Simultaneous regulation of tomato golden mosaic virus coat protein and AL1 gene expression: expression of the AL4 gene may contribute to suppression of the AL1 gene

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The tomato golden mosaic virus (TGMV) coat protein and AL1 genes are located in opposite directions on either side of an intergenic region. To enable the effects of the AL1, AL2 and AL3 gene products on expression of the coat protein and AL1 genes to be studied simultaneously, a plasmid was constructed, containing the intergenic region linked on one side to a 5'-terminal portion of the AL1 gene fused to a β-glucuronidase (GUS) reporter gene (to replace most of the AL1 gene) and on the other side to a neomycin phosphotransferase (NEO) reporter gene (to replace the coat protein gene). This GUS–NEO plasmid was mixed with plant expression plasmids containing the AL1, AL2 or AL3 coding regions, the DNA was transformed into Nicotiana benthamiana protoplasts and GUS activities and NEO protein levels were measured. Control transformations were carried out with the GUS–NEO plasmid mixed with the AL1, AL2 or AL3 plasmids in which mutations were introduced to prevent translation of the open reading frames (ORFs). The results showed that trans-activation of the coat protein gene by the AL2 gene product and suppression of the AL1 gene by the expression of AL1 DNA (both reported previously) can occur simultaneously. It was also shown that expression of AL4, a small ORF contained within AL1 DNA but in a different reading frame, as well as expression of ORF AL1, can cause significant suppression of AL1 gene expression. Neither the AL1 nor the AL3 gene products affected the expression of the coat protein gene.

Introduction

Tomato golden mosaic virus (TGMV) belongs to subgroup III of the geminiviruses, members of which are characterized by a genome of two circular ssDNA components, A and B (Francki et al., 1991). Both DNA A and DNA B are required for infection and symptom development of plants (Hamilton et al., 1983), but DNA A can replicate alone in single cells (Rogers et al., 1986). DNA A has four open reading frames (ORFs) with the potential to encode proteins of more than 100 amino acids, which are transcribed in a rightwards (AR1) or leftwards (AL1, AL2 and AL3) direction (Hamilton et al., 1984; Sunter & Bisaro, 1989) (Fig. 1a). ORF AR1 encodes the capsid protein (Kallender et al., 1988), AL1 is an absolute requirement for viral DNA replication (Elmer et al., 1988), AL2 is needed for systemic infection of plants (Elmer et al., 1988) and AL3 is required for normal levels of DNA replication, but is not an absolute requirement (Elmer et al., 1988; Sunter et al., 1990). ORFs AL1 and AR1 are separated by a 313 nucleotide (nt) intergenic region (nt 14 to 326). The major transcriptional start sites for the AL1 and AR1 genes have been mapped within the intergenic region at nt 62 and 319 respectively (Sunter & Bisaro, 1989) and TATA boxes which may form part of the promoters for these genes are located about 30 nt upstream of these transcriptional start sites. Deletion of the latter TATA box abolishes coat protein promoter activity (Hayes & Buck, 1989).

The products of the AL2 gene of TGMV and the related geminivirus African cassava mosaic virus (ACMV) have been shown to trans-activate transcription of the respective coat protein gene (Sunter et al., 1990; Sunter & Bisaro, 1991, 1992; Haley et al., 1992). Conversely expression of AL1 DNA was shown to suppress expression of the AL1 gene (Haley et al., 1992; Sunter et al., 1993). However expression of the coat protein and AL1 genes were studied separately. Furthermore it was not shown whether suppression of the AL1 gene was due to expression of ORF AL1 or to expression of AL4, a small ORF contained within AL1 DNA but in a different reading frame (or to both). Here we describe the use of a bidirectional expression cassette...
to study the expression of the coat protein and AL1 genes simultaneously in a transient expression protoplast system. We show that expression of ORF AL4, as well as that of ORF AL3, can suppress AL1 gene expression. We also show that expression of ORFs AL1(AL4) and AL3 have no effect on coat protein gene expression and that trans-activation of the coat protein gene by AL2 and suppression of the AL1 gene by expression of the AL1(AL4) ORFs can occur simultaneously.

