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Transient expression of heterologous RNAs using tomato golden mosaic virus

Linda Hanley-Bowdoin*, J.Scott Elmer and Stephen G.Rogers

Plant Molecular Biology, Corporate Research Laboratories, Monsanto Company AA2G, 700 Chesterfield Village Parkway, St Louis, MO 63198, USA

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ABSTRACT

The genome of the geminivirus tomato golden mosaic virus (TGMV) consists of two circular DNA molecules designated as components A and B. The A component contains the only virally-encoded function required for autonomous replication in infected plant cells. We used agroinoculation of petunia leaf discs with the A component to develop a transient expression system which permits direct examination of viral transcripts by S1 nuclease protection. The AR1 gene, which encodes the TGMV coat protein, was transcribed transiently in leaf discs after agroinoculation of TGMV A DNA. Synthesis of AR1 RNA was dependent on T-DNA transfer and TGMV DNA replication, demonstrating that it is a plant transcription product. The AL open reading frames of TGMV A were also expressed transiently in leaf discs. The ratio between AR1 RNA and the major leftward RNA was constant and was used to normalize AR1 transcription for viral DNA copy number. The bacterial genes encoding chloramphenicol acetyltransferase (CAT) and beta-glucuronidase (GUS) were transiently expressed in leaf discs from the AR1 promoter in TGMV A. The levels of AR1 and GUS RNAs were similar in leaf discs after adjusting for viral DNA copy number, while CAT RNA was less abundant. The geminivirus transient expression system allows rapid analysis of RNAs transcribed from foreign genes and can serve as a preliminary screen in the construction of transgenic plants.

INTRODUCTION

Transient expression systems have been instrumental in defining the mechanisms involved in eucaryotic gene expression. They provide information rapidly and with minimal effort in comparison to analogous experiments in transgenic organisms. In animal cells, transient expression has been used to characterize promoters (1), RNA processing signals (2), transcript stability (3), trans acting factors (4), and translational signals (5). The broad application of transient systems in animal cells has been facilitated by the ability to analyze expression at the levels of RNA, protein, and enzymatic activity. Although transient expression systems for plants are not as well developed, they have been used to characterize plant promoter elements that activate transcription (6,7), confer tissue specificity (8) or respond to environmental stimuli (9). The effects of introns (10) and antisense RNA (11) on plant gene expression have also been examined in transient assays. Most plant transient expres-

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sion systems rely on the capacity of protoplasts to transiently express genes introduced by electroporation (12,13) or $CaPO_4$-polyethylene glycol (14). Transient expression has also been detected in plant tissue inoculated with *Agrobacterium* (15) or bombarded by microprojectiles coated with DNA (16). In all cases, expression was assayed at the level of enzymatic activity of the protein encoded by a reporter gene on the introduced DNA. The relationship between plant transcript levels and expression has only been determined in two studies (8,10). Further application of plant transient expression systems to RNA analysis has been limited by the difficulty in isolating sufficient quantities of the RNA of interest. In animal cell systems, this problem was overcome by using viral replication functions to amplify the number of DNA templates available for transient transcription (17,18). In plants, cauliflower mosaic (19) and tobacco mosaic viruses (20,21) have been used to express nonviral genes. However, these viruses replicate via RNA intermediates and have genome size constraints that limit their potential as plant transient expression vectors. The geminiviruses, tomato golden mosaic virus (TGMV; 22,23) and casava latent virus (24), have also been used to express nonviral genes in plants and are better candidates for a transient expression system.

Geminiviruses are a group of plant DNA viruses that infect both dicots and monocots and include TGMV, maize streak virus (MSV), and beet curly top virus (25,26). They fall into two classes characterized by their insect vectors and by their genomes, which exist in either single or two component configurations. Their genomes are small, circular DNAs, which occur as single strands in virions and plants and as double strands in plants. They are localized in plant nuclei where they can assemble into nucleosome-like structures (27). Geminivirus DNA can produce infection at low efficiency when mechanically inoculated (28) or at high efficiency when inoculated via *Agrobacterium* (29-31). The nucleotide sequences of several geminivirus genomes have been determined and indicate that they can encode four to six polypeptides (32-34). Geminivirus transcription is bidirectional, originating in a noncoding intergenic region and progressing divergently around the genome to polyadenylation sites located on the opposite side (35,36). The genes of TGMV, a two component geminivirus, have been characterized functionally by determining the phenotypes conferred by mutations in each open reading frame (37-39). The A component encodes the only viral function (AL1) required for replication and can replicate autonomously. Replication of the A component has been observed in plants transgenic for TGMV A (40,41) and in leaf discs shortly after co-culture with *Agrobacterium* carrying TGMV A DNA (37). The TGMV A component also encodes the viral coat protein (AR1), which can be replaced by neomycin phosphotransferase (22) and CAT (23) coding sequences without loss of infectivity. These results suggest that a TGMV

A replicon could be used to amplify nonviral genes to a copy number sufficiently high that RNAs transcribed from them would be readily detected. We report that viral and heterologous transcripts can be detected in leaf discs 2 days after agroinoculation of the TGMV A component.

MATERIALS AND METHODS

Enzymes.

RQI deoxyribonuclease and RNasin ribonuclease inhibitor were from Promega. Nuclease S1, polynucleotide kinase and calf intestine alkaline phosphatase were from Boehringer Mannheim Biochemicals. All other enzymes were from New England Biolabs, Inc.

Plasmids.

