Identification of sequences required for AL2-mediated activation of the tomato golden mosaic virus-yellow vein BR1 promoter

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A 108 bp sequence has been identified in the tomato golden mosaic virus-yellow vein (yvTGMV) B component that is necessary and sufficient for AL2-mediated activation of the BR1 promoter. The sequence appears to have a bipartite arrangement, with elements located between −144 to −77 and −59 to −36 from the transcription start site, with both being required for activation by AL2. These sequences are located upstream of a TATA box and bind nuclear proteins from spinach, tomato and Arabidopsis. These sequences are also capable of binding Arabidopsis PPD2, which has been shown previously to interact with the yvTGMV coat protein (CP) promoter. We have identified two putative transcription factor-binding sites (CCAAT and GTGANTG10) that are conserved in sequences necessary for activation of the yvTGMV BR1, as well as the yvTGMV and cabbage leaf curl virus (CabLCV) CP promoters, which are all activated by AL2. The yvTGMV BR1 promoter exhibits AL2-independent expression in vascular tissue, similar to the yvTGMV, CabLCV and spinach curly top virus CP promoters. Together, this further confirms a common regulatory mechanism for AL2-mediated activation of bipartite begomovirus promoters.

INTRODUCTION

Plant viruses of the family Geminiviridae have small ssDNA genomes, which are replicated in the nuclei of infected plant cells (Preiss & Jeske, 2003; Stenger et al., 1991). Viral dsDNA replicative form (RF) intermediates are produced that provide templates for transcription. As geminiviruses do not encode an RNA polymerase, they rely heavily on interactions with the host transcription machinery for viral gene expression (Lacatus & Sunter, 2009). In begomoviruses, including Tomato golden mosaic virus-yellow vein virus (yvTGMV) and Cabbage leaf curl virus (CabLCV), the genome consists of two DNA components (A and B), both of which are required for infectivity (Hamilton et al., 1983). Viruses in this genus encode a transcriptional activator protein (AL2, also known as TrAP) that is required for expression of the coat protein (CP) gene and the BR1 gene, which encodes a nuclear shuttle protein (NSP) (Lacatus & Sunter, 2008; Sunter & Bisaro, 1991, 1992). Using transgenic lines containing CP promoter-reporter constructs, it has been demonstrated that AL2 induces CP expression through activation in mesophyll cells and derepression in vascular tissue (Lacatus & Sunter, 2008; Sunter & Bisaro, 1997). In both yvTGMV and CabLCV, sequences required for activation of the CP promoter in mesophyll are located within the common region upstream of the CP transcription start site (Lacatus & Sunter, 2008; Sunter & Bisaro, 2003). Similarly, sequences required for repression lie between 1.2 and 1.5 kbp upstream of the CP transcription start site and are probably located within the AL2 gene (Lacatus & Sunter, 2008; Sunter & Bisaro, 1997). It is currently believed that CP expression is repressed through the action of regulatory proteins binding to the repressor element, although the exact mechanism is unknown (Sunter & Bisaro, 1997).

The AL2 protein encoded by begomoviruses exhibits several properties typical of a transcription factor: an acidic-type activation domain (Hartitz et al., 1999); localization to both the nucleus and the cytoplasm of yvTGMV-infected Nicotiana benthamiana cells (Wang et al., 2003); and self-interaction, which correlates with nuclear localization and efficient transcriptional regulation (Yang et al., 2007). However, AL2 does not specifically bind dsDNA, indicating that interactions with cellular proteins rather than direct recognition of specific regulatory elements target the protein to responsive promoters (Hartitz et al., 1999; Noris et al., 1996). Evidence for this mechanism has been provided recently. A plant DNA-binding protein, Arabidopsis PEAPOD2 (AtPPD2), was identified that specifically binds sequences mediating activation of the CP promoter of both yvTGMV and CabLCV (Lacatus & Sunter, 2009). AtPPD2 is localized to the nucleus, as expected for a transcription factor, but does not appear to activate transcription directly. It is, however,

One supplementary table is available with the online version of this paper.
capable of interacting with the AL2 proteins of several different begomoviruses (Lacatus & Sunter, 2009), confirming previous observations that the transcriptional activation function of AL2 is not virus specific (Sunter et al., 1994). In contrast, AtPPID2 does not appear to bind to sequences mediating repression of the CP promoters in yvTGMV or CabLCV (Lacatus & Sunter, 2009). This is again consistent with previous studies demonstrating that although AL2 can interact with both elements, different nuclear proteins bind regulatory sequences responsible for activation and repression of the geminivirus CP promoter (Lacatus & Sunter, 2008; Sunter & Bisaro, 1997).

