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Evidence for Activation and Derepression Mechanisms

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Tomato golden mosaic virus (TGMV) is a bipartite member of the subgroup III Geminiviridae. Like all geminiviruses, TGMV replicates in the nucleus of susceptible cells by rolling circle replication (RCR). Double-stranded replicative form DNA generated during RCR serves as template for the transcription of viral genes by RNA polymerase II and the associated cellular transcription machinery. Previous studies in tobacco protoplasts and *Nicotiana benthamiana* leaf discs have shown that the viral *AL2* gene product transactivates expression of the coat protein (*CP*) and *BR1* movement protein genes, and that activation occurs at the level of transcription. Because of its function and properties, we propose the name TrAP, transcriptional activator protein, for the *AL2* gene product. Using transgenes consisting of complete and truncated versions of the *CP* promoter fused to the GUS reporter gene, we show in the studies presented here that TrAP is required for *CP* gene expression in both mesophyll and phloem tissues. Surprisingly, TrAP appears to induce *CP* expression by different mechanisms in different cell types: it may activate the *CP* promoter in mesophyll cells, and acts to derepress the promoter in phloem tissue. In addition, TrAP is clearly capable of inducing the expression of responsive chromosomal promoters and could, in principle, activate host genes. Distinct viral sequence elements mediate expression and derepression in phloem and activation in mesophyll, suggesting that TrAP interacts with different components of the cellular transcription machinery to accomplish *CP* gene expression in different cell types, and underscoring the intricacy and complexity of virus–host interactions.

INTRODUCTION

The Geminiviridae is a diverse family of plant infectious agents characterized by circular, single-stranded DNA (ssDNA) genomes and a unique paired particle morphology. These viruses amplify their DNA in the nuclei of host cells by rolling circle replication (RCR), using host DNA polymerases and replication machinery. Viral transcription occurs from double-stranded DNA (dsDNA) replicative forms (RF) generated during RCR and is accomplished by host RNA polymerase II (for review see Stanley, 1991; Lazarowitz, 1992; Bisaro, 1996). Because of their relatively simple genome structure and extensive reliance on host biosynthetic machinery, the geminiviruses are ideal model systems for the study of cellular DNA replication and transcription.

DNA virus transcription programs usually employ a strategy in which an early viral gene product induces the expression of viral genes required later in the multiplication cycle. In this way, the proper temporal regulation of transcription required for a successful productive infection is achieved. The geminiviruses appear to conform to this general strategy, although viruses belonging to different subgroups execute their transcription programs in very different ways. In subgroup I geminiviruses (including wheat dwarf virus and maize streak virus), the viral replication initiator protein (Rep) is multifunctional, and is required not only for replication but also for coat protein (*CP*) promoter activity (Hofer *et al.,* 1992). The use of a single protein for replication and for the regulation of late gene (coat protein) expression suggests a simple and elegant mechanism for coordinating the viral multiplication cycle. In contrast, the gene regulation circuitry is more complex in the dicot-infecting viruses belonging to subgroup III. In these agents, Rep is also necessary for replication (Elmer *et al.,* 1988a; Hanley-Bowdoin *et al.,* 1990). However, it is not required for the expression of late genes but instead negatively regulates its own expression (Sunter *et al.,* 1993; Eagle *et al.,* 1994). Subgroup III virus genomes, which may be either monopartite (e.g., tomato yellow leaf curl virus; TYLCV) or bipartite (e.g., tomato golden mosaic virus; TGMV, and African cassava mosaic virus; ACMV), characteristically contain an *AL2* gene (also known as *AC2* or *C2*) that is required for the expression of CP and BR1 movement protein (Sunter *et al.,* 1990; Sunter and Bisaro, 1991; Sunter and Bisaro, 1992; Gröning *et al.,* 1994). Although the precise mechanisms by which these genes respond to the *AL2* gene product are not yet known, transcriptional run-on

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experiments have shown that activation occurs at the level of transcription (Sunter and Bisaro, 1992). Because it functions to activate viral gene expression, we propose the name TrAP, transcriptional activator protein, for the *AL2* gene product.

The means by which transcription in subgroup II geminiviruses (specifically beet curly top virus; BCTV) is controlled has not been investigated in any detail, but it is clear that regulation differs from subgroup III viruses because mutations which inactivate the *L2* gene (an apparent *AL2* homologue) do not abolish CP synthesis (Stanley *et al.,* 1992; Hormuzdi and Bisaro, 1995). Further, while several different subgroup III geminiviruses produce a protein that can transactivate the *CP* promoter of a TGMV *AL2* mutant in tobacco protoplasts, BCTV is unable to complement the same mutant (Sunter *et al.,* 1994). In more recent studies, TGMV DNA A was shown to complement noninfectious ACMV and potato yellow mosaic virus *AL2* mutants *in planta* (Saunders and Stanley, 1995; Sung and Coutts, 1995), reinforcing the conclusion that this gene is to some extent functionally interchangeable among the subgroup III viruses.

We are studying the mechanisms by which TrAP activates the *CP* promoter in TGMV. That TrAP function is not virus-specific within subgroup III suggests this protein acts either through conserved viral DNA sequences, through conserved interactions with host factors, or both. Comparative DNA sequence analysis has revealed the presence of a conserved sequence, called the conserved late element (CLE), within most *CP* and *BR1* gene promoters that may mediate activation by TrAP (Argüello-Astorga *et al.,* 1994). However, studies in this and other laboratories have so far failed to provide evidence that TrAP binds the CLE or any dsDNA sequence in a specific manner, although it does bind dsDNA and ssDNA nonspecifically (Noris *et al.,* 1996; Sung and Coutts, 1996; M. D. Hartitz, G. Sunter, and D. M. Bisaro, manuscript in preparation).