The A component of TGMV was cloned into the binary T-DNA vector pMON505 (15) as a one and one half copy tandem repeat to give pMON337 (30; Fig. 1). The same TGMV A repeat was cloned into pMON506, a derivative of pMON505 that lacks a T-DNA border sequence, to give pMON403 (30). Plasmid pMON337 was modified by mutagenesis of the AL1 open reading frame (Fig. 1), resulting in pMON441 and pMON446 (37). In pMON417 and pMON1520, the CAT and GUS coding sequences, respectively, were cloned downstream of the AR1 promoter in place of the AR1 coding sequence (Elmer et al., in preparation). Plasmid pMON417 was constructed by insertion of a 750bp BglII-AsuII fragment containing the CAT coding sequence into the XhoII and AsuII sites of the AR1 gene (23). Plasmid pMON1520 was constructed by insertion of a 1.9kb BglII-BamHI fragment with the GUS coding sequence (42) into the BglII site of pMON458, an AR1 deletion construct (38).

Plant inoculation and leaf disc transformation.

Infected *Nicotiana benthamiana* plants were produced by inoculating the transgenic line 3427, which contains three tandem copies of the TGMV B component, with *Agrobacterium* carrying pMON337 (30). Leaf discs from Mitchell diploid petunia (*Petunia hybrida*), *N. benthamiana* and *Nicotiana tabacum* var. Samsun or cotyledon explants from tomato UC82B were inoculated with *A. tumefaciens* containing the disarmed pTiB6S3-SE plasmid and binary vectors carrying one and one-half copies of wild type or mutated TGMV A DNA (37). The plant tissue and *Agrobacterium* were co-cultured for 3 days on MS104 agar medium (43) prior to RNA analysis.

RNA isolation and analysis.

Total RNA was isolated from infected leaves or 30 leaf discs (1-2 g; 44). Total RNA was isolated from *A. tumefaciens* by resuspending the bacteria in 6M urea, 0.36M NaCl, 1% SDS, 20mM EDTA, 10mM Tris-HCl pH8.0 followed by extraction with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and ethanol precipitation. After isolation, the

plant and bacterial RNA samples were treated with 100 units/ml RQ1 DNase in 40mM Tris-HCl pH8.0, 10mM NaCl, 5mM $MgCl_2$, 100 units/ml RNasin ribonuclease inhibitor for 60 min at 37°C. The RNA was extracted twice with phenol/$CHCl_3$/isoamyl alcohol and precipitated with ethanol. Total RNA was assayed by S1 nuclease protection (45) using single-stranded DNA probes that were [^{32}P]5' end-labeled (46). The DNA probes were used in at least 100-fold excess relative to RNA. The S1-protected products were resolved electrophoretically on 6% polyacrylamide-8.3M urea DNA sequencing gels (47) using [^{32}P]ΦX174-*Hae*III DNAs as size markers. The S1-protected products were quantitated using an LKB Ultrascan XL laser densitometer.

Biosafety Considerations.

Transgenic plants with tandem repeats of the geminivirus components in their genomes were propagated in limited access growth chambers. No *Agrobacterium* strains were used that contained more than one TGMV DNA component (ca. 50% of the genome) in accordance with the NIH Guidelines for cloning of virus genomes.

RESULTS

The TGMV AR1 gene is transiently expressed in leaf discs after agroinoculation.

The A component of the TGMV genome can replicate to high copy number in leaf discs after inoculation with *A. tumefaciens* carrying tandem copies of A (37). We investigated the possibility that amplification of viral DNA also increased TGMV A transcription and resulted in detectable levels of viral RNAs. Petunia leaf discs were precultured for two days, agroinoculated with pMON337 containing one and one half tandem copies of TGMV A (Fig. 1), and sampled over two weeks. Transcripts of the AR1 gene (48), which encodes the viral coat protein, were analyzed by S1 nuclease protection of total RNA from each time point (Fig. 2). A protected product whose size corresponds to an AR1 RNA with its 5' end at position 319 of TGMV A (Sunter et al. in preparation) was observed 2 days after co-culture (lane 3). AR1 RNA was present at similar levels during days 2 to 6 after co-culture (lanes 3-7) and then slowly declined through the second week (lanes 8-12). The same transcript was detected in the leaves of TGMV-infected *N. benthamiana* plants (lane 13); northern analysis verified that AR1 RNAs in leaf discs and infected plants are the same size (data not shown). S1 protection of AR1 RNA resulted in a doublet. We observed the same doublet when AR1 RNA was subjected to primer extension analysis (data not shown), indicating that the 5' end of AR1 RNA is heterogeneous in plants. A second RNA species (*), which was 20 nt longer at its 5' end, was also observed in leaf discs. This RNA was present at one day after inoculation, peaked at days 2 and 3 (lanes 3 and 4), and subsequently disappeared (lanes 5-12). The rapid disappearance of the longer RNA correlated with the application of carbenicillin selection at day 3, suggesting that it is an *Agrobacterium* transcript.