Previous studies have shown that AL2 activates the BR1 gene at the level of transcription, explaining the lack of infectivity for al2 mutants (Sunter & Bisaro, 1992). Despite this, little information is available concerning regulation of the BR1 promoter in different tissues. In this study we extend our observations on regulation of geminivirus virion sense promoters and present evidence that BR1 promoter activity is regulated in vascular tissue in a manner similar to that of the CP promoter. The relevance of this regulation to the life cycle of begomoviruses is discussed.

RESULTS

Identification of sequences required for TrAP-mediated activation of the BR1 promoter

The genome arrangement of the yvTGMV B genome component is similar to the A component with bidirectional transcription units diverging from an ~230 nt common region (CR) containing viral promoters as well as the origin of replication, which is designated nucleotide 1 (Fig. 1a). The yvTGMV BR1 transcription unit comprises a single unspliced mRNA initiating around nucleotide 307 and terminating approximately 20 nt downstream of the translation termination codon (Sunter et al., 1989). Previous studies demonstrated that sequences important for TrAP-mediated activation lie within 1090 bp of the BR1 transcription start site (Sunter & Bisaro, 1992). To further define sequences necessary for TrAP-mediated activation a series of truncated promoter–reporter constructs were generated, starting with BR1p[−445]:GUS (Fig. 1b) and including −330, −144, −77 and −52. The rationale for using an initial deletion end point at −445 was based on previous results demonstrating that AL2-mediated activation of the CP promoter is dependent on sequences within the CR (Sunter and Bisaro, 2003), which we anticipated would be the same for the BR1 promoter. Truncated BR1 promoters are indicated by the position of the 5′ end point, with the transcription start site defined as +1. In each construct the β-glucuronidase (GUS) reporter is linked as a translational fusion to the first four amino acids of BR1, and flanked at the 3′ end by the nopaline synthase (nos) polyadenylation signal. Promoter–reporter constructs were introduced into Agrobacterium and cultures used to infuse leaves of N. benthamiana in the presence of Agrobacterium containing either the binary vector pMON521 (Rogers et al., 1987) as a negative control, or a cassette capable of expressing the yvTGMV AL2 from the cauliflower mosaic virus (CaMV) 35S promoter (Lacatus & Sunter, 2008). Extracts were prepared 3 days post-infusion and promoter activity was measured by fluorometric GUS assay as described previously (Sunter & Bisaro, 1991). Extracts from leaves co-infused with promoter–reporter constructs and the negative control (vector alone) exhibited basal GUS activity up to eightfold greater than the vector-alone background, depending on the amount and context of remaining promoter sequence (data not shown). The level of basal GUS activity was similar to that observed with CP promoter–reporter constructs in the absence of AL2, where activity was up to 10-fold greater than background (Sunter & Bisaro, 2003). However, significantly greater activity was detected in extracts from leaves co-infused with Agrobacterium containing the 35S-AL2 expression plasmid and constructs containing promoter 5′ end points at −445, −330 and −144 (Fig. 2). In these cases expression levels were 30- to 100-fold greater than basal activity (Fig. 2). This is again similar to results observed for CP promoter activity (Fig. 2) where expression levels were 50-fold greater than basal activity. In contrast, no significant increase over basal activity was observed in leaves co-infused with 35S-AL2 and promoter–reporter constructs with 5′ end points at −77 or −52. These results allow us to conclude that sequences required for BR1 promoter activation lie within 144 bp of the transcription start site, and that an element (or elements) required for TrAP activation lies between −144 and −77. The results also indicate that the level of AL2 activation of the promoter–reporter construct with a 5′ end point at −445 was approximately threefold higher than that detected with the promoter–reporter construct with a 5′ end point at −330 (Fig. 2). Although this may indicate additional elements that play a role in AL2 activation of the BR1 promoter, sequences between −144 and −77 represent the element absolutely required for activation.