It is known that the transcriptional activator proteins of several mammalian DNA viruses do not bind to specific DNA sequences, but instead are targeted by specific interactions with host transcription factors that recognize sequence elements within responsive promoters (e.g., adenovirus E1A, the herpesvirus VP16 activator, and EBNA2 of Epstein–Barr virus) (Gerster and Roeder, 1988; Triezenberg *et al.,* 1988; Flint and Shenk, 1989; Kristie *et al.,* 1989; Hsieh and Hayward, 1995). It is possible that TrAP is targeted to responsive promoters by similar protein–protein interactions. If so, then TrAP may also be capable of activating host genes, provided it has access to them. Because activation of host chromosomal genes could play a significant role in viral pathogenesis, one goal of this study was to determine whether TrAP is able to activate a transgene driven by the *CP* promoter during the course of TGMV infection. A second goal of this study was to address questions of tissue specificity and developmental regulation. Previous studies of TrAP function were performed in protoplasts or leaf discs, and so it was not known if TrAP is required for *CP* expression in all tissues, or whether the promoter might be active only in cells of a particular developmental stage.

In this study, we present evidence which indicates that TrAP is required for expression of the TGMV *CP* promoter in all tissues, and that it is capable of activating a chromosomal promoter. Surprisingly, however, the mechanism by which expression is achieved is different in different cell types: TrAP apparently activates the *CP* promoter in mesophyll cells and derepresses it in vascular tissue. We also show that the *CP* promoter may be sensitive to developmental influences.

MATERIALS AND METHODS

DNA techniques

The map locations of restriction endonuclease sites noted here are from the wild-type TGMV sequence (Hamilton *et al.,* 1984) or from modified TGMV genomes containing the GUS reporter in place of the *CP* gene (Sunter and Bisaro, 1991; Sunter and Bisaro, 1992). All restriction endonucleases and DNA modifying enzymes were used as recommended by the manufacturers. Other techniques were performed according to Ausubel *et al.* (1987) unless otherwise stated. Sequence alterations were confirmed by restriction analysis and/or sequencing.

Cloning of promoter/reporter gene constructs

The constructs pTGA26 (TGMV A), pTGA44 (TGMV A ($AL2^-$)), pTGA79 (35S-AL2), pTGA61 (TGMV A (CP:GUS-nos3′)), pTGA55 (TGMV A (CP:GUS/$AL2^-$)), pTGA35 (TGMV A (CP:GUS)), pCLV 1.3A (ACMV A), pLOGAN (BCTV), and pMON9749 (35S-GUS) have been previously described (Klinkenberg *et al.,* 1989; Stenger *et al.,* 1991; Sunter and Bisaro, 1991, 1992; Sunter *et al.*, 1994). These were used in biolistic experiments and/or to construct the transgenes described below.

Plasmid pTGA75, used to create transgenic *Nicotiana benthamiana* plants carrying the A75 transgene, was constructed by inserting the 2609-bp *Eco*RI–*Xho*I fragment from pTGA61 (Sunter and Bisaro, 1992), containing the TGMV *CP* promoter region (−657) fused to the β-glucuronidase (GUS) coding sequence (CP:GUS), into the *Eco*RI and *Xho*I sites of the binary Ti plasmid vector pMON521 (Rogers *et al.,* 1987) to create pTGA74. An 887-bp *Xho*I–*Not*I fragment containing the nopaline synthase (nos) polyadenylation signal from pMON530 (Rogers *et al.,* 1987) was then inserted into the *Xho*I/*Not*I sites of pTGA74 to create pTGA75. Plasmid pTGA55M, used to create transgenic plants carrying the A55M transgene, was constructed by inserting a TGMV A genome con-

taining the CP:GUS replacement and an *AL2* frameshift mutation as a 3831-bp *Eco*RI fragment from pTGA55 (Sunter and Bisaro, 1991) (Fig. 1) into the *Eco*RI site of pMON521. Plasmid pTGA35M, used to create transgenic plants carrying the A35M transgene, was constructed from pTGA35 (Sunter and Bisaro, 1991) in the same fashion (Fig. 1). The TGMV A genome in pTGA35 also contains the CP:GUS replacement but is wild-type with respect to *AL2.* Plasmid pTGA95M, used to create transgenic plants carrying the A95M transgene, was created by cloning the 895-bp *Eco*RI–*Bam*HI fragment of TGMV A from pTGA26 into pGEM 5Zf(+), followed by insertion of the 2932-bp *Eco*RI–*Bam*HI fragment of pTGA35 (Sunter and Bisaro, 1991) (Fig. 1), creating a monomeric TGMV A (CP:GUS/AL2$^-$) construct linearized at the *Bam*HI site in *AL2/3*. Plasmids pTGA400 and pTGA401, used to generate transgenic plants carrying the A400 and A401 transgenes (Fig.1), were constructed by replacing the 1969-bp *Bgl*II–*Xho*I fragment of pTGA74 with the 2268-bp *Bgl*II–*Bam*HI and 2572-bp *Bgl*II–*Nhe*I fragments, respectively, of pTGA55.

Protoplast transfection and analysis

Protoplasts were prepared from *Nicotiana tabacum* var. Wisconsin 38 suspension cells or *N. benthamiana* leaves (Potrykus and Shillito, 1986; Bilang *et al.,* 1994) and transfected as described (Sunter *et al.,* 1990; Brough *et al.,* 1992). DNAs used in transfection experiments included pTGA75, pTGA55M, and pTGA95M, either alone or together with a 35S-AL2 expression construct (pTGA79) (Sunter and Bisaro, 1992). Fluorometric GUS assays were performed according to Jefferson (1987) using equivalent amounts of protein.

Transgenic plants

Ti plasmid constructs were mobilized by triparental mating into *Agrobacterium tumefaciens* strain GV3111SE containing the disarmed Ti plasmid pTiB36SE, and cultures were used to transform *N. benthamiana* leaf discs (Horsch and Klee, 1986). Transformants were selected on the basis of kanamycin resistance (300 μg/ml). The presence and integrity of the transgene in transformed lines was confirmed by genomic Southern blot hybridization and by PCR amplification of integrated transgene sequences (data not shown).