**Methods**

Construction of pGUS-NEO. An EcoRI-BamHI fragment of pTAneo2 (Petty et al., 1986), comprising an N-terminal sequence of AL1, the intergenic region of TGMV DNA A, a bacterial neomycin phosphotransferase (NEO) gene and the TGMV coat protein terminator, was cloned into the corresponding sites of the vector pEMBL9+ (Dente et al., 1985) to give pTAneo3. An EcoRI linker (CCGAATTCGCG) was ligated to a Smal-EcoRI fragment of pBl212 (Jefferson et al., 1987), containing a bacterial β-glucuronidase (GUS) gene linked to a nopaline synthase (nos) terminator. The product was cleaved with EcoRI and cloned into the EcoRI site of pTAneo3 to give pGUS-NEO (7 kb) (Fig. 1b). Nucleotide sequencing was used to identify clones with the GUS gene in the desired orientation and to confirm that the GUS gene and the 5′-terminal part of the AL1 ORF were in the same reading frame.

Construction of pAL1, pAL2 and pAL3. Plasmid pCaP35J (Yamaya et al., 1988) contains a cauliflower mosaic virus (CaMV) 35S promoter, modified to produce a Stul site at the transcriptional start site, and cloned between the BamHI and HindIII sites of pUC18. Plasmid pCamBSEn (A. G. Day, personal communication) was created by cloning the 0.35 kb ClaI-HindIII CaMV 35S promoter fragment (Odell et al., 1985) (blunt-ended) into the Smal site of the pBS+ phagemid (Stratagene) to give pCamBS. The Eco-Rev-KpnI fragment (blunt-ended) of pCamBS containing the upstream region of the 35S promoter, which acts as a transcriptional enhancer (Kay et al., 1987), was then cloned into the SacI site (blunt-ended) of pCamBS to give pCamBSEn. Plasmid pRH100, which contains the 35S promoter with the additional enhancer and the Stul site at the transcriptional start site, was created by replacing the Eco-Rev-PstI fragment of pCamBSEn with the equivalent fragment of pCaP35J. The Ω translational enhancer from tobacco mosaic virus (TMV; Gallie et al., 1987) was introduced into pRH100 as follows. A Ndel site was introduced at the 3′ end of the Ω sequence using PCR (Saiki et al., 1988) with a cloned Ω sequence, pJH101 (Gallie et al., 1987) at the template, GTATTATTACAAAC which corresponds to the 3′ end of the Ω sequence as the forward primer and GAAGCTTGCGGATCCATATGTAATTGTA-AATAG which contains HindIII, Smal, BamHI and Ndel sites linked to a sequence complementary to the 3′ end of the Ω sequence as the reverse primer. The amplified fragment was cleaved with HindIII and cloned between the HindIII and Stul sites of pRH100 to give pRH101. A nos terminator was isolated from pNeo6, a clone of a NEO gene flanked by a nos promoter and terminator (M. W. Bevan, personal communication). pNeo6 was cleaved with EcoRI and the product was made blunt-ended and cleaved with BamHI. The 0.35 kb fragment containing the nos terminator was then ligated between the Smal and BamHI sites of pRH101 to give pRH102. This vector contains the enhanced 35S promoter linked to the Ω sequence and the nos terminator. It has unique Ndel and BamHI sites to enable ORFs to be cloned between the Ω sequence and the nos terminator.

Ndel and BglII sites were introduced by in vitro mutagenesis at the ATG translational initiation codon and just downstream of the termination codon respectively of the AL1 ORF in pAX, a clone of TGMV DNA A in pEMBL9X (Brough et al., 1988) to give pAX1. The AL1 ORF was excised from pAX1 with Ndel and BglII and cloned between the Ndel and BamHI sites of pRH102 to give pAAX1. Similarly Ndel and BglII sites were introduced at the translational initiation codon and just downstream of the termination codon of the AL2 and AL3 ORFs in pAX to give pAX2 and pAX3 respectively. The AL2 and AL3 ORFs were excised from pAX2 and pAX3 with Ndel and BglII and cloned between the Ndel and BamHI sites of pRH102 to give pAAX2 and pAAX3 respectively.

Construction of mutants. Mutations were introduced into pAAX1, pAAX2 and pAAX3 by the method of Künkel et al. (1987) using the following oligonucleotides: for mutation A, CGGTTTTGGATGC-GATGGCA (frameshift after the first ATG of ORF AL1); for mutation B, AGGGGGGAGTGGAGAAGACG (frameshift after the first ATG of ORF AL2); for mutation C, GTGAGGTGTCCTCATGTAAGCCT (frameshift after the first ATG of ORF AL3); for mutation D, GTGAGGTGTCCTCATGTAAGCCT (frameshift after the first ATG of ORF AL4 and elimination of the second ATG of ORF AL4); for mutation E, AGGCCATGTCATCTGGCTG (frameshift after the second ATG of ORF AL1); for mutation F, GAAAGGCGAC-ACATAGTCTTTCCCG (frameshift after the third ATG of ORF AL1). The mutant plasmids were designated pAAX1-mut1 (mutation A), pAAX2-mut1 (mutation B), pAAX3-mut1 (mutation C), pAAX1-mut2 (mutations A and E), and pAAX3-mut4 (mutations A, D, and E).