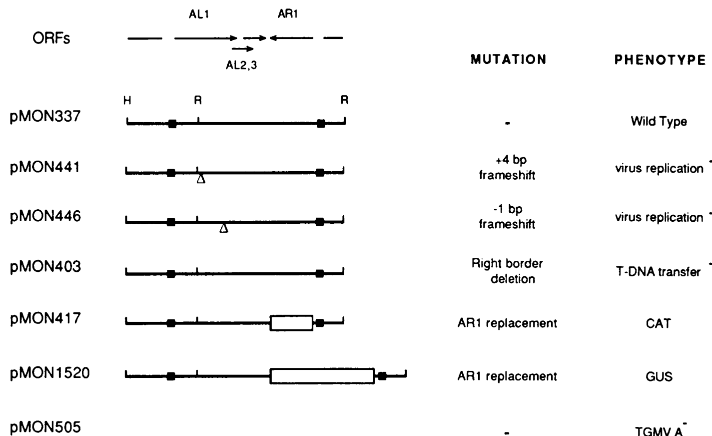

Figure 1. Diagram of cloned TGMV A DNAs used for transient expression. Plasmid pMON337 contains one and one half tandem copies of wild type TGMV A DNA with the full copy cloned as an EcoRI (E) fragment and the half copy as a HindIII (H) to EcoRI fragment (37). Plasmid pMON337 includes two copies of the TGMV A common region (filled boxes). The open reading frames AL1, AR1, and AL2,3 that are not interrupted in pMON337 and their orientations are shown by the arrows. The lines indicate incomplete open reading frames. Plasmids pMON441 and pMON446 contain mutations (Δ) in the AL1 open reading frame. The mutation in pMON446 does not overlap the AL2,3 open reading frame. In pMON417 and pMON1520, the AR1 open reading frame was replaced by CAT and GUS coding sequences (open boxes), respectively. The nature of the mutation and resulting phenotype of each plasmid are listed.

Full length protection of the single-stranded S1 probe was observed with leaf disc (lanes 3-12) and infected leaf RNAs (data not shown). The full length protection was strand-specific (data not shown) and sensitive to DNase digestion (lane 13), establishing that it was due to single-stranded TGMV A DNA. The kinetics for single-stranded viral DNA accumulation in leaf discs were similar to that of authentic AR1 RNA accumulation. The accumulation of double-stranded TGMV A DNA also paralleled AR1 RNA (data not shown). Three longer AR1 RNA species (←; lane 13), which traverse the TGMV A common region, were detected in infected leaves. These AR1 RNAs were sensitive to RNase digestion and alkaline hydrolysis, confirming that they were not due to single-stranded TGMV A DNA contamination. The larger AR1 RNAs were also detected in leaf discs upon long exposure of the autoradiogram.

A plant RNA polymerase transcribes the AR1 gene.

Agrobacterium can persist in leaf disc cultures for several weeks in the presence of carbenicillin selection. Some plant promoters, including TGMV AR1 (49), can support transcription in bacteria. We examined AR1 RNA synthesis in leaf discs inoculated with TGMV A replication mutants (AL1) and a T-DNA transfer mutant and in

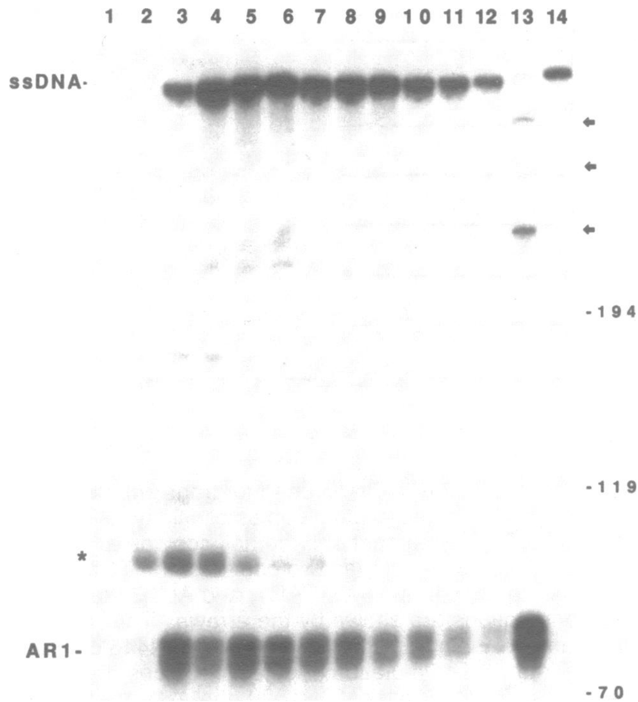

Figure 2. Time course of AR1 transient expression. Total RNA isolated from petunia leaf discs inoculated with *Agrobacterium* carrying pMON337 and from TGMV infected *N. benthamiana* leaves were assayed by S1 nuclease protection using a 746nt probe 5' end-labeled at a XhoI site (lane 14; Fig. 4B). Lanes 1-12. The single-stranded probe was used to protect RNA (40 µg) from leaf discs at 0 (lane 1), 1 (lane 2), 2 (lane 3), 3 (lane 4), 4 (lane 5), 5 (lane 6), 6 (lane 7), 7 (lane 8), 8 (lane 9), 10 (lane 10), 12 (lane 11) and 14 (lane 12) days after co-culture. The leaf discs were transferred to medium containing carbenicillin (500 mg/l) and kanamycin (300 mg/l) at day 3 (lane 4). The leaf disc RNAs were not digested with DNase. The S1 probe was used to protect RNA (1 µg) from infected leaf tissue and digested with DNase (lane 13). The S1-protected product which corresponds to the major plant AR1 transcript is indicated. Reaction products which reflect longer AR1 transcripts (⬅), an *Agrobacterium* transcription product (*), and single-stranded TGMV A DNA (ssDNA) are also identified. The numbers correspond to the positions and sizes of DNA markers.