Transgenic F1 seed resulting from self-fertilization of primary transformants was surface sterilized and germinated on MS media containing kanamycin (300 μg/ml) for up to 35 days. Seedlings were analyzed for GUS expression or transferred to soil for further analysis. Callus induction was performed by cutting discs from surface sterilized leaves of F1 progeny and allowing the discs to form callus on MS media.

In some experiments, 6 to 7-week-old transgenic plants were agroinoculated (Grimsley *et al.,* 1987) with TGMV or BCTV as previously described (Elmer *et al.,* 1988b; Briddon *et al.,* 1989). Systemically infected leaves showing disease symptoms or comparable leaves from mock-inoculated plants were histochemically assayed for GUS activity (described below) 10–20 days postinoculation.

Histochemical staining

Histochemical staining for GUS activity in transgenic plant tissue, or following biolistic delivery of promoter constructs to nontransformed plant tissue, was performed essentially as described (Jefferson, 1987; Jefferson *et al.,* 1987). Tissues were surface sterilized and infiltrated with X-Gluc substrate and incubated at 37° overnight. The following day the stained samples were fixed as described (Bhattacharyya-Pakrasi *et al.,* 1993) and stored in 100% ethanol. Tissue samples were photographed directly under a dissecting microscope. Selected tissues were hand sectioned and photographed under a light microscope.

Biolistic assays

Transient expression assays were performed by introducing DNA constructs into nontransgenic *N. benthamiana* tissue by high velocity particle bombardment (Klein *et al.,* 1988). Leaves from nontransgenic plants were surface sterilized and placed on MS media lacking hormones. M10 tungsten particles were sterilized, coated with DNA, and delivered to the leaves using a particle inflow gun (Finer *et al.,* 1992). Following incubation at room temperature for 24–72 hr, tissue was stained for GUS activity as described above.

RESULTS

TrAP-independent expression of truncated *CP* promoters in vascular tissue

In order to investigate whether TrAP is capable of activating a chromosomal gene, and to ascertain whether its activity is required for *CP* promoter expression in all tissues, a number of constructs were prepared and used to create transgenic *N. benthamiana* plants. The constructs consisted of varying amounts of TGMV sequence upstream or downstream of the *CP* gene, which was replaced in a transcriptional fusion with the β-glucuronidase (GUS) coding sequence (Jefferson, 1987). For each construct, at least three independent transgenic lines were established using *Agrobacterium*-mediated transformation, and Southern blot hybridization analysis and PCR amplification was used to verify the presence of the transgene (data not shown). Data were collected from the F1 or F2 progeny of primary regenerants, although

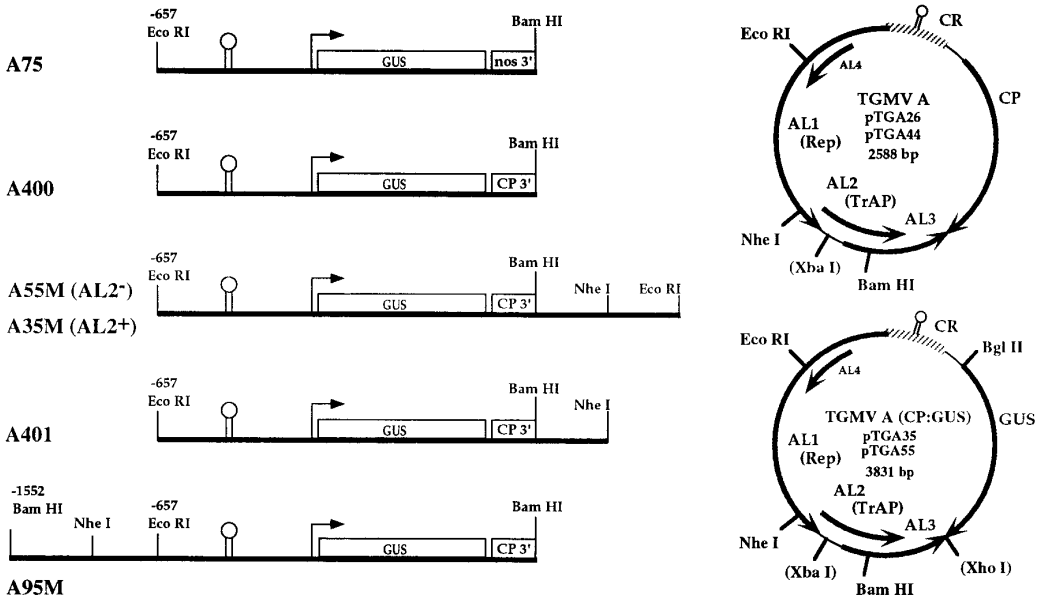
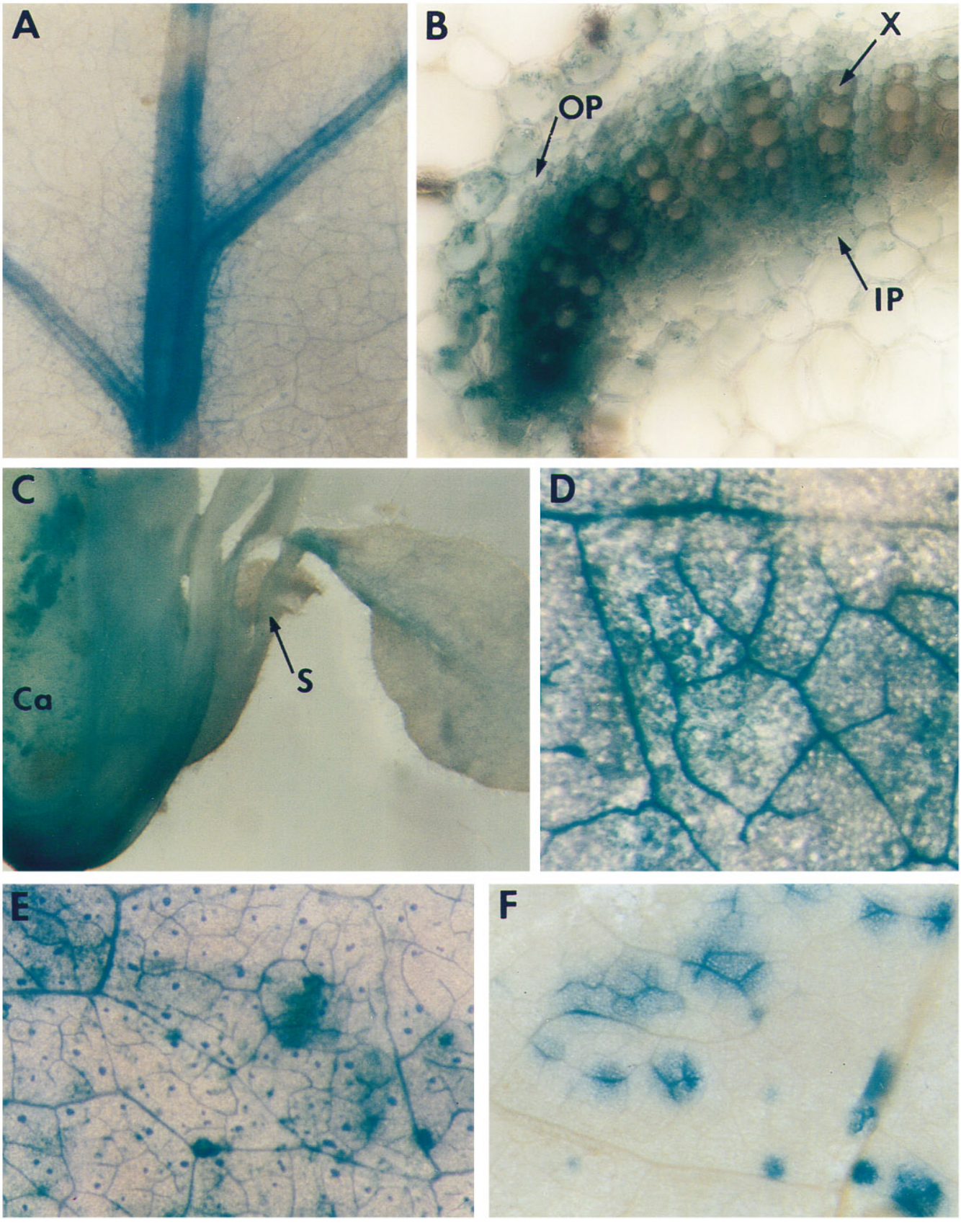