Protoplast isolation and transformation. Nicotiana benthamiana plants were grown under sterile conditions in Murashige and Skoog medium MS5519 (Sigma) with 3% (w/v) sucrose and 0.8% agar at 25 °C with lighting for 16 h per day provided by fluorescent and tungsten lamps (4000 to 5000 lux) and propagated from cuttings. Protoplasts were prepared by cutting leaves from 10 cm high plants into 1 mm strips, followed by digestion overnight with 1% cellulase and 0.5% macerozyme in CPW salts (Power & Chapman, 1985), supplemented with 10% (w/v) mannitol and 3 mm-MES pH 5.8 (Tan et al., 1987) at 25 °C in the dark. The protoplast suspension was filtered through 75 μm nylon gauze and washed twice with W5 medium (Menczel et al., 1981).

For transformation (Negrotto et al., 1987), protoplasts (0.8 × 10^6 to 1.2 × 10^7) were incubated in MM9 solution (9% mannitol, 15 mM-MgCl₂, 5 mm-MES pH 5.8, 50 μg of pGUS-NEO, 25 μg of the appropriate expression plasmid, 50 μg of carrier DNA (sheared salmon sperm DNA) and 15% (w/v) polyethylene glycol 8000 in a volume of 400 μl at room temperature for 30 min. The protoplasts were then washed with the culture medium (MS5519) with the addition of 3% (w/v) glucose, 6% (w/v) mannitol, 0.5 mg/l 6-benzylaminopurine and 2 mg/l 1-naphthaleneacetic acid, pH 5.8, suspended in the culture medium at a concentration of 0.4 × 10^5 to 0.5 × 10^6 protoplasts/ml, incubated in the dark at 25 °C in 3 cm diameter Petri dishes for 1 or 2 days and then assayed for GUS activity and NEO levels. Background levels in extracts of untransformed protoplasts were subtracted. As there was some variation between values obtained with different batches of protoplasts, control transformations were carried out each time by transforming protoplasts with pGUS-NEO mixed with expression plasmids (pAL1-mut1, pAL2-mut1 or pAL3-mut1) in which mutations had been introduced to prevent translation of the ORFs. Expression plasmids with mutated ORFs were used in preference to an expression plasmid completely lacking the ORF, so that transcripts of the same size would be produced by both the wild-type and control (mutant) plasmids. In this way, any transcriptional competition would be eliminated and any observed effects could be ascribed entirely to translation of the ORF. When the ratios of pGUS-NEO+pAL1, pAL2 or pAL3 to pGUS-NEO+pAL1-mut1, pAL2-mut1 or pAL3-mut1 GUS enzyme activities or NEO protein levels were compared,
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variation between different batches of protoplasts was minimized. Values obtained after 1 and 2 days incubation, which were similar, were averaged.

GUS assay. Protoplasts were lysed and GUS activity was determined as described by Jefferson (1987) and Jefferson et al. (1987). Fluorescence was measured using Fluoroplates (Titerkit, Flow Laboratories) with a Fluoroscan fluorimeter (Flow Laboratories) with excitation at 365 nm and emission at 455 nm.

NEO assay. NEO levels were determined by ELISA using an NPTII ELISA kit (5 Prime-3 Prime, Inc.) according to the manufacturer’s instructions.

Statistical methods. Tests for significance of differences in the expression of the GUS and NEO genes were carried out using a two-tailed t-test (Fisher, 1954; Fisher & Yates, 1963).

Results and Discussion

Construction of expression cassettes

To study expression of the TGMV AL1 and coat protein genes simultaneously, a cassette (GUS–NEO, Fig. 1b) was constructed, consisting of the intergenic region of DNA A linked on one side to a 5’-terminal portion of the AL1 gene fused to a GUS reporter gene (to replace most of the AL1 gene) and on the other side to a NEO gene (to replace the coat protein gene). The GUS and NEO genes were linked to nos and TGMV coat protein transcriptional terminators respectively. The plasmid containing the GUS–NEO cassette was designated pGUS–NEO.