Agrobacterium to establish that the AR1 gene is transcribed by a plant RNA polymerase (Fig. 3). No RNA corresponding to AR1 RNA from infected plants (lane 2) was detected in petunia leaf discs inoculated with pMON441 (lane 4) and pMON446 (lane 5), which both include AL1 mutations. No authentic AR1 RNA was detected in leaf discs inoculated with pMON403 (lane 6), which contains wild type TGMV DNA but lacks a T-DNA right border sequence. AR1 RNA was observed in leaf discs inocu-

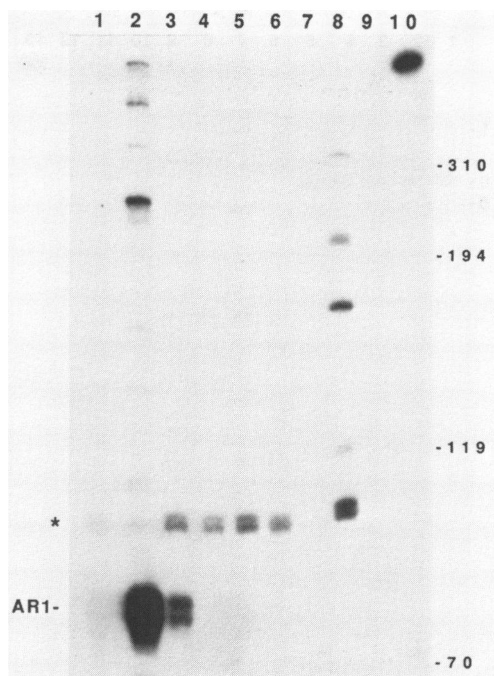

Figure 3. Transient expression of AL1 mutant and T-DNA transfer defective vectors. Total RNA (40 µg) isolated from petunia leaf discs inoculated with *Agrobacterium* carrying pMON337 (lane 3), pMON441 (lane 4), pMON446 (lane 5), pMON403 (lane 6) and pMON505 (lane 7) were assayed by S1 nuclease protection using a 746nt probe 5' end-labeled at a XhoI site (lane 10; Fig. 4B). Total RNA (40 µg) from *Agrobacterium* carrying pMON337 was assayed in lane 8. Total RNA (5 µg) from uninfected (lane 1) and TGMV infected (lane 2) *N. benthamiana* leaves were assayed. In lane 9, no RNA was included in the S1 reaction. The products that corresponds to the major plant AR1 transcript and the major *Agrobacterium* RNA (*) are indicated. The numbers correspond to the positions and sizes of DNA markers.

lated with pMON337 (lane 3). These results show that the synthesis of AR1 RNA in leaf discs is dependent on T-DNA transfer and TGMV A replication and demonstrate that it is a plant transcript. A longer RNA species (*) was seen with all the vectors, but not in infected plants (cf. lane 2 and lanes 3-6). The same RNA was present in *Agrobacterium* containing pMON337 (lane 8), indicating that it is a bacterial transcript.

Transcription of AR1 and AL genes is proportional.

The level of AR1 RNA synthesis is dependent on the copy number of TGMV A DNA, which can vary widely in agroinoculated leaf discs (J.S. Elmer, unpublished results). We investigated the possibility of using a transcript from the AL1 open reading frame (Fig. 1) as an internal standard for TGMV A DNA copy number and AR1