FIG. 1. Diagrams of *CP* promoter:GUS transgenes, TGMV DNA A, and replicating TGMV A (CP:GUS) constructs. In all cases except wild-type TGMV A, the *CP* gene was replaced in a transcriptional fusion with the GUS reporter at the *CP* transcription start site (nucleotides 319–320; arrow shown on linear transgenes only) (Petty *et al.,* 1988; Sunter *et al.,* 1989). All constructs contain the conserved geminivirus hairpin located in the common region (CR), which is nearly identical in the A and B components of bipartite viruses (CR, hatched lines, indicated only in circular genomes). Linear constructs A75, A400, A55M, A35M, A401, and A95M (left) were used to generate transgenic plants. A75 and A400 differ only in having either the nopaline synthase polyadenylation signal (nos 3′) or the wild-type TGMV polyadenylation signal (CP 3′). A diagram of the wild-type, circular TGMV DNA A (generated in plant cells from pTGA26) is shown at the upper right. The positions of genes are indicated by solid arrows (Rep, replication initiator protein; TrAP, transcriptional activator protein; CP, coat protein). The *Xba*I site in parenthesis is present only in TGMV *AL2* mutants (e.g., pTGA44). The mutation was created by insertion of a C residue, which generated the restriction site and moved a termination codon in frame with the *AL2* coding sequence. The *AL2* mutants could produce a truncated TrAP consisting of the 23 N-terminal amino acids (Elmer *et al.,* 1988a). The circular genomes generated in plant cells from pTGA35 and pTGA55, shown at the lower right, were used in biolistic transient expression experiments. Both of these circular genomes contain the GUS replacement of the *CP* gene, and pTGA55 is also an *AL2* mutant and contains the *Xba*I site (in parenthesis). The *Xho*I site (in parenthesis) is present only in pTGA61, which was used in construction of the A75 transgene (see Materials and Methods).

no differences in transgene expression were observed between regenerants and selfed progeny.

Previous studies demonstrated that in tobacco protoplasts or leaf discs cut from *N. benthamiana* plants, expression of the *CP* promoter is dependent on the presence of an intact *AL2* gene (Sunter and Bisaro, 1991, 1992). In accordance with these results, the A75 construct (pTGA75), which lacks the *AL2* gene, did not express GUS above background levels in protoplasts unless it was cotransfected with an expression plasmid containing the *AL2* gene driven by the cauliflower mosaic virus 35S promoter (pTGA79) (data not shown). As shown in Fig. 1, the A75 construct contains a truncated *CP* promoter consisting of 657 bp of upstream sequence fused to the GUS gene, which is bordered on its 3′ end by the nopaline synthase polyadenylation signal. Surprisingly, as illustrated in Figs. 2A and 2B, when *N. benthamiana* plants transgenic for the A75 *CP* promoter were histologically stained for GUS activity, strong expression was visible in phloem tissue, although no expression was evident in mesophyll or other cell types. Similar vascular-specific expression

FIG. 2. *CP* promoter-GUS activity in *N. benthamiana* tissue. Tissue samples were treated and prepared as described under Materials and Methods. Leaves, stems, and hand cut sections from *N. benthamiana* plants were photographed under a light microscope. (A) Leaf from a plant containing the A75 transgene. Magnification 25×. (B) Transverse section through a vascular bundle from a plant containing the A75 transgene. The section is through the petiole mid-rib region of the leaf. Note that staining is confined to phloem tissue adjacent to unstained xylem elements. Magnification is 150×. IP, inner phloem; OP, outer phloem; X, xylem. (C) Shoot arising from callus containing the A75 transgene. Note that the phloem-restricted expression pattern is reestablished soon after shoot organization. Magnification 25×. Ca, callus; S, shoot. (D) Systemically infected leaf from a plant containing the A75 transgene 2 weeks after inoculation with TGMV. Note that GUS staining appears in both phloem and mesophyll cells surrounding a chlorotic lesion (upper right) in which staining is much reduced or absent. Magnification 25×. (E) Systemically infected leaf from a plant containing the A55M transgene 2 weeks after inoculation with TGMV. Note that GUS staining appears in both phloem and mesophyll cells. Magnification 25×. (F) Nontransgenic *N. benthamiana* leaf bombarded with pTGA35. Note staining in phloem and vascular tissue. The expression of GUS in a large number of contiguous cells is probably the result of limited cell to cell spread of DNA A (released from pTGA35), which can occur in the absence of DNA B (Klinkenberg and Stanley, 1990). Magnification is 25×.