Identification of a minimal sequence required for AL2-mediated activation of a heterologous promoter core

To test the ability of viral sequences to activate expression, putative BR1 promoter fragments were placed upstream of the heterologous CaMV 35S promoter core (−46 to +8; Benfey & Chua, 1990). Chimeric promoters containing sequences from −144 to +15, −77 to +15, −144 to −59 or −144 to −36 of the BR1 promoter were linked as transcriptional fusions to the GUS reporter gene and introduced into Agrobacterium. N. benthamiana leaves were infected with each promoter–reporter construct in conjunction with Agrobacterium containing a 35S-AL2 expression cassette, or a negative control with the pMON521 vector alone (521). Increases in GUS activity of 30- to 40-fold above basal levels (P<0.05) were observed in extracts prepared from leaves co-infused with 35S-AL2
and BR1 : 35S core promoter–reporter constructs containing sequences from −144 to +15, and −144 to −36 of the BR1 promoter (Fig. 3). With the other core promoter–reporter constructs minimal increases of two- to threefold were evident in extracts containing AL2 as compared with extracts co-transfected with the same promoter–reporter constructs and the negative control plasmid (Fig. 3). This confirms that a sequence important for maximal activation by AL2 lies between −144 and −36 of the BR1 translation start site, and identifies a 108 bp fragment of the BR1 promoter as a minimal sequence necessary and sufficient to mediate TrAP activation in N. benthamiana leaves. Consistent with the deletion analysis (Fig. 2), activity of the BR1 : 35S core promoter containing sequences from −77 to +15 did not exhibit a response to AL2 (Fig. 3). Together, this indicates that a sequence(s) between −144 and −77 is necessary for AL2-mediated activation of the BR1 promoter. Interestingly, a BR1 : 35S core promoter containing sequences from −144 to −59 was minimally responsive to AL2 (Fig. 3). When sequences between −59 and −36 are present (−144 to −36) the promoter is able to respond to AL2 (Fig. 3). Thus, it appears that two elements, one located between −144 and −77 and a second between −59 and −36, are necessary for AL2-mediated activation of the BR1 promoter in N. benthamiana.

**Binding of nuclear proteins to the BR1 minimal promoter sequence**

Previous experiments have demonstrated that sequences mediating AL2 activation of the CabLCV CP promoter are capable of binding nuclear proteins from different crop species (Lacatus & Sunter, 2008). To investigate whether DNA sequences shown to be required for activation of the yvTGMV BR1 promoter were capable of interacting with nuclear proteins, electrophoretic mobility shift analysis was performed using sequences from −144 to +15. Proteins from spinach, tomato and Arabidopsis bound to the BR1.
activator element, as evidenced by a shift in the labelled probe (Fig. 4a, lanes 2, 5 and 8), which was efficiently competed using unlabelled DNA containing the yvTGMV BR1 activator sequence (Fig. 4a, lanes 3, 6 and 9). Binding was also efficiently inhibited using unlabelled DNA containing the yvTGMV CP activator sequence (Lacatus & Sunter 2009) (Fig. 4a, lanes 4, 7 and 10), which suggests that the same proteins are capable of binding the BR1 and CP activator sequences. The formation of complexes with each of the protein extracts, as judged by the intensity of the signal, appeared to be different. This could reflect different affinities of the proteins within each extract for the promoter sequence or levels of the proteins within each extract. AtPPD2 has been shown to specifically interact with sequences mediating activation of the yvTGMV CP promoter (Lacatus & Sunter, 2009). We therefore tested whether DNA sequences shown to be required for activation of the yvTGMV BR1 promoter were also capable of interacting with AtPPD2. A GST-tagged version of the PPD2 protein was expressed in Sf9 insect cells and purified using glutathione-agarose as previously described (Hartitz et al., 1999). As shown (Fig. 4b, lane 2), the addition of purified GST-PPD2 leads to the formation of a complex, indicating binding to sequences necessary for activation of the BR1 promoter by AL2. Binding was efficiently inhibited using unlabelled DNA containing the yvTGMV BR1 activator sequence (−144 to −36) (Fig. 4b, lane 3). However, binding was not inhibited (Fig. 4b, lane 4) using viral sequences containing the AL1629 promoter (Tu & Sunter, 2007). This confirms that AtPPD2 exhibits some specificity with respect to binding yvTGMV promoter sequences, given that PPD2 almost certainly binds to host sequences as well as the BR1 promoter.