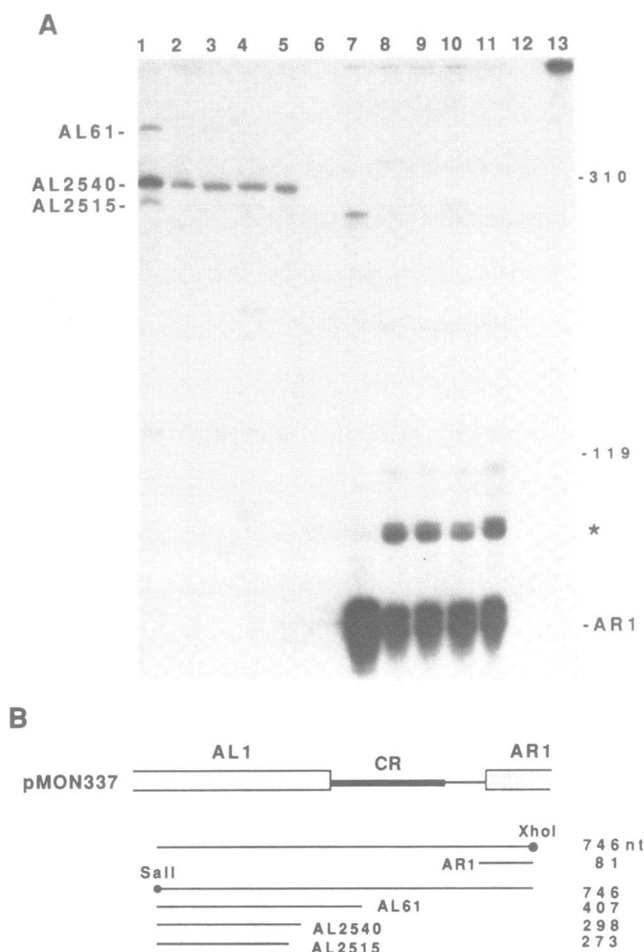

Figure 4. Constant ratio of AL2540 and AR1 RNAs. (A) Total RNA isolated from petunia leaf discs (40 μg) inoculated with *Agrobacterium* carrying pMON337 and from uninfected and TGMV infected *N. benthamiana* leaves (2.5 μg) were assayed by S1 nuclease protection using the complementary strands of a 746bp DNA fragment (lane 13; panel B). The strand that was labeled at the SalI site was protected from digestion by AL1 RNAs in infected leaves (lane 1), inoculated leaf discs (lanes 2-5) and uninfected leaves (lane 6). The strand that was labeled at the XhoI site was protected from digestion by AR1 RNA in infected leaves (lane 7), inoculated leaf discs (lanes 8-11) and uninfected leaves (lane 12). The protected products corresponding to AL61, AL2540, AL2515 and AR1 plant RNAs are indicated. The *Agrobacterium* AR1 transcript product is also designated (*). The numbers correspond to the positions and sizes of DNA markers. (B) The single-stranded S1 probes used to analyze TGMV AL and AR1 RNAs and the reaction products are compared to pMON337. The 5' ends of the AL1 and AR1 open reading frames and the TGMV common region are indicated. The positions of the [^{32}P]5'-ends of the probes are marked (•). The sizes of the probes and the reactions products are listed.

TABLE 1. Ratio of AR1 and AL2540 RNAs

Tissue	Construct	Relative Amount of RNA[a]		AR1/AL2540 Ratio
		AR1	AL2540	
Infected leaf		1406 (62.2)	55.9	25.2
Leaf disc -1	pMON337	22.6 (1.00)	1.0	22.6
-2	pMON337	23.7 (1.05)	1.04	22.8
-3	pMON337	26.7 (1.18)	1.14	23.4
-4	pMON337	28.7 (1.27)	1.20	23.9

[a] The relative amount of RNA was determined by normalizing the areas under the scans of the autoradiograms of the S1-protected products (Fig. 4A). All of the RNAs were normalized against leaf disc-1 AL2540 RNA. The AR1 RNAs were also normalized against leaf disc-1 AR1 RNA in the parentheses.

transcription. Three RNA species which overlap the AL1 open reading frame with their 5' ends located at positions 61-62, 2540 and 2515 on the TGMV A genome were identified (Fig. 4, lane 1; Sunter et al. in preparation). These RNAs are designated as AL61, AL2540 and AL2515, respectively. (AL1 transcription is counter-clockwise, beginning in the common region and passing through site 0/2588, with positions 61-62 being more 5' than 2540 and 2515) The same RNAs were detected by primer extension (data not shown), indicating that all three RNA species are primary transcripts. We compared the levels of AL and AR1 RNAs synthesized in four parallel, independent transient assays of petunia leaf discs agroinoculated with pMON337. The complementary strands of a TGMV A SalI-XhoI fragment labeled to the same specific activity were used to protect the AL and AR RNAs (Fig. 4B). For the comparisons, we used AL2540 RNA since it is 8-fold more abundant than AL61 and AL2515 RNAs in infected leaf tissue and leaf discs. The AL2540 RNA was readily detected in leaf discs (Fig. 4A, lanes 2-5) while detection of the AL61 and AL2515 RNA species required long exposures of the autoradiograms (data not shown). The ratios of AL2540 and AR1 RNAs were very reproducible in the leaf disc assays (Table 1; Fig. 4, cf. lanes 2-5 and 8-11) and similar to the ratio in infected leaf tissue (cf. lanes 1 and 7). Consequently, the AL2540 to AR1 ratio can be used to adjust the levels of transient RNA transcribed from wild type and modified AR open reading frames for variations in viral DNA copy number.

The GUS and CAT genes are transcribed in leaf discs from the AR1 promoter.

TGMV A DNA can replicate in leaf discs when the AR1 open reading frame is replaced by either the bacterial sequence encoding CAT as in pMON417 or GUS as in pMON1520 (Elmer et al., manuscript in preparation). In these constructs, the AR1 promoter directs transcription of the heterologous genes in the transient expression

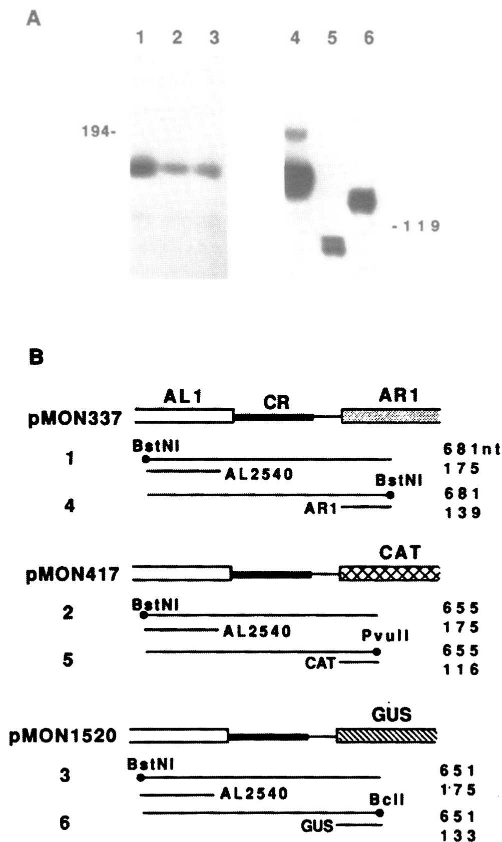

Figure 5. Transient expression of heterologous coding sequences using vectors derived from TGMV A. (A) Total RNA (40 µg) isolated from petunia leaf discs inoculated with *Agrobacterium* carrying pMON337 (lanes 1 and 4), pMON417 (lanes 2 and 5), or pMON1520 (lanes 3 and 6) was assayed by S1 nuclease protection using the single-stranded probes in panel B. The probes were protected by AL2540 (lanes 1-3), AR1 (lane 4), CAT (lane 5), or GUS (lane 6) RNA. The numbers indicate the positions and sizes of DNA markers. (B) The single-stranded S1 probes used to analyze AL2540, AR1, CAT and GUS RNAs and the reaction products are compared to pMON337, pMON417 and pMON1520. The 5' ends of the AL1 open reading frame, the rightward open reading frames, and the TGMV common region are indicated. The number of each probe corresponds to the lane in panel A in which it was used. The positions of the [^{32}P]5'-ends of the probes are marked (●). The sizes of the probes and the reactions products are listed.