A
B
X
OP
IP
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S
Ca
D
E
F

TABLE 1

TGMV Coat Protein Promoter Expression in Transgenic *N. benthamiana* Plants

	TrAP-Independent expression[a]		TrAP Activation of expression[b]	
Construct	Phloem	Mesophyll	Phloem	Mesophyll
A75	+	−	na[c]	+
A400	+	−	na	+
A55M	−	−	+	+
A35M	−	−	+	+
A95M	−	−	+	+
A401	−	−	+	+

[a] For each promoter construct, at least 20 plants from each of at least 3 independent transgenic lines were examined histochemically for GUS expression after 21 days postgermination as described under Materials and Methods.

[b] For each promoter construct, at least 10 plants from each of at least 3 independent transgenic lines were inoculated with TGMV to provide TrAP function, and systemically infected leaves were examined histochemically for GUS expression 10–21 days postinoculation as described under Materials and Methods.

[c] na, not applicable.

has been observed with truncated *CP* promoters from pepper huasteco virus (PHV; R. Ruiz-Medrano and R. F. Rivera-Bustamante, personal communication). Clearly, the truncated A75 promoter contains sequence elements capable of directing expression in vascular tissue in a TrAP-independent fashion. More recent experiments have shown that transgenic plants containing promoter constructs with as few as 163 bp of upstream sequence show essentially the same expression pattern as A75 plants, indicating that sequence elements responsible for phloem expression are located in this relatively small region (F. A. Meyer and D. M. Bisaro, manuscript in preparation). Vascular expression from the A75 transgene may be developmentally regulated, since it was not observed in seedlings prior to 14 days postgermination (data not shown). The onset of expression was typically between 14 and 21 days. In contrast, expression in control seedlings harboring a 35S-GUS construct was evident at the earliest time point examined (3 days).

The TrAP-independent expression observed in A75 transgenic plants was not an artifact resulting from use of the heterologous nopaline synthase polyadenylation signal. A similar transgene construct, A400, consisting of the GUS gene fused to the same 657-bp upstream sequence but bordered by the native *CP* 3′ polyadenylation signal (Petty *et al.,* 1988; Sunter *et al.,* 1989) also displayed TrAP-independent expression that was confined to phloem tissue (Fig. 1 and Table 1). This expression was not observed before 14 days post-germination.

TrAP is required for expression of the complete *CP* promoter in all cell types

In an attempt to reconcile the fact that *CP* expression in protoplasts depends on TrAP with the observation of TrAP-independent expression in plants harboring the truncated A75 promoter, transgenic plants containing a complete *CP* promoter (A55M) were generated (Fig. 1). The complete promoter is defined here as all DNA A sequences except those present in the *CP* coding region. Further, because the TGMV genome is circular, it was assumed that locating promoter elements upstream or downstream of the transcription start site would have little effect on their function, and so the additional sequences in the linear A55M are downstream of the *CP* gene. This assumption was validated by experiments with the A95M construct discussed below. Further, it is important to note that A55M contains a frameshift mutation within the *AL2* gene and could produce only a truncated TrAP consisting of 23 N-terminal amino acids (Elmer *et al.,* 1988a; Sunter *et al.,* 1990). As a consequence, the A55M construct (pTGA55M) did not express GUS above background levels in protoplasts unless it was cotransfected with an *AL2* expression plasmid (data not shown).

In obvious contrast to A75 and A400 transgenic plants containing truncated *CP* promoters, no GUS expression was observed in any tissues of plants transgenic for the complete A55M promoter (Table 1). Therefore, the additional sequences present in the complete promoter must contain a repressor element capable of masking the TrAP-independent phloem expression seen with the truncated promoters. When leaf discs cut from A55M plants were placed on culture medium and allowed to callus, intense TrAP-independent GUS expression was observed in the callus tissue. This result verified that the chromosomally inserted A55M *CP* promoter is viable and potentially active and revealed an additional aspect of developmental regulation, namely that repression is not maintained in unorganized tissue. Interestingly, the GUS expression seen in unorganized callus disappeared in shoots arising from A55M callus tissue, showing that repression is rapidly reestablished following tissue organization. Analogous results were obtained when leaf discs cut from A75 plants were allowed to callus. Shoots which organized from the A75 callus lost GUS expression in mesophyll and other tissues, but GUS continued to be expressed in the phloem (Fig. 2C).

To confirm that repression of *CP* promoter activity in phloem tissue was not dependent on repressor location (at least in the context of linear transgenes), construct A95M was built and used to generate transgenic *N. benthamiana* plants (Fig. 1). A95M is similar to A55M in that it contains a complete promoter and a disruption of the *AL2* gene, which could theoretically allow the synthesis

of a truncated TrAP consisting of the N-terminal 48 amino acids fused to unrelated sequence. However, in A95M, the repressor-containing *Bam*HI–*Eco*R1 fragment is located upstream rather than downstream of the *CP* gene. The A95M promoter construct (pTGA95M) did not express GUS above background levels in the absence of *AL2* function in protoplasts (data not shown) and behaved identically to A55 in transgenic plants; that is, TrAP-independent GUS expression was not observed (Table 1).