### DISCUSSION

The two DNA components of begomoviruses have similar arrangements of the virion sense transcription units, with a single RNA spanning either the CP or the BR1 ORFs (Sunter & Bisaro, 1989; Sunter et al., 1989). Previous analysis has shown that the CP and BR1 genes of yvTGMV were regulated by AL2 at the level of transcription (Sunter & Bisaro, 1992). Subsequent work on the yvTGMV CP promoter has shown that AL2 induces CP gene expression in different cell types through distinct viral sequence elements. In mesophyll tissue AL2 activates the promoter but in phloem AL2 acts to de-repress the promoter (Sunter & Bisaro, 1997). To determine whether AL2-mediated activation was conserved for the BR1 promoter we identified sequence elements within yvTGMV DNA B capable of promoter activity. Using 3’-truncated promoters we have shown that the yvTGMV BR1 promoter exhibits AL2-dependent regulation in leaf infusion assays (Fig. 2). Sequences necessary for this activity are located within 144 bp of the transcription start site for BR1, and we have shown that sequences between −144 and −36 are sufficient

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**Fig. 2.** AL2-mediated activation of BR1 promoter–reporter constructs. The activity of promoter–reporter constructs containing different BR1 5’ flanking sequences in the presence or absence of yvTGMV AL2 was determined. N. benthamiana leaves were infused with Agrobacterium cultures containing promoter–reporter constructs along with vector alone or a construct capable of expressing yvTGMV AL2 from the CaMV 35S promoter. GUS activity was measured in extracts isolated 3 days post-transfection. Columns represent the mean fold activation [ratio (AL2 activated/basal) GUS activity] for each construct, where basal activity is defined as the activity for each construct in the presence of vector alone, which was arbitrarily assigned a value of 1. Error bars represent the SEM from three independent experiments. Activities were compared with basal levels using Student’s t-test, to identify significant increases in expression over background (P<0.05).
for AL2-mediated activation (Fig. 3). This supports the results of promoter–reporter analyses, which showed that deletion of sequences between −144 and −77 resulted in a loss of activation by AL2 (Fig. 2). However, removal of sequences from −59 to −36 also resulted in a loss of AL2 activation, indicating that sequences within this region are also necessary (Fig. 3). Thus, it appears that the BR1 promoter has a bipartite arrangement (Fig. 6) with respect to AL2 activation, which is similar to the yvTGMV CP promoter, where two elements, between −125 and −107 and −96 and −60, confer AL2 activation (Sunter & Bisaro, 2003). Sequences required for AL2 activation of the BR1 promoter lie outside of the ~230 bp CR (Fig. 6) that is highly conserved between DNA A and B. This is in contrast to sequences required for AL2 activation of the CP promoter, which in part are located within the CR.

Having defined a region necessary and sufficient for AL2-mediated activation of the BR1 promoter we carried out a direct comparison between sequences necessary for AL2-mediated activation of the BR1 (−144 to −36) and CP (−125 to −60) promoters. Very little sequence homology was detectable (data not shown). This lack of conserved sequence elements within promoters activated by AL2 reiterates the hypothesis that AL2 could interact with several host factors, although this has not been shown to date. Previous work had found little or no homology between CR sequences of different begomoviruses, with only a conserved late element (CLE) being identified (Argüello-Astorga et al., 1994). This element was shown to be important for activation in PHYVV (Ruiz-Medrano et al., 1999), but is not required for activation of the CP promoter in yvTGMV (Sunter & Bisaro, 2003). In addition, the CLE in the yvTGMV BR1 CR is located outside the region identified as necessary and sufficient for AL2 activation (Fig. 6). The CLE is also absent from the CP and BR1 promoters of many begomoviruses, including bean golden yellow mosaic virus-Puerto Rico and CabLCV, and yet these promoters can be