system (Fig. 5). CAT (lane 5) and GUS (lane 6) RNAs were detected in petunia leaf discs 3 days after agroinoculation. The 5' ends of the heterologous RNAs correspond to the AR1 transcription start site, verifying that the RNAs were transcribed from the

TABLE 2. Ratio of Right ORF and AL2540 RNAs in Petunia Leaf Discs

Right ORF	Construct	Relative Amount of RNA[a]		RNA Ratio
		Right ORF	AL2540	
AR1	pMON337	89.2	2.42	36.9
CAT	pMON417	16.9	1.05	16.1
GUS	pMON1520	28.9	1.0	28.9

[a] The relative amount of RNA was determined by normalizing the areas under the peaks of scans of autoradiograms of the S1-protected products (Fig. 5A). The levels were adjusted for specific activity by comparing the amounts of AL2540 RNA detected with AL2540 probes from pMON417 and pMON1520 to levels detected with the AL2540 probe from pMON337.

AR1 promoter. The levels of CAT and GUS RNAs were reduced relative to AR1 RNA (Fig. 5A, cf. lane 4 and lanes 5-6). This difference was due in part to lower template copy number since AL2540 RNA was also reduced in leaf discs inoculated with pMON417 (lane 2) and pMON1520 (lane 3) relative to pMON337 (lane 1). The rightward open reading frame to AL2540 ratios are given in Table 2. The AR1 to AL2540 ratio for leaf discs inoculated with pMON337 in this experiment differed from those in Table 1 because different probes were used. The lengths of the AL2540 and AR1 hybrids formed by the two sets of probes differed by 123 and 58 nt, respectively (cf. Fig. 4B and Fig. 5B). Consequently, the efficiencies of hybridization and the resulting ratios also differed. The AL2540 hybrids in Fig. 5 were identical and the hybrids corresponding to AR1, CAT and GUS RNAs varied in length by only 23 nucleotides, minimizing the variation in hybridization efficiency due to hybrid length. The pMON337 and pMON1520 transient assays showed similar ratios, indicating that the AR1 promoter supported transcription of the GUS gene nearly as efficiently as its own coding sequence. The ratio for the pMON417 assay was lower, possibly because of less CAT transcription or CAT RNA instability. CAT and GUS enzyme activities were detected in leaf discs agroinoculated with pMON417 and pMON1520 (data not shown), demonstrating that the CAT and GUS mRNAs transcribed from the TGMV A vector are functional. The time course for the appearance of GUS activity was similar to the time course of AR1 accumulation (Fig. 2).

TGMV A genes are transcribed in leaf discs of other solanaceous species.

Several solanaceous species are hosts for TGMV. We inoculated tissue from tomato, *N. tabacum*, and *N. benthamiana* with *Agrobacterium* carrying pMON337 to determine if AR1 RNA is synthesized in other solanaceous species shortly after co-culture (Fig. 6) Similar amounts of AR1 RNA were observed in leaf discs from petunia (Fig. 6A, lane 2) and tomato cotyledons (lane 3) three days after co-culture. Three-

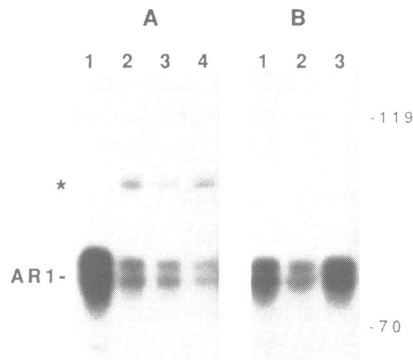

Figure 6. Transient expression of AR1 in different solanaceous species. Total RNA isolated from plant tissues (40 μg) inoculated with *Agrobacterium* carrying pMON337 and from TGMV infected *N. benthamiana* leaves (5 μg) were assayed by S1 nuclease protection using a 746 nt probe 5' end-labeled at a XhoI site (Fig. 4B). (A) The single-stranded probe was used to protect AR1 RNA from infected leaves (lane 1), petunia leaf discs (lane 2), tomato cotyledons (lane 3), and *N. tabacum* leaf discs (lane 4). (B) The probe was used to protect AR1 RNA from infected leaves (lane 1), wild type *N. benthamiana* leaf discs (lane 2), and TGMV B transgenic *N. benthamiana* leaf discs (lane 3). The autoradiogram in panel A was exposed 10-fold longer than the film in panel B. The S1-protected product that corresponds to the major plant AR1 transcript is indicated. The *Agrobacterium* transcription product is also marked (*). The numbers indicate the positions and sizes of DNA markers.