The effect of placing the wild-type *AL2* coding sequence in the context of the complete promoter was also examined. In this experiment, transgenic plants containing A35M, the wild-type (with respect to *AL2*) parent of A55M (Fig. 1), were examined for GUS activity after 14 days postgermination. Surprisingly, in no case was GUS expression observed in these plants (Table 1), suggesting that either the *AL2* gene is not expressed in this linear context or that *AL2* expression levels from low copy number chromosomal transgenes is insufficient to support expression of the *CP* promoter. It should be noted that the lack of *CP* promoter activity in phloem of plants transgenic for A35M, which contains an intact *AL2* coding sequence, effectively rules out the possibility that a truncated 23 or 48 amino acid AL2 polypeptide produced by A55M and A95M, respectively, was responsible for repression.

One additional transgene construct, A401, was analyzed as a first step toward mapping the repressor element. The A401 *CP* promoter is similar to A55M, except that it lacks the 592-bp *Nhe*I–*Eco*RI fragment (Fig. 1). Because A401 transgenic plants do not show GUS expression in any tissue (Table 1), it appears that the repressor element resides within the 300-bp *Bam*HI–*Nhe*I fragment that is present in A401, but absent in A75. Thus, the repressor is located within or near the *AL2* gene, and almost directly opposite the *CP* transcription start site on the circular genome (Fig. 1).

It was concluded from these experiments that a repressor element is present in the A55M, A95M, A35M, and A401 *CP* promoter constructs, but not in the A75 and A400 promoters. The repressor is responsible for the lack of phloem expression in A55M, A95M, A35M, and A401 transgenic plants. Repression is not maintained in unorganized callus tissue, where the *CP* promoter is expressed at a high level in the absence of TrAP.

Activation and derepression of *CP* promoters by TGMV infection

To determine whether TrAP is capable of activating a chromosomal gene, transgenic plants containing *CP* promoter transgenes were agroinoculated with TGMV. In healthy or mock inoculated A75 transgenic plants, GUS expression remained confined to the phloem for up to four months postgermination (or approx. 2 months after mock inoculation), when expression gradually declined with the onset of senescence (data not shown). In contrast, by 10 days after TGMV inoculation, abundant mesophyll expression was clearly evident in systemically infected leaves of A75 plants, primarily in cells surrounding chlorotic lesions (Fig. 2D). Expression was usually absent within the lesions, probably as a result of the cytopathic effects of virus infection. Similar mesophyll expression was seen following TGMV inoculation of A400 transgenic plants, demonstrating that the polyadenylation signal is not a factor in mesophyll activation of these truncated *CP* promoters (Table 1).

Inoculation of A55M transgenic plants with TGMV yielded similar results. While GUS expression was never observed in any tissue of healthy or mock-inoculated A55M transgenic plants, TGMV infection resulted in expression from the complete *CP* promoter in phloem and mesophyll cells by 10 days postinoculation (Fig. 2E). Expression in systemically infected leaves was strongest in vascular and mesophyll tissues surrounding chlorotic lesions, whereas the lesions themselves often showed little or no GUS activity. *CP* promoter activity was also evident in phloem and mesophyll tissues of A95M, A35M, and A401 transgenic plants following agroinoculation with TGMV (Table 1).

Expression from *CP* promoters in transgenic plants following TGMV infection is not due to a general stress response, because no GUS expression was observed in A55M plants or in mesophyll cells of A75 plants following agroinoculation with BCTV, which does not activate the TGMV *CP* promoter (10 plants each examined) (Sunter *et al.,* 1994). Likewise, no expression was observed in leaves wounded with forceps.

From these experiments, it was concluded that a specific virus-coded factor, probably TrAP, is responsible for activation of the *CP* promoter in mesophyll tissue and for derepression of the promoter in phloem tissue. The viral factor is clearly capable of inducing expression from responsive chromosomal promoters.

Activation and derepression of the *CP* promoter is mediated by TrAP

GUS expression was not observed in A55M plants or in mesophyll cells of A75 plants following biolistic delivery of an *AL2* expression plasmid (pTGA79), probably because expression from chromosomal *CP* transgenes does not reach levels sufficient for detection by the histochemical assay when the expression of TrAP is transient. To circumvent this difficulty and to verify that TrAP is indeed the viral factor responsible for activation and derepression, a replicating TGMV construct containing the A55M *CP* promoter was employed in biolistic experiments. The replicating construct, pTGA55 (TGMV A

TABLE 2

TGMV CP Promoter Activation Following Biolistic Delivery to *N. benthamiana* Leaves

Construct	GUS Expression[a] in mesophyll and vascular tissue
pUC118	–
pTGA55	–
TGMV A (CP:GUS/AL2$^-$)	
pTGA35	+
TGMV A (CP:GUS)	
pTGA55 + pTGA44	–
TGMV A (CP:GUS/AL2$^-$) + TGMV A (AL2$^-$)	
pTGA55 + pTGA26	+
TGMV A (CP:GUS/AL2$^-$) + TGMV A	
pTGA55 + pCLV1.3A	+
TGMV A (CP:GUS/AL2$^-$) + ACMV A	
pTGA55 + pLOGAN	–
TGMV A (CP: GUS/AL2$^-$) + BCTV	
pTGA55 + pTGA79	+
TGMV A (CP:GUS/AL2$^-$) + 35S-AL2	
35S-GUS	+

[a] For each construct or pair of constructs, DNA was delivered biolistically to 2–3 leaves of nontransgenic *N. benthamiana* plants in each of at least 3 independent experiments. Bombarded leaves were examined histochemically for GUS expression as described under Materials and Methods.

