be used to study the activities of the proteins separate from other viral activities. We have constructed transgenic lines that only express AL1 (L. Hanley-Bowdoin, J.S. Elmer, and S.G. Rogers, manuscript submitted) and are currently using these lines to characterize AL1 selectively. The transgenic lines described here will also enable us to examine the functions of BL1 and BR1, especially as they relate to AL gene expression. The transgenic plants may also provide insight into the identity and the character of plant cells that replicate TGMV and how the virus moves through the plant.

METHODS

Enzymes

RQ1 deoxyribonuclease and RNasin ribonuclease inhibitor were from Promega. Nuclease S1, polynucleotide kinase, calf intestine alkaline phosphatase, and AMV reverse transcriptase were from Boehringer Mannheim Biochemicals. Sequenase T7 DNA polymerase and *Escherichia coli* DNA polymerase (Klenow fragment) were from United States Biochemical Corporation and Pharmacia LKB Biotechnology, Inc., respectively. All other enzymes were from New England Biolabs, Inc.

Plasmids

The A and B components of TGMV were cloned into the binary T-DNA vector pMON505 (Horsch and Klee, 1986) as one and one-half copy tandem repeats to give pMON337 and pMON393, respectively (Elmer et al., 1988b). Derivatives of plasmid pMON337, modified by site-directed mutagenesis of the AL1 open reading frame (Elmer et al., 1988a), were made and designated as pMON421 (12-bp deletion from positions 1810 to 1821), pMON441 (4-bp insertion at position 2242), and pMON446 (1-bp deletion at position 1602). Derivatives of pMON337 with mutations in the AL2 and AL3 open reading frames and designated as pMON422 and pMON438, respectively, were also constructed. Plasmid pMON422 contains a 1-bp insertion at position 1530, and pMON438 has an 84-bp deletion from positions 1124 to 1208.

Figure 1A shows TGMV A DNA modified by insertion of the trinucleotides, GTA and TCT, into the AL1 RNA leader region at map coordinates 16 and 28 to create NdeI and BglII sites, respectively (Elmer et al., 1988a). A HindIII linker was also inserted into the ScaI site at position 791 in the AR1 open reading frame (Elmer et al., 1988b). The AL1, AL2, and AL3 open reading frames were excised on a 1.8-kb BglII-HindIII fragment and cloned into the binary plant transformation vector pMON530 (Rogers et al., 1987) to give pMON450 (Figure 1B). In pMON450, the AL1, AL2, and AL3 open reading frames are located downstream of the CaMV 35S promoter and upstream of the TGMV A polyadenylation signal and the nopaline synthase (NOS) 3′ end.

Transgenic Plants, Whole Plant Infectivity Assay, and Leaf Disc Assay

Nicotiana benthamiana was transformed by *Agrobacterium tumefaciens* containing the disarmed pTiB6S3-SE plasmid and pMON450 (Rogers et al., 1986). Transgenic plants were identified by their kanamycin-resistant phenotype. All transgenic plants containing geminivirus components in their genomes were propagated in limited access growth chambers. Wild-type and transgenic *N. benthamiana* plants were stem-inoculated using mixed cultures of *Agrobacterium* containing pTiB6S3-SE and binary vectors carrying one and one-half tandem copies of wild-type or mutated TGMV A DNA and/or wild-type TGMV B DNA (Elmer et al., 1988b). The plants were scored for the appearance and severity of symptoms. AL1 and AL2 complementation was scored for wild-type symptoms as compared with no symptoms for AL1 and AL2 mutants. AL3 complementation was scored for wild-type symptoms that developed 7 days to 14 days after inoculation as compared with the attenuated and delayed (21 days to 28 days) symptoms seen with AL3 mutants inoculated onto wild-type plants. Complementation of TGMV B DNA by AL1, AL2, and AL3 was assessed by the appearance of attenuated symptoms. Crude virus was isolated and mechanically inoculated onto transgenic and wild-type plants as described elsewhere (L. Hanley-Bowdoin, J.S. Elmer, and S.G. Rogers, manuscript submitted). Leaf discs from wild-type and transgenic *N. benthamiana* plants were agroinoculated with pMON393 and assayed for replication of TGMV B DNA (Elmer et al., 1988a).

DNA Isolation and Analysis

Total DNA was isolated from 0.5 g of infected leaves or leaf discs (Dellaporta, Wood, and Hicks, 1983). The DNA (about 10 μg/lane) was electrophoresed in 1.2% agarose/0.4 M Tris-acetate, pH 8, 1 mM EDTA gels and analyzed by DNA gel blot hybridization (Thomashow et al., 1980). TGMV A- and B-specific probes were radiolabeled using random oligonucleotides and *E. coli* DNA polymerase (Klenow fragment).

RNA Isolation and Analysis

Total RNA was isolated from infected leaves and treated with RQ1 DNase as described previously (Nagy et al., 1986; Hanley-Bowdoin et al., 1988). TGMV A RNAs were analyzed by S1 nuclease protection (Berk and Sharp, 1977) using single-stranded DNA probes that were labeled with ^{32}P at their 5′ ends (Hanley-

Bowdoin, Orozco, and Chua, 1985). The reaction products were resolved and quantitated as described previously (Hanley-Bowdoin et al., 1988). The 5′ ends of TGMV A RNAs were mapped by primer extension (Day et al., 1987) using 21-bp synthetic oligonucleotide primers that were labeled with ^{32}P at their 5′ ends. RNasin (500 units/mL) was included in the primer extension reactions (de Martynoff, Pays, and Vassart, 1980). The primer extended products were co-electrophoresed with dideoxy sequencing ladders generated from TGMV A DNA using the same primers.

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