Fig. 3. Activation of BR1:35S core promoters. N. benthamiana leaves were infused with Agrobacterium cultures containing BR1:35S core promoter constructs along with vector alone or a construct capable of expressing yvTGMV AL2 from the CaMV 35S promoter. Values represent the ratio of activated relative to basal activity detected with the core promoter alone, which was arbitrarily assigned a value of 1. BR1:35S core promoter–reporter constructs tested included −144 to +15, −77 to +15, −144 to −36 (three experiments) and −144 to −59 (two experiments). Error bars represent the SEM and asterisks represent statistically significant differences (P<0.05) as determined by ANOVA followed by Newmann–Keuls multiple range test.
activated by cognate or heterologous AL2 (Hung & Petty, 2001; Qin & Petty, 2001).

Analysis of potential transcription factor-binding sites within the BR1 promoter, using the PLACE database (http://www.dna.affrc.go.jp/PLACE/), revealed many sites that were present in BR1 promoter fragments exhibiting AL2 activation, but absent from fragments that failed to respond to AL2 (data not shown). Interestingly, sequences corresponding to CCAAT, GTGANTG10 and WRKY710S are present between -144 and -36 and are also found in the promoters of other genes activated by AL2 (Table 1).

The ability of AL2 to activate the BR1 promoter is lost when sequences between -144 and -77 are deleted. As the sequences deleted include the GTGANTG10 and WRKY710S elements this suggests they may play an important role in regulation of the BR1 promoter by AL2. The CCAAT and GTGANTG10 motifs are present in the CP promoters of both yvTGMV and CabLCV, whereas the WRKY710S motif is only found in the yvTGMV BR1 and CabLCV CP promoters (Table 1). As all three promoters are activated by AL2, this could indicate that the GTGANTG10 motif is critical for AL2-mediated activation. The GTGA motif of GTGANTG10 is important for modulating transcription of pollen-specific promoters (Rogers et al., 2001). How this is relevant to activation by AL2 is currently unknown.

Lastly, a CCAAT box element is present at -36, which when deleted in chimeric BR1:35S core promoters results in loss of AL2-mediated activation. This element is also found within the CP promoters of yvTGMV and CabLCV, again suggesting a possible role in AL2 activation. This is supported by evidence that when sequences within the yvTGMV CP promoter containing the CCAAT box are deleted, chimeric 35S:CP promoters are no longer AL2-responsive (Sunter & Bisaro, 2003). The CCAAT box is a conserved transcription factor-binding site found in numerous eukaryotic promoters. However, the sequence appears to bind numerous different transcription factors, which may be important given the lack of functional conservation in promoters dependent on AL2 for activity (Ruiz-Medrano et al., 1999; Sunter & Bisaro, 2003). There are also transcription factor-binding sites conserved between the yvTGMV and CabLCV CP promoters, but absent from the yvTGMV BR1 promoter (Table 1). The relevance of these sites is currently unknown.
We had previously demonstrated that the yvTGMV and CabLCV CP promoters bound AtPPD2, a plant-specific DNA-binding protein (Lacatus & Sunter, 2009). We expected that sequences mediating activation of the BR1 promoter by AL2 would also interact with AtPPD2. This was shown to be the case, where sequences between −144 and +15 bound GST-AtPPD2 purified from Sf9 insect cells (Fig. 4b). Binding was efficiently competed using unlabelled sequences between −144 and +15, but not with sequences containing the AL1629 promoter from yvTGMV DNA A, which was used as a non-specific competitor. This is consistent with the BR1 promoter being activated through an interaction of AtPPD2 with AL2.