(CP:GUS/AL2$^-$)), consists of ~1.5 tandemly repeated copies of A55M inserted into a plasmid vector (Sunter and Bisaro, 1991). In plant cells, unit-length circular genomes are generated from the tandem repeats by a replicative release mechanism and replicate extrachromosomally to high copy number (Stenger *et al.,* 1991; Kanevski *et al.,* 1992). Thus, pTGA55 produces a circular, replicating viral genome that is in all other respects identical to the linear A55M construct: it contains the same GUS reporter insertion and the same *AL2* frameshift mutation (Fig. 1). A replicating version of the A35M construct, pTGA35 (TGMV A (CP:GUS)), which is identical to pTGA55 except that it lacks the *AL2* frameshift mutation, was also used (Fig. 1). Both pTGA35 and pTGA55 replicate to similar extents in tobacco protoplasts, and pTGA35 expresses GUS to very high levels (Sunter and Bisaro, 1991; Brough *et al.,* 1992). Because pTGA55 does not make functional TrAP, it does not express GUS above background levels in protoplasts, despite its high copy number (Sunter and Bisaro, 1991).

Biolistic delivery of pTGA35 to healthy, nontransgenic *N. benthamiana* leaf tissue resulted in numerous blue spots indicating GUS expression (Table 2). Spots were observed in the mesophyll and in cells that were clearly part of the vascular bundle, indicating that the *CP* promoter was active in both mesophyll and phloem tissues (Fig. 2F). In contrast, no expression in mesophyll or phloem tissue was ever seen following biolistic delivery of pTGA55.

That expression was observed with pTGA35 and not with the *AL2* mutant pTGA55 suggested that TrAP is necessary for *CP* promoter activity in all tissues. To explore this point more fully, pTGA55 was cobombarded to nontransgenic *N. benthamiana* leaves with TGMV DNA A, a TGMV *AL2* mutant, ACMV DNA A, BCTV DNA, and an expression plasmid containing the TGMV *AL2* gene under the control of the 35S promoter. In these experiments, expression in mesophyll and phloem tissue was observed only when pTGA55 was codelivered with DNAs capable of expressing a complementing TrAP protein; namely TGMV DNA A, ACMV DNA A, and the *AL2* expression construct. Expression was not observed when pTGA55 was cobombarded with a TGMV *AL2* mutant, or with BCTV DNA (Table 2).

Finally, the pTGA35 and pTGA55 constructs were used to generate transgenic *N. benthamiana* plants. In transgenic plants, unit-length circular molecules representing these modified viral genomes are expected to be released from the tandem copies and to replicate extrachromosomally (Rogers *et al.,* 1986). Following histochemical staining, GUS expression was visible in isolated clusters of mesophyll and vascular cells in transgenic pTGA35 plants (data not shown). GUS expression was never observed in pTGA55 transgenic plants, even though Southern blot hybridization analysis confirmed that they contained replicating, unit genome length pTGA55 DNA. Taken together, these experiments unequivocally demonstrated that TrAP is both necessary and sufficient for activation of the *CP* promoter in mesophyll cells, and for derepression of the *CP* promoter in the phloem.

DISCUSSION

In the studies presented here, transgenic *N. benthamiana* plants containing complete or truncated versions of the TGMV *CP* promoter fused to the GUS reporter gene were used to investigate issues of tissue specificity and developmental regulation and to examine whether TrAP is required for the expression of this promoter in all tissues. We found that the requirement for TrAP depends on the tissue and on the extent of the promoter examined. Certain truncated versions of the *CP* promoter are active in phloem cells in the absence of TrAP, and this phloem-specific expression may be developmentally regulated since it is not observed prior to 14 days postgermination. However, in the context of the complete promoter (which contains all potential promoter elements except those which might be present in the *CP* gene itself), phloem expression is repressed and no TrAP-independent expression is seen in any tissue, with the exception of unorganized callus. Upon inoculation of transgenic plants containing *CP* promoter constructs with TGMV,

we learned that TrAP is capable of activating responsive chromosomal promoters and that this viral protein induces *CP* promoter expression in both phloem and mesophyll cells. The data suggest that TrAP derepresses the complete *CP* promoter in phloem tissue, and the possibility that TrAP also acts by a derepression mechanism in mesophyll cannot be ruled out at this time. However, as TrAP-independent expression of the *CP* promoter has yet to be observed in mesophyll or in protoplasts in this and other studies, we hypothesize that the promoter is activated by TrAP in these cells. Further analysis of *CP* promoter activity in protoplasts and transgenic plants will be required to clarify this issue.

Experiments in which a replicating version of the complete (but *AL2*$^-$) *CP* promoter was codelivered biolistically to nontransgenic *N. benthamiana* leaves along with several viral DNAs and expression constructs proved that TrAP is the viral factor responsible for promoter activation and derepression. Specifically, expression from the complete *CP* promoter in mesophyll and phloem tissues was observed only when it was codelivered with TGMV DNA A, ACMV DNA A, or a 35S-*AL2* expression construct, all of which can provide functional, complementing TrAP. No expression was seen when the promoter construct was codelivered with a TGMV *AL2* mutant or with BCTV DNA.

A rationale for the dependence of *CP* and *BR1* promoters on TrAP may be seen by examining the viral replication process. A key step in the control of viral DNA synthesis follows the production of circular plus strand DNA by RCR (Kornberg and Baker, 1992). The nascent ssDNA may either reenter the replication/transcription pool following priming and minus strand synthesis (RF→RF synthesis), or it may be removed from the pool by encapsidation or other means (RF→SS synthesis). In the subgroup III geminiviruses, this step may be regulated by TrAP. Both CP and BR1 movement protein bind ssDNA (Pascal *et al.,* 1994), and placing their expression under the control of TrAP may ensure that these proteins are not made prematurely, allowing sufficient amounts of the double-stranded RF to accumulate early in the replication cycle. However, the possibility that TrAP may also play a more direct role in regulating a switch from RF→RF to RF→SS synthesis cannot be ruled out at this time.

