Pathogenicity of Alternanthera mosaic virus is affected by determinants in RNA-dependent RNA polymerase and by reduced efficacy of silencing suppression in a movement-competent TGB1

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Four biologically active cDNA clones were derived from the Alternanthera mosaic virus (AltMV; genus Potexvirus) isolate, AltMV-SP, which differ in symptoms in infected Nicotiana benthamiana plants. Two clones induced necrosis and plant death; a mixture of all four clones induced milder symptoms than AltMV-SP. Replication of all clones was enhanced by a minimum of fourfold at 15 °C. A mixture of clones 4-7 (severe) and 3-1 (mild) was indistinguishable from AltMV-SP, but the ratio of 4-7 to 3-1 differed at 25 and 15 °C. RNA copy numbers of mixed infections were always below those of 4-7 alone. Determinants of symptom severity were identified in both Pol and TGB1; the mildest (4-1) and most severe (3-7) clones differed at three residues in the ‘core’ Pol domain [R(1110)P, K(1121)R, R(1255)K] and one [S(1535)P] in the C-terminal Pol domain of RNA-dependent RNA polymerase, and one in TGB1 [P(88)L]. Pol [P(1110)R(1121)K(1255)] + TGB1L(88) always induced systemic necrosis at 15 °C. Gene exchanges of Pol and TGB1 each affected replication and symptom expression, with TGB1P(88) significantly reducing silencing suppression. The difference in silencing suppression between TGB1P(88) and TGB1L(88) was confirmed by an agroinfiltration assay. Further, co-expression of TGB1P(88) and TGB1L(88) resulted in interference in the suppression of silencing by TGB1L(88). Yeast two-hybrid analysis confirmed that TGB1P(88) and TGB1L(88) interact. These results identify a TGB1 residue that significantly affects replication and silencing suppression, but maintains full movement functions.

INTRODUCTION

Alternanthera mosaic virus (AltMV) is a potexvirus first described from Alternanthera pungens, closely related to Papaya mosaic virus (Geering & Thomas, 1999). AltMV also infects the species Phlox, and the 6607 nt sequence of AltMV-PA has been determined previously (Hammond et al., 2006). Symptom differences between isolates have been reported; in Nicotiana benthamiana, AltMV-SP (the isolate studied here) and AltMV-PA produce chlorotic lesions on inoculated leaves and systemic chlorotic mosaic, while AltMV-Po produced milder symptoms (Hammond et al., 2006).

Occurrence of multiple sequence types, or mixed isolates, within a single plant has been reported for multiple virus groups including potexviruses. For example, six distinct subisolates of Plantago asiatica mosaic virus (PlAMV) were recovered from a single lily (Komatsu et al., 2008). As RNA-dependent RNA polymerase (RdRp) lacks proof-reading activity, RNA virus populations accumulate variations; population variation is limited by selection pressure for variants that interact successfully with host and viral proteins necessary to complete the infection cycle (Garcia-Arenal et al., 2001; Liang et al., 2002; Rico et al., 2006; Schneider & Roossinck, 2001). Changing environmental conditions such as temperature can affect
The potexvirus TGB1 is a multifunctional protein, with activities important for cell-to-cell movement, increasing plasmodesmal size exclusion limit, RNA silencing suppression and RNA helicase (Verchot-Lubicz et al., 2007). TGB1 is also the elicitor for resistance mediated by the Nb gene of potato (Malcuit et al., 1999). Bayne et al. (2005) utilized random mutagenesis to generate two classes of potato virus X (PVX) TGB1 mutants defective in silencing suppression. One class supported cell-to-cell movement if complemented by a heterologous suppressor of RNA silencing, while the second class was also defective in a second function required for movement.