To enable the effects of the AL1, AL2 and AL3 gene products on GUS and NEO expression from the GUS–NEO cassette to be studied, expression cassettes were constructed, consisting of a CaMV 35S RNA promoter plus an additional transcriptional enhancer (Kay et al., 1987) linked to the Ω translational enhancer from TMV (Gallie et al., 1987), followed by the AL1, AL2 or AL3 coding region and a nos terminator (Fig. 1c). The plasmids containing the AL1, AL2 and AL3 expression cassettes were designated pAL1, pAL2 and pAL3 respectively.

Suppressive effect of ORF AL4 on expression of the GUS gene by the GUS–NEO cassette

It has been shown for TGMV in N. tabacum protoplasts that, whereas the AL2 and AL3 gene products have no significant effect on the expression of the AL1 gene, expression of AL1 DNA suppressed expression of the AL1 gene (Sunter et al., 1993). We found similar results when N. benthamiana protoplasts were transformed with a mixture of pGUS–NEO and pAL2, pAL3 or pAL1. Neither pAL2 and pAL3, nor pAL2-mut1 and pAL3-mut1, which contained frameshift mutations after the initiation codons of the AL2 and AL3 ORFs respectively, had any significant effect on GUS activity. In contrast pAL1 caused almost complete suppression of GUS activity (Table 1). The suppressive effect of AL1 DNA on GUS (and hence AL1) expression could be due to expression of ORF AL1, ORF AL4 or both. To study this further, mutations were introduced into the AL1 expression cassette and their effect on expression of GUS activity by pGUS–NEO was measured (Table 1).

Table 1. Regulation of GUS expression from pGUS–NEO by wild-type and mutant AL1, AL2 and AL3 expression plasmids*

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Potential ORFs expressed</th>
<th>Mean GUS activity†</th>
<th>n‡</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAL1</td>
<td>AL1 + AL4</td>
<td>2.8</td>
<td>22</td>
<td>0.5</td>
</tr>
<tr>
<td>pAL1-mut1</td>
<td>AL4 + truncated AL1</td>
<td>24.0</td>
<td>19</td>
<td>2.3</td>
</tr>
<tr>
<td>pAL1-mut2</td>
<td>AL4</td>
<td>42.3</td>
<td>9</td>
<td>4.8</td>
</tr>
<tr>
<td>pAL1-mut3</td>
<td>Truncated AL1</td>
<td>77.2</td>
<td>9</td>
<td>5.1</td>
</tr>
<tr>
<td>pAL1-mut4</td>
<td>Truncated AL1</td>
<td>109.9</td>
<td>9</td>
<td>7.8</td>
</tr>
<tr>
<td>pAL2</td>
<td>AL2 + truncated AL3§</td>
<td>97.3</td>
<td>4</td>
<td>17.3</td>
</tr>
<tr>
<td>pAL2-mut1</td>
<td>Truncated AL3§</td>
<td>93.6</td>
<td>4</td>
<td>14.9</td>
</tr>
<tr>
<td>pAL3</td>
<td>AL3</td>
<td>99.3</td>
<td>3</td>
<td>7.7</td>
</tr>
<tr>
<td>pAL3-mut1</td>
<td>–</td>
<td>112.9</td>
<td>4</td>
<td>15.2</td>
</tr>
</tbody>
</table>

* N. benthamiana protoplasts were transformed with pGUS–NEO mixed with equal amounts of expression plasmids.
† Expressed relative to the value obtained with pGUS–NEO alone, which was assigned a value of 100. The mean value for pGUS–NEO alone was 9300 picomoles of 4-methylumbelliferone produced per milligram of protein.
‡ n, Number of protoplast experiments.
§ ORFs AL2 and AL3 overlap; hence pAL2 contains the 5’-terminal part of ORF AL3.
pAL1-mut1, which contained a frameshift mutation after the first ATG of ORF AL1, had a lower suppressive effect on GUS expression than did pAL1 (Table 1). Statistical analysis showed that this difference was significant at the 99% confidence level ($P = 0.01$). Therefore the AL1 protein is required for the full suppressive effect of pAL1. Nevertheless, the GUS activity obtained with pGUS-NEO+pAL1-mut1 was significantly ($P = 0.01$) lower than that obtained with pGUS-NEO alone, pGUS-NEO+pAL2-mut1 or pGUS-NEO+pAL3-mut1. There are several possible causes of this partial suppression of GUS activity by pAL1-mut1. Transcriptional competition could occur between pGUS-NEO and pAL1-mut1, e.g. for RNA polymerase or transcription factors. This seems unlikely in view of the absence of such competition with pAL2-mut1 and pAL3-mut1 (Table 1). Suppression could be caused by expression of ORF AL4 or by initiation of translation at internal methionine codons within the AL1 ORF at amino acid positions 196 or 230 to produce truncated AL1 proteins of 150 or 122 amino acids respectively.

