**Functional equivalence of late gene promoters in bean golden mosaic virus with those in tomato golden mosaic virus**

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In the bipartite geminivirus tomato golden mosaic virus (TGMV), the activity of late gene promoters is up-regulated by the multifunctional viral protein AL2. Cis-acting sequences required for AL2-mediated promoter responses have not been well characterized. However, nucleotide sequence analysis has implicated a motif termed the conserved late element (CLE). The CLE is present in TGMV and many other begomoviruses, although it is not ubiquitous. Here we analysed the regulation of late gene expression in bean golden mosaic virus (BGMV), one of the begomoviruses which lacks the CLE. Transient reporter gene assays showed that BGMV late gene promoters were trans-activated in *Nicotiana benthamiana* protoplasts, both by the homologous BGMV AL2 protein and by the heterologous TGMV AL2 protein. The BGMV AL2 protein also trans-activated TGMV late gene promoters. Consistent with these results, we found that hybrid viruses with the late gene promoters exchanged between BGMV and TGMV were viable in planta.

Although details of their transcriptional regulation are incompletely known, considerable progress has been made towards understanding the activation of late gene expression in certain bipartite geminiviruses of the genus *Begomovirus*. One such virus is tomato golden mosaic virus (TGMV). However, gene expression in the related bean golden mosaic virus (BGMV), the type member of the genus, remains relatively uncharacterized. The A and B DNA components of begomoviruses are transcribed bidirectionally from promoters contained within a large intergenic region (Fig. 1a). The intergenic region is divided into a common region (CR) of near identical sequence in the A and B components of a given virus, and unique regions *ARi*, *BRi* and *BLi* which lie between CR and the *AR1*, *BR1* and *BL1* open reading frames (ORFs), respectively.

In TGMV, expression of the late genes, *AR1* and *BR1*, has been shown to be up-regulated by the viral AL2 protein through cell type-specific trans-activation or de-repression mechanisms (Sunter & Bisaro, 1992, 1997). However, the identification of cis-acting sequences required for this transcriptional control has been hampered because AL2 proteins lack sequence-specific DNA-binding activity in *vivo* (Noris et al., 1996; Sung & Coupts, 1996; Hartitz et al., 1999). Nevertheless, AL2 proteins from diverse begomoviruses can trans-activate the TGMV *AR1* promoter in protoplasts (Sunter et al., 1994), and *al2* mutants can be complemented by a heterologous wild-type A component in planta (Saunders & Stanley, 1995; Sung & Coupts, 1995). Thus, essential cis-acting sequences required for the response of late gene promoters to AL2 must be well conserved among the begomoviruses. Comparative genome sequence analysis was used to identify a candidate motif, termed the conserved late element (CLE) (Arguello-Astorga et al., 1994), which was subsequently shown to confer a degree of AL2-responsiveness on a heterologous promoter (Ruiz-Medrano et al., 1999).