We report here multiple distinct biologically active cDNA clones (hereafter 'infectious clones') derived from AltMV-SP, and use of these clones and chimeric derivatives to identify determinants contributing to symptom severity and RNA accumulation. Both the RdRp Pol domain and TGB1 contribute to symptom severity, and significantly affect RNA accumulation. Instead of emergence of variation and subsequent selection, we observed stable co-existence in N. benthamiana of two dominant sequence variants within AltMV-SP, resulting in modulation of symptom severity and RNA accumulation. Two single amino acid variants of TGB1 differ significantly in the ability to suppress RNA silencing, and interact with inhibitory effects on silencing suppression, differentiating silencing suppression from movement functions of TGB1 in fully infectious clones of AltMV.

**RESULTS**

**Four distinct clones infectious as transcripts were recovered from AltMV-SP-infected N. benthamiana**

The AltMV genome was amplified as separate 5' and 3' fragments, each containing a unique MluI site at nt 3125, upstream of the RdRp Pol domain. Four 5' clones (nt 1–3143; RdRp clones 1–4) and seven 3' clones (nt 3117–6607; [Pol+TGB+CP] clones 1–7) were ligated in all combinations to produce 28 full-length AltMV clones. Only clones 3-1, 3-7, 4-1 and 4-7, combining 5'[RdRp] clones 3 or 4 and 3'[Pol+TGB+CP] clones 1 or 7 (Fig. 1a), were infectious. For each infectious clone, 3/3 transcript-inoculated plants were infected as detected by RT-PCR using primers PP12/PP15 and Western blotting using AltMV-specific antibodies. No plants were infected by any other full-length transcripts.

Infectious clones AltMV 3-1 and 4-7 were fully sequenced and submitted to GenBank (accession nos GQ179646 and GQ179647). Clones 3-7 and 4-1 are identical to 3-1 and 4-7 from nt 1 to 3125, and to 4-7 and 3-1 from nt 3126 to 6607, respectively.

Symptoms of infectious clones inoculated separately varied from very mild to severe depending upon the combination of 5' and 3' regions; none produced symptoms typical of AltMV-SP (Fig. 1b). At 25 °C, AltMV 3-1 produced mild systemic mosaic 10 days post-inoculation (p.i.). However, plants recovered with symptoms rarely visible at 30 days p.i. No symptoms were observed in 4-1-infected plants at 25 °C. When [Pol+TGB+CP] was derived from clone 7 (3-7 and 4-7), symptoms were more severe than AltMV-SP; 3-7 always induced localized systemic necrosis by 10 days p.i. and growth was inhibited. Necrosis observed with 4-7 was delayed with respect to 3-7; symptoms were initially mild, but necrosis developed by 20 days p.i., a response not observed with AltMV-SP at 25 °C. AltMV-SP and mixtures of all four infectious clones produced mild mosaic symptoms without necrosis (Fig. 1b, upper panels).

All clones induced more severe symptoms at 15 than at 25 °C. Clones 3-7 and 4-7 induced necrosis and killed plants by 30 days p.i.; with 3-1 and 4-1, necrosis was detected in inoculated and upper leaves and the stem. AltMV-SP and the mixture of all clones developed severe symptoms at 15 °C, but without necrosis until about 30 days p.i. (Fig. 1b, lower panels).

**Quantification of viral RNA**

Differences in AltMV RNA accumulation were observed by quantitative real-time PCR (Q-RT-PCR) using CP- (Fig. 1c) or RdRp-specific primers (Supplementary Table S1, available in JGV Online) in N. benthamiana plants infected with each clone, suggesting that both [RdRp] and [Pol+TGB+CP] regions affect replication and/or systemic movement. Viral RNA levels were significantly increased in infections including 3' clone 7 compared with clone 1; and were similarly increased by 5' clone 3 compared with clone 4. RNA levels were correlated with symptom severity (Fig. 1).

Viral RNA accumulation of all clones at 15 °C was >fivefold that at 25 °C, as shown by Q-RT-PCR with either RdRp or CP primers (Fig. 1c and data not shown).

**Sequence analysis of mild and severe clones**

A total of 240 nt differences (three in the 5' UTR and 237 in RdRp) between 5' clones 3 and 4 result in 45 predicted amino acid differences within RdRp. These included five in the methyl transferase (MT) domain, 24 between MT and AlkB, four in AlkB and RNA helicase, and four