fold less AR1 RNA was detected in *N. tabacum* leaf discs (lane 4). This difference reflects variation in the TGMV A DNA copy number as demonstrated by the similar AR1 to AL2540 ratios for petunia, tomato and *N. tabacum* (Table 3). In contrast to these

TABLE 3. AR1 RNA in Solanaceous Species

Tissue	Construct	Relative AR1 RNA[a]	AR1/AL2540 Ratio
Petunia leaf discs	pMON337	1.0	25.5
Tomato cotyledon	pMON337	0.72	25.9
N. tobacum leaf discs	pMON337	0.35	28.9
N. benthamiana			
infected leaf		68.0	28.7
leaf disc			
-wild type	pMON337	6.6	24.4
-transgenic TGMV B	pMON337	21.0	23.0

[a] The relative amount of AR1 RNA was determined by normalizing the areas under the of scans of the autoradiograms of the S1-protected products (Fig. 6) against petunia leaf discs. The values were adjusted for the 10-fold difference in exposure between Fig. 6A and 6B.

results, 6-fold more AR1 RNA was detected in inoculated leaf discs from wild type *N. benthamiana* (Fig. 6B, lane 1) relative to petunia leaf discs. The level of AR1 RNA was 20-fold greater in inoculated leaf discs from a transgenic *N. benthamiana* line containing tandem inserts of the TGMV B component (Fig. 6B, lane 3) than from petunia and only 3 to 4-fold less than infected leaf tissue. More AL2540 RNA was also detected in both types of *N. benthamiana* leaf discs. The *N. benthamiana* AR1 to AL2540 ratios were similar to those obtained for petunia, tomato and *N. tabacum* (Table 3), indicating that the TGMV A copy number is higher in *N. benthamiana* leaf discs. The *Agrobacterium* RNA (*) was readily seen in the petunia, tomato, and *N. tabacum* assays in Fig. 6A, but could only be detected in *N. benthamiana* leaf disc RNA upon long exposure of the autoradiogram.

DISCUSSION

We have developed a rapid and convenient system for studying the relationship between gene expression and RNA in plants. The transient expression system is based on two important features of the geminivirus TGMV. The A component of the TGMV genome replicates autonomously in leaf discs (37), amplifying the number of DNA template molecules available for transcription and resulting in high levels of viral RNA in leaf discs. The TGMV coat protein is dispensable for replication and its open reading frame can be replaced by nonviral coding sequences (22,23). We used TGMV A-based vectors to express the heterologous genes, GUS and CAT, in leaf discs.

The TGMV A transient expression system depends on *Agrobacterium* to transfer DNA into plant cells. *Agrobacterium*-mediated transfer is more efficient and reliable than mechanical inoculation of cloned viral DNA (30) even though the total number of plant cells in leaf discs that are T-DNA recipients is very small. Leaf discs co-cultured with *Agrobacterium* carrying tandem copies of TGMV A DNA in the T-DNA express AR1 RNA two days after inoculation. Part of this lag period reflects the 12 h required to activate *Agrobacterium* for transfer (50). After transfer, the TGMV A DNA can replicate or recombine directly out of the T-DNA. Alternatively, the TGMV A DNA can integrate via the T-DNA into the plant nuclear genome and then be released (40). A circular TGMV A DNA, which is able to replicate autonomously to high copy number and support high levels of RNA synthesis, is formed in both instances. Our results showed that TGMV A DNA and RNA accumulate for one week and then decline. The decrease may be due to cytotoxic effects of TGMV A (51), resulting in the death of the plant cells replicating and expressing this genome component.

The synthesis of authentic plant AR1 RNA in leaf discs requires both T-DNA transfer and viral replication. A second AR1 RNA, whose synthesis is not dependent

on T-DNA transfer or viral replication, was observed in the S1-protection experiments. We have identified this RNA as an *Agrobacterium* transcript. The AR1 promoter region of TGMV also supports transcription in *Escherichia coli* (49). A sequence with strong homology to the *E. coli* consensus promoter (52) is located immediately upstream of the 5' end of AR1 gene, with a putative bacterial -10 promoter element overlapping the TATA box of the AR1 promoter. Consequently, the AR1 promoter region cannot be easily engineered to eliminate bacterial transcription without also affecting plant transcription. We overcame this problem by examining the 5' ends of RNAs, which allows us to distinguish between the plant and bacterial transcripts.

In order for a transient expression system to be reproducible and quantitative, there must be a mechanism to adjust for variations in template copy number. One method is to normalize transcription of the gene of interest against transcription of a reference gene that is subject to the same variations in gene dosage. The organization of TGMV A is well suited to this approach since RNA transcribed from the AL1 gene can be used to standardize the levels of RNA derived from the AR transcription unit. The ratio of AR1 to AL2540 RNA deviated by less than 10% in four parallel petunia leaf disc assays using pMON337 at three days post-inoculation. The AR1 to AL2540 RNA ratios from petunia, tomato, *N. tabacum*, wild type *N. benthamiana* and TGMV B transgenic *N. benthamiana* transient assays were within 21% even though these species transform with different frequencies. The transient assay ratios were very similar to those determined for TGMV infected leaf tissue, indicating that TGMV A promoters function normally in leaf discs. The results show that the ratio of AR1 and AL2540 transcription is very reproducible and independent of transformation efficiency, establishing that it is a reliable and consistent control for DNA copy number.