Interestingly, although a recognizable CLE-like sequence is found upstream from the *AR1* and/or *BR1* ORFs in many begomoviruses, including TGMV, it is not universally present. One of the viruses which lacks a CLE is BGMV. Whether the late gene promoters of CLE-containing and CLE-lacking viruses are functionally equivalent has not previously been tested. However, *al2* mutants of TGMV or BGMV can be complemented to some extent by the heterologous A component during co-infection of their common host *Nicotiana benthamiana*, and hybrid viruses with the *AL2* ORF exchanged between BGMV and TGMV are viable (Gillette et al., 1998). These observations suggested that the AL2 proteins of TGMV and BGMV can function equivalently, despite the absence of a CLE in BGMV. Here we have evaluated the relationship between the late gene promoters of BGMV and those of the better characterized virus, TGMV. Promoter activities were measured directly in the presence or absence of each AL2 protein using transient gene expression assays in protoplasts. We also analysed the phenotypes *in planta* of hybrid viruses in which the *ARi* or *BRi* non-coding regions were exchanged between BGMV and TGMV.
Fig. 1. (a) Schematic illustration of BGMV and TGMV genome organization. The location and direction of transcription of functional ORFs on each DNA component are indicated by open arrows. For clarity, the apparently non-functional AL4 ORF is not shown. Intergenic sequences which are conserved between the A and B components of a given virus, the common region (CR), are shown as solid rectangles. The position of the stem–loop structure in the replication origin is also depicted. Unique non-coding sequences between CR and the AR1, BR1 or BL1 ORFs are designated ARi, BRi and BLi, respectively. The locations of SspI, and XbaI or SnaBI restriction sites used to exchange the ARi or BRi non-coding regions in hybrid viruses are also shown. Solid arcs denote regions of each DNA component which were used to drive the expression of a luciferase reporter gene in place of the AR1 or BR1 ORF. (b) Trans-activation of BGMV AR1 and BR1 promoters by homologous and heterologous AL2 proteins. N. benthamiana leaf protoplasts were transfected with reporter plasmids in which the firefly luciferase gene was under the control of the indicated promoters. Transfections contained the reporter plasmid, and either no additional plasmid (−) or an effector plasmid from which TGMV AL2 (pTGA79) or BGMV AL2 (pGAA79) was expressed. Luciferase specific activity (S.A.) is given in relative light units/µg total protein in protoplast lysates. Each data point shown for samples with co-transfected AL2 represents the mean and standard error of five replicate transfections. The transfection experiments with different reporter plasmids shown were done with independent preparations of protoplasts, so luciferase S. A. cannot be compared directly between them.
To test directly whether the AL2 proteins of BGMV or TGMV could trans-activate the BGMV late gene promoters, which lack the CLE, we used a reporter gene expression assay. Reporter plasmids were constructed in which restriction fragments that encompassed the ARi or BRi non-coding sequences from TGMV or BGMV, together with CR and additional upstream flanking DNA (solid arcs in Fig. 1a), were used to drive expression of a firefly luciferase gene. These DNA sequences are similar in extent to those tested in other, comparable studies (Sunter & Bisaro, 1992). Protoplasts were prepared from N. benthamiana leaves (Jones et al., 1990) and aliquots of 2·5 × 10^5 cells were transfected with 2·5 μg of a
reporter plasmid, either alone or together with 10 µg of a plasmid from which either BGMV AL2 or TGMV AL2 was constitutively expressed (Sunter & Bisaro, 1992). After incubation for 72 h under continuous light, the protoplasts were lysed, and luciferase activity and total protein concentration were determined. Each promoter was analysed in at least three independent experiments, and representative results are presented (Fig. 1b). This analysis showed directly that BGMV is similar to TGMV in that its AR1 and BR1 promoters are trans-activated by the AL2 protein. We also confirmed that the AL2 proteins of each virus were able to trans-activate the AR1 and BR1 promoters of the other. These results extend previous demonstrations of heterologous complementation between begomovirus AL2 proteins and promoters (Sunter et al., 1994) to include those of BGMV, the type member of the genus. Although other studies have implicated the CLE in trans-activation (Ruiz-Medrano et al., 1999), our results show that this motif per se is not a necessary component of all AL2-responsive begomovirus promoters.

The transient reporter gene expression assays indicated that overall the AR1 and BR1 promoters of TGMV and BGMV functioned similarly in leaf mesophyll protoplasts. To confirm these results and extend them to virus-infected whole plants, we also constructed and analysed hybrid viruses in which the ARi or BRi non-coding DNA sequences were exchanged on SspI–XbaI or SspI–SmaI restriction fragments, respectively (Fig. 1a). These non-coding sequences contain proximal elements of the AR1 and BR1 promoters (Petty et al., 1988; Sunter & Bisaro, 1989, 1992). Whether the adjacent upstream sequences also contribute to promoter activity is currently unknown, but their exchange was not attempted because CR contains virus-specific elements of the replication origin (Fontes et al., 1994). Each hybrid DNA component was constructed in a recombinant plasmid as a partial tandem dimer. Hybrids in the genetic background of TGMV were designated TB-ARi (plasmid pTBARSX) and TB-BRi (plasmid pTG1.2BNY), and those in the background of BGMV were designated BT-ARi (plasmid pGTAARSX) and BT-BRi (plasmid pGTBRSS). Plasmids containing partial tandem dimers of wild-type TGMV and BGMV DNA components were described previously (Fontes et al., 1994).

The phenotypes of the resulting hybrid viruses were determined after inoculation of N. benthamiana by micro-projectile bombardment with recombinant plasmids, as described previously (Schaffer et al., 1995). Each hybrid A component was co-inoculated with wild-type DNA B from the appropriate virus, and each hybrid B component was co-inoculated with wild-type DNA A. Wild-type TGMV elicits strong symptoms of infection in N. benthamiana, and by comparison those induced by the TGMV-based hybrid viruses TB-ARi and TB-BRi were attenuated. On directly inoculated leaves, both hybrids produced smaller chlorotic lesions than wild-type TGMV, and on systemically infected leaves the symptoms consisted of mild rugosity, epinasty and vein yellowing. In contrast to TGMV, wild-type BGMV produces asymptomatic systemic infections of N. benthamiana (Petty et al., 1995). Similar to wild-type BGMV, the BT-ARi hybrid produced completely asymptomatic systemic infection in this host. In contrast, BT-BRi gave rise to small, pale chlorotic lesions on directly inoculated leaves, and also occasionally to some slight epinasty of the systemically infected leaves.