AL2-dependent activation of several CP promoters has been demonstrated for a number of begomoviruses, including yvTGMV, CabLCV, PHYVV, African cassava mosaic virus and Mungbean yellow mosaic virus (Haley et al., 1992; Lacatus & Sunter, 2008; Ruiz-Medrano et al., 1999; Shivaprasad et al., 2005; Sunter & Bisaro, 1991). In contrast, AL2-independent expression has only been reported for yvTGMV (Sunter & Bisaro, 1997), PHYVV (Ruiz-Medrano et al., 1999) and CabLCV (Lacatus & Sunter, 2008). We have extended this observation to the BR1 promoter of yvTGMV, which also exhibits AL2-independent vascular expression (Fig. 5). Sequences necessary for AL2-independent BR1 promoter activity in vascular tissue are located within 445 bp of the BR1 coding region, whereas no expression is detectable in mesophyll tissue (Fig. 5). It is interesting to note that the CP promoter of Spinach curly top virus (SpCTV) also exhibits activity in vascular tissue in the absence of any viral protein (Rao & Sunter 2012). No CP promoter activity was detectable

### Table 1. Transcription factor binding sites within begomovirus promoters activated by AL2

<table>
<thead>
<tr>
<th>Name</th>
<th>Consensus sequence</th>
<th>yyTGMV CP Position</th>
<th>yyTGMV BR1 Position</th>
<th>CabLCV CP Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAATBOX1</td>
<td>CCAAT</td>
<td>+ (−102)</td>
<td>+ (−52)</td>
<td>+ (−85)</td>
</tr>
<tr>
<td>GTGANTG10</td>
<td>GTGA</td>
<td>+ (−103)</td>
<td>+ (−133)</td>
<td>+ (−13)</td>
</tr>
<tr>
<td>EBOXBNNAPA</td>
<td>CANNTG</td>
<td>+ (−108)</td>
<td>−</td>
<td>+ (−80, −16)</td>
</tr>
<tr>
<td>MYCONSENSUS</td>
<td>CANNTG</td>
<td>+ (−108)</td>
<td>−</td>
<td>+ (−80, −16)</td>
</tr>
<tr>
<td>ROOTMOTIF</td>
<td>ATATT</td>
<td>+ (−67, −89)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>SORLIP2AT</td>
<td>GGGCC</td>
<td>+ (−98)</td>
<td>−</td>
<td>+ (−31)</td>
</tr>
<tr>
<td>WRKY710S</td>
<td>TGAC</td>
<td>−</td>
<td>+ (−132, −120, −93)</td>
<td>+ (−69)</td>
</tr>
</tbody>
</table>

*(+)* Presence and (−) absence of each element within the given promoter. The position of the site is given with reference to the transcription start site, except for CabLCV CP where it is relative to the translation start codon.
outside of the vascular tissue (Rao & Sunter, 2012). This may suggest that SpCTV in particular, and curtoviruses in general, may regulate CP expression in vascular tissue through repression and subsequent de-repression, although this has yet to be demonstrated. It is therefore possible that begomoviruses and curtoviruses have evolved a common regulatory circuit for CP and BR1 expression in the vascular tissue that involves repression of the promoter. Relief of this repression is mediated through activity of the BR1 model for replication.

Methods

Cloning of promoter-reporter gene constructs. A series of truncated BR1 promoters was generated by replacing the CaMV 35S promoter in pBI221 with different amounts of 5′ flanking sequences of the yvTGMV BR1 gene. These 5′-truncated promoter constructs were linked to the GUS reporter in a translational fusion consisting of the N-terminal four amino acids of the BR1 protein, and flanked at the 3′ end by the nos 3′ polyadenylation signal. PCRs containing cloned yvTGMV DNA B as template and different primer combinations (for sequences see Table S1 available in JGV Online) amplified DNA fragments of 472 bp (TGMV BR1 –144F and TGMV BR1 Rev), 171 bp (TGMV BR1 –144F and TGMV BR1 Rev), 104 bp (TGMV BR1 –77F and TGMV BR1 Rev) and 79 bp (TGMV BR1 –52F and TGMV BR1 Rev). Following restriction with HindIII–BamHI, the resulting fragments were used to replace the 873 bp HindIII–BamHI fragment of pBI221 to generate cloned DNA containing promoter-reporter constructs or empty vector (pMON521) were delivered to N. benthamiana leaves by leaf infiltration as described previously (Lacatus & Sunter, 2008). Three leaves from four individual plants were infused with Agrobacterium containing each promoter-reporter construct. Following incubation for 3 days, the infused area from each leaf (total of 12 leaves) was harvested using a ½-inch cork borer and fluorometric GUS assays performed using equivalent amounts of protein as described (Sunter & Bisaro, 2003).