It seems clear that distinct mechanisms exist for the expression of the *CP* promoter in different cell types, requiring a number of cellular factors to interact with this promoter. We found that the truncated A75 *CP* promoter, which contains 657 bp of TGMV sequence upstream of the transcription start site, was strongly expressed in vascular tissue in the absence of TrAP. Sequence elements required for this vascular expression have been localized to a small region within 163 bp of the transcription start site (F. A. Meyer and D. M. Bisaro, manuscript in preparation). Similar results, although different in detail, have been obtained with truncated *CP* promoters of pepper huasteco virus, a related subgroup III geminivirus (R. Ruiz-Medrano and R. L. Bustamante, personal communication). Thus, in an abbreviated context, the activity of the *CP* promoter appears to be tissue specific and TrAP-independent. Because it is likely that all transcription in eukaryotic cells requires activation *in vivo* (Zawel and Reinberg, 1995), *CP* promoter activity in the phloem no doubt depends on cellular transcriptional regulatory proteins, which bind positive regulatory elements near the start site. These proteins may be limited to the phloem, where they appear approximately 14 days postgermination in *N. benthamiana* leaves. The same or similar factors may be present in unorganized callus tissue. The identity of these proteins is unknown.

In the context of the complete promoter (A55M *CP* promoter), which includes all DNA A sequences except those that comprise the *CP* open reading frame, TrAP-independent expression was never observed. This and similar results with the somewhat smaller A401 promoter allowed us to localize a repressor element to a 300-bp sequence within or near the *AL2* gene, almost directly opposite the *CP* transcription start site on the circular TGMV A component. That repression is mediated by a cis-acting element and is not an artifact due to the expression of an interfering, truncated AL2 protein from A55M and A401 was confirmed by the absence of phloem expression in transgenic plants harboring the A35M promoter, which contains an intact but apparently unexpressed *AL2* coding sequence.

Phloem expression normally activated by regulatory proteins binding near the *CP* transcription start site appears to be blocked by a factor which binds the repressor element, although how these positive and negative regulatory proteins interact with other, or with general transcription factors (GTFs), is not known. To alleviate repression in vascular tissue, TrAP apparently interacts with the repressor to mask or abolish its activity. The mechanism by which this occurs is also not known, although it seems likely that it involves a specific protein–protein interaction. The repressor is (at least) present in phloem tissue, but the experiments presented here did not allow us to determine whether it is also present in mesophyll cells. It is likely that the repressor does not accumulate in unorganized tissue, because the complete *CP* promoter is expressed in a TrAP-independent manner in callus.

Repression is increasingly recognized as an important eukaryotic gene control strategy, and there are several examples of plant promoters which are controlled by interactions between factors binding positive and negative regulatory elements (Bruce *et al.,* 1991; Harrison *et al.,* 1991; Guevara-Garcia *et al.,* 1993; Sun *et al.,* 1993). TGMV appears to have adopted this strategy to control

the *CP* promoter in the phloem; a strategy which in this case may be described as regulated repression.

It is interesting to note that the negative regulatory element located in the *AL2* gene does not appear to affect transcription of the *AL1* and *AL3* genes, which are required for viral replication, or the *AL2* gene itself, which directs the synthesis of TrAP. Clearly, the repressor is specific in its repression of the *CP* promoter, but how this specificity is achieved is not known. Whether the repressor is capable of functioning in a heterologous promoter in an appropriate context is under investigation.

While many geminiviruses are reported to be phloem-restricted, TGMV is not and is able to invade mesophyll and other tissues of *N. benthamiana* (Rushing *et al.,* 1987). Consequently, this virus must have evolved mechanisms for expressing its promoters in cell types outside the vasculature. Evidence presented in this report clearly shows that this is the case, and that mesophyll expression of all *CP* promoters used in this study requires TrAP.

Herpes simplex virus (HSV) VP16 is a particularly relevant model for TrAP, given some of the properties these proteins share. VP16 activates the expression of HSV immediate early viral genes through a sequence element present in all responsive promoters. However, by itself VP16 has no substantial affinity for dsDNA. Rather, the response element contains a binding site for the cellular transcription factor Oct-1, and it is the formation on the response element of a multiprotein complex consisting of Oct-1, VP16, and other cellular proteins that potentiates transcription (Gerster and Roeder, 1988; Triezenberg *et al.,* 1988; Kristie *et al.,* 1989). Another useful model for TrAP is also found in the herpesviridae; namely the Epstein–Barr virus (EBV) EBNA2 activator. EBNA2 is expressed early after EBV infection and activates genes, both viral and cellular, which are required for virus latency and B cell immortilization. Again, EBNA2 does not bind DNA directly, but instead is targeted to regulated promoters via an interaction with the DNA binding protein CBF-1, a transcriptional repressor. The interaction of EBNA2 with CBF-1 appears to occlude a repression domain in the latter, thereby activating transcription (Hsieh and Hayward, 1995).

A number of viral and cellular factors are known to repress transcription by a number of different mechanisms, and several can act either as activators or repressors depending on promoter context and/or the presence or absence of other specific proteins (Shi *et al.,* 1991; Roberts and Green, 1995; Zawel and Reinberg, 1995). However, repression in general is not well understood. In TGMV, we have found a repressor element that behaves in a promoter-specific fashion, and we have identified a viral protein, TrAP, that can counter repression. This same protein can also activate transcription in some cell types. It will be interesting to elucidate the nature of the repressor, and to understand how a small, 15-kDa viral protein specifically interacts with it and with other elements of the transcription machinery to alternatively activate or derepress gene expression.

Any model that invokes cellular transcription factors for targeting TrAP to responsive promoters implies that TrAP can activate host genes. Indeed, evidence provided in this report conclusively demonstrates that TrAP is capable of activating a responsive chromosomal promoter, and this is also suggested by the recent work of Stanley and colleagues with the ACMV system (Hong *et al.,* 1996). Several mammalian virus transactivators are known to activate host as well as viral genes, and in some cases this is important in pathogenesis. EBNA2 (EBV), E1A (adenovirus), and Tax (human T cell leukemia virus), for example, are oncoproteins that function in part by activating cellular genes (Flint and Shenk, 1989; Matthews *et al.,* 1992; Franklin *et al.,* 1993; Wagner and Green, 1993). We consider it possible that activation (or derepression) of host genes by TrAP plays an important role in geminivirus pathogenesis, and experiments to address this question are in progress.

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