Nucleic acids were extracted from systemically infected leaves and analysed as described previously (Jeffrey et al., 1996). Briefly, DNA concentrations were determined by fluorimetry in the presence of Hoechst 33258 dye, and 2.5 µg aliquots were resolved by electrophoresis and Southern blotting. The blots were hybridized with 32P-labelled viral component-specific probes (Fig. 2a), and the accumulation of viral DNA (expressed as ng viral DNA/µg total leaf DNA) was determined by PhosphorImager comparison with cloned TGMV double-stranded (ds) DNA standards (Fig. 2b). This analysis confirmed that each of the hybrid viruses was capable of infecting N. benthamiana systemically. Infectivity of both TGMV and BGMV in N. benthamiana requires BR1 expression (Brough et al., 1988; Schaffer et al., 1995), and systemic movement of BGMV in this host additionally requires expression of AR1 (Pooma et al., 1996), so the heterologous non-coding sequences in TB-BRi, BT-ARi and BT-BRi were clearly functional. Although TGMV ar1 mutants can spread systemically in N. benthamiana (Brough et al., 1988; Gardiner et al., 1988), their ability to accumulate single-stranded (ss) DNA is severely compromised (Jeffrey et al., 1996). In contrast, TB-ARi was efficiently able to accumulate ssDNA (Fig. 2a, b), which is consistent with AR1 gene expression being driven by the heterologous BGMV non-coding sequences in this hybrid virus as well.

In addition to confirming the viability of hybrid viruses, the Southern blotting and PhosphorImager analysis revealed the surprising result that, under the experimental conditions used here, the A and B DNA components of wild-type TGMV accumulated in a molar ratio of approximately 1:3. Although differences in cell-to-cell movement efficiency can lead to excess accumulation of DNA A over DNA B in infected tissues (Frischmuth & Stanley, 1991; Schaffer et al., 1995), the reverse effect cannot occur because DNA B is completely dependent for its replication on proteins encoded by DNA A (Rogers et al., 1986). Instead, the excess accumulation of DNA B observed here suggests that TGMV DNA B has an (as yet uncharacterized) intrinsic replication advantage over DNA A. Such a replication advantage could also explain why the subgenomic, defective DNA species which accumulate during TGMV infection are derived predominantly from DNA B (Fig. 2a, and MacDowell et al., 1986).

In comparison with wild-type TGMV, changes in the relative molar accumulation of DNAs A and B showed that the TGMV-based hybrid DNA components exhibited significant cis-acting defects in DNA accumulation (Fig. 2b). In contrast to the wild-type TGMV A:B ratio of 1:3, the molar A:B ratio
for TB-ARi was ~ 1:10, and for TB-BRi it was ~ 1:1. These altered ratios reflect a decrease of ~ 3-fold in the accumulation of the hybrid A component TB-ARi, and a similar ~ 3-fold decrease in the accumulation of the hybrid B component TB-BRi, relative to wild-type DNAs A and B. Previous studies have identified roles in TGMV DNA replication for CR sequences upstream from and including the stem-loop structure (Orozco et al., 1998). Our results suggest that the efficiency of TGMV DNA replication may also depend on sequences downstream from the stem-loop structure, which were replaced in the TB-ARi and TB-BRi hybrid DNA components. Because the relative molar accumulation of the A and B components of the BGMV-based hybrids BT-ARi and BT-BRi did not differ significantly from wild-type BGMV (Fig. 2b), it appears that determinants of BGMV replication efficiency are not similarly located in this region. It may be significant that in TGMV, but not in BGMV, the sequences which are conserved between DNAs A and B (i.e. CR) extend downstream from the stem-loop structure, although the sequence conservation in this region is imperfect (Hamilton et al., 1984). Further experiments designed specifically to address DNA replication efficiency will be required to test this possibility.

In conclusion, we have shown here that non-coding sequences adjacent to the AR1 and BR1 ORFs of TGMV and BGMV contain functionally equivalent promoter sequences which respond to the AL2 proteins of either virus in transient gene expression assays in N. benthamiana protoplasts. Analysis of hybrid viruses in which the AR1 or BR1 non-coding regions were exchanged between BGMV and TGMV confirmed that the promoters are also functionally equivalent, at a gross level at least, in virus-infected plants. Taken together, these results suggest that comparison of the BGMV and TGMV promoters may provide a useful experimental system with which to define cis-acting elements required for their common response to AL2.

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References


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