Generation of transgenic plants. Agrobacterium containing BR1p[-445]:GUS was used to transform N. benthamiana leaf discs and primary transformants selected and grown as described previously (Sunter et al., 2001). From kanamycin-resistant T1transformants, T2 plants were generated by self-crossing of three to five independent primary transformants. Three to five individual plants from each T2 line were chosen for subsequent analysis.

Leaf infiltration and analysis. Agrobacterium cultures containing promoter-reporter constructs or empty vector (pMON521) were delivered to N. benthamiana leaves by leaf infiltration as described previously (Lacatus & Sunter, 2008). Three leaves from four individual plants were infused with Agrobacterium containing each promoter-reporter construct. Following incubation for 3 days, the infused area from each leaf (total of 12 leaves) was harvested using a ½-inch cork borer and fluorometric GUS assays performed using equivalent amounts of protein as described (Sunter & Bisaro, 2003).

Agro-inoculation and histochemical staining. For inoculation of transgenic and non-transgenic N. benthamiana, 6- to 7-week-old plants were inoculated with Agrobacterium cultures containing tandemly repeated copies of the yvTGMV (FJ694490) or BCTV (AF379637) genomes as previously described (Lacatus & Sunter, 2008). Systemically infected leaves showing disease symptoms or comparable leaves from mock-inoculated plants were assayed for GUS activity by histochemical staining 10–20 days post-inoculation essentially as described (Jefferson et al., 1987; Lacatus & Sunter, 2008; Sunter & Bisaro, 1997). Tissues were surface sterilized, fixed, infiltrated with X-Glc substrate and incubated at 37 °C overnight. The following day stained samples were cleared and stored in 100 % (v/v) ethanol. Tissue samples were photographed using a light microscope (Axioskop; Carl Zeiss) under a ×10 objective.

Protein expression and purification. Arabidopsis PPD2 was expressed as a GST-6× histidine (His)-tagged protein in insect cells using the Bac-to-Bac baculovirus system (Invitrogen). The GST-tagged protein was expressed in baculovirus-infected cells and purified as described previously (Lacatus & Sunter, 2009).

Electrophoretic mobility shift assay. PCR was used to amplify the yvTGMV BR1 (primers TGMV BR1 –144F and TGMV BR1 Rev) and CP (primers previously described in Lacatus & Sunter, 2008) ORF, which was defined as 52F and including 52F and TGMV BR1 Rev). Following restriction with HindIII–BamHI, the resulting fragments were used to replace the 873 bp HindIII–BamHI fragment of pBI221 to generate cloned DNA containing promoter-reporter constructs with deletion end points at –445 and including –159, –92 and –67 (relative to the transcription start site for the BR1 ORF, which was defined as +1). DNAs were then restricted with HindIII–EcoRI and the resulting 2580, 2278, 2211 and 2186 bp fragments were cloned into the binary plasmid vector pMON521 (Rogers et al., 1987) to generate BR1p[-445]:GUS, BR1p[-144]:GUS, BR1p[-77]:GUS and BR1p[-52]:GUS, respectively. An additional promoter-reporter construct with a deletion end point at –330 (BR1p[-330]:GUS) was generated by restriction of BR1p[-445]:GUS with HindIII and PmII. Following Klenow treatment, DNA was self-ligated. All binary plasmid constructs were mobilized into Agrobacterium tumefaciens strain GV3110SE by triparental mating (Rogers et al., 1987) and used for agro-inoculation. Agrobacterium cultures containing tandemly repeated copies of the wild-type yvTGMV A and B genome components, wild-type Beet curly top virus (BCTV), and 35S-AL2 (pTGA89) have been described previously (Elmer et al., 1988; Lacatus & Sunter, 2008).

BR1:35S core promoters were generated by cloning PCR fragments upstream of the CaMV 35S core promoter sequence from –46 to +8 (Benfey & Chua, 1990) at a PmII site. PCRs containing cloned yvTGMV DNA B as template and different primer combinations (for sequences see Table S1 available in JGV Online) amplified DNA fragments of 159 bp (TGMV BR1 –77F and BR1 Min1R), 85 bp (TGMV BR1 –144F and BR1 Min2R) and 108 bp (TGMV BR1 –144F and BR1 Min3R).

References