TGMV A can be used as a vector for the expression of heterologous coding sequences in plant cells. We detected CAT and GUS RNAs in leaf discs when their coding sequences were substituted for the AR1 open reading frame. The levels of AL2540 RNA were 60% lower with both heterologous gene constructs as compared to pMON337. Since both substitutions produced similar reductions, the lower levels of AL2540 RNA were not due to the presence of a particular heterologous DNA sequence that interferes with transcription. The reduction might reflect the loss of an upstream activating sequence for AL2540 transcription that is located in AR1 open reading frame. However, a more likely explanation is that fewer DNA templates were available for AL2540 transcription with the heterologous vectors. Less TGMV A DNA was detected in leaf discs agroinoculated with pMON417 or pMON1520 than with pMON337 (J. S. Elmer, unpublished results). The lower levels of TGMV A DNA seen with the heterologous vectors cannot be attributed to variations in the size of the replicons since the TGMV A DNAs derived from pMON417 and pMON337 differed by only

25 bp. Replication of pMON1520 TGMV A DNA, which is 1.1 kbp larger than the wild type A component, demonstrated that TGMV A replication is not subject to tight size constraints in agroinoculated cells. The reduced levels of TGMV A DNA seen with the heterologous vectors may be due to the deletion of a cis-acting element located in the AR1 open reading frame that enhances virus replication. The GUS and AR1 to AL2540 ratios differed by only 21%, indicating that the AR1 promoter drives transcription of the GUS gene almost as efficiently as its own coding sequence. In contrast, the CAT to AL2540 ratio is 43% of the corresponding AR1 ratio indicating that there is significantly less CAT RNA than AR1 RNA at a constant DNA copy number. This difference probably reflects the relative instability of CAT RNA in plant cells (53).

The expression of many genes has been examined in transgenic plants (54). These studies demonstrated that there are strong position effects which affect gene expression in transgenic plants (55,56). The expression of a transferred gene can vary more than 25-fold between independent stable transformants, making it is necessary to examine many individuals for quantitative analysis. Since TGMV A is not integrated into the plant genome, TGMV A expression is not susceptible to position effects. Each leaf disc assay also reflects many independent transfer events, eliminating any cell to cell variation in expression. Consequently, fewer assays are necessary to quantitate expression. However, it is necessary to confirm the results obtained with the TGMV A transient expression system in transgenic plants, especially if the gene of interest is regulated in a tissue-specific manner.

The sensitivity of the TGMV transient expression system can be increased by the choice of host and the use of transgenic lines. We observed a 6 to 19-fold higher level of AR1 and AL2540 RNAs in leaf discs from wild type *N. benthamiana* as compared to the other solanaceous species after inoculation with pMON337. This result may reflect a higher efficiency of agroinoculation of *N. benthamiana* or it may indicate that host-specific factors affect the efficiency of TGMV A replication and expression in the transient system. *N. benthamiana* displays more severe symptoms than petunia, tomato and *N. tabacum* upon infection by the virus. Viral functions can also affect the efficiency; the level of TGMV A gene expression increased 3-fold more in leaf discs from a transgenic *N. benthamiana* line containing TGMV B. The B component encodes functions involved in symptom development and viral movement (40) and when both TGMV genome components are present, the virus can move systemically (57). In TGMV B transgenic leaf discs, the cell population capable of expressing TGMV A includes primary transformants and probably adjacent cells where the virus has spread. In wild type leaf discs, the only cells that can express TGMV A are direct T-DNA recipients. TGMV B may also encode a trans acting function which enhances TGMV A replication and/or transcription, but there is no evidence for a B-encoded

trans acting factor. One important consequence of enhanced TGMV expression in *N. benthamiana* is that the bacterial RNA signal is reduced relative to the plant transcripts and no longer contributes significantly to the total AR1 RNA signal. Since *Agrobacterium* transcription can be disregarded in *N. benthamiana* leaf discs, particularly those transgenic for TGMV B, it is the plant species of choice for TGMV A transient expression.

The TGMV A transient expression system has several advantages. It is fast, very sensitive, and allows direct RNA analysis. It is possible to detect TGMV A transcripts in total RNA isolated from the equivalent of one leaf disc within two days after co-culture with *Agrobacterium*. The transient assay, from pre-culture to S1 nuclease protection, can be completed in one week. The assay is semi-quantitative because the levels of TGMV A transcripts can be standardized with respect to DNA copy number. The expression of TGMV A genes is not subject to position effects. The AR1 gene is not a complex transcription/translation unit and, consequently, it is straight forward to clone heterologous coding sequences downstream of the AR1 promoter. There are no known replication constraints on the size of the heterologous sequences that can be inserted into TGMV A. The plant tissue used in the assay is readily available and requires minimal preparation. The major disadvantage of the TGMV A expression system is its dependence on viral host range. It should be possible to extend the range of the transient expression system by using TGMV A as a model for the development of analogous vectors from other geminiviruses with different host ranges. Recently, CAT activity was demonstrated in maize leaves agroinoculated with a MSV variant containing the CAT coding sequence (58).

The TGMV A transient assay system is a powerful tool for the study of plant gene expression, especially when it is coupled with parallel studies in transgenic plants. It can serve as a preliminary screen for the expression of heterologous genes in plant cells at the level of RNA prior to the construction of transgenic plants. The TGMV A system can be used to ask questions concerning RNA processing and transcript stability. We are currently testing its efficacy for promoter analysis by characterizing the expression of a variety of heterologous promoters in place of the AR1 promoter. In all of these experiments, it will be necessary to validate the expression observed using the TGMV A vector with gene expression data from transgenic plants.

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*To whom correspondence should be addressed

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