The two possibilities described above were distinguished using further mutations. pAL1-mut4 has frameshift mutations after all three methionine codons of ORF AL1. It also has a frameshift mutation after the first ATG of ORF AL4 which also eliminates the second ATG. This mutant did not have a significant effect on expression of GUS activity (Table 1), showing that the suppressive effect of pAL1-mut1 was due to expression of a protein and not to transcriptional competition. Construct pAL1-mut2, which has frameshift mutations after all three methionine codons of ORF AL1, but can potentially express ORF AL4, caused an approximately 60% reduction in GUS activity (Table 1). Hence expression of ORF AL4 can suppress expression of the AL1 gene. Whether this ORF is expressed in wild-type TGMV DNA A is not known. Elmer et al. (1988) showed that introduction of an amber mutation near the N terminus of the putative AL4 protein did not affect virus replication or symptom development in N. benthamiana. However an equivalent ORF exists in several geminiviruses (Stanley et al., 1992) and mutations in the equivalent C4 ORF of beet curly top virus cause symptom alterations (Stanley & Latham, 1992) suggesting expression. If expression of the TGMV AL4 ORF occurs, translation probably initiates from the second ATG (two codons downstream from the first ATG), since its sequence context AAGATGG matches the consensus sequence (A/GNNATGG) for translational initiation in plants (Lütcke et al., 1987). In comparison, the first ATG of the AL4 ORF has the sequence context TTCATGA and is likely to be poorly expressed. If both the AL1 and AL4 ORFs are expressed, the effect of pAL1 in almost completely suppressing AL1 gene expression may be due to the combined effects of the AL1 and AL4 proteins.

Since pAL1-mut1 had a significantly ($P = 0.01$) more suppressive effect on GUS activity than did pAL1-mut2, it is likely that one or both of the truncated AL1 proteins can also exert a suppressive effect on AL1 gene expression. Support for this conclusion came from the ability of pAL1-mut3, which has the potential to express the truncated AL1 proteins but not the AL4 gene, to cause a reduction of about 20% in GUS activity by pGUS-NEO (Table 1) (significant at the 95% confidence level, $P = 0.05$). Although it is unlikely that such truncated proteins are produced in TGMV-infected plants, the result shows that the suppressive effect of the AL1 protein lies, at least in part, in its C-terminal region.

**Absence of effect of the AL1(AL4) and AL3 gene products on expression of the NEO gene by the GUS–NEO cassette**

It has been reported that the ACMV AC3 (equivalent to AL3), but not the AC1 (equivalent to AL1), gene product stimulated ACMV coat protein gene expression by 60% (Haley et al., 1992). Equivalent experiments have not been reported for TGMV, although TGMV AL3 mutants are capable of synthesizing coat protein (Sunter et al., 1990). The effects of TGMV AL1(AL4), AL2 and AL3 gene products on expression of the NEO gene by pGUS–NEO are shown in Fig. 2. As reported previously for both TGMV (Sunter et al., 1990; Sunter & Bisaro, 1991, 1992) and ACMV (Haley et al., 1992), the AL2 gene product trans-activated expression of the coat protein gene, as shown by an eightfold increase in NEO activity. However the pAL1 or pAL3 expression plasmids had no significant effect on NEO activity. The difference in effects of AL3 between TGMV and ACMV could possibly be the result of expression by the bidirectional cassette, differences between the two viruses or differences in the protoplast systems (N. benthamiana for TGMV, N. clevelandii for ACMV).

**Simultaneous regulation of GUS and NEO expression in the GUS–NEO cassette by a mixture of pAL1 and pAL2**

To determine whether the AL1 and AL2 gene products could regulate GUS and NEO expression simultaneously, N. benthamiana protoplasts were transformed with a mixture of pGUS–NEO, pAL1 and pAL2, and GUS activity and NEO protein levels were measured. As controls, protoplasts were also transformed with mixtures of pGUS–NEO, pAL1 and pAL2-mut1 and pGUS–NEO, pAL2 and pAL1-mut4. It was found that
Simultaneous suppression of AL1 gene expression by pAL1 and trans-activation of the coat protein gene by pAL2 suggests that the DNA-binding sites of the AL1 protein (and possibly the AL4 gene product) and the AL2 protein are largely distinct. The binding site of the AL1 protein has been localized to a part of the intergenic region which includes the AL1 putative core promoter and the AL1 major transcriptional initiation site (Fontes et al., 1992), but isolation of, or binding studies with, the AL2 or AL4 gene products has not yet been reported.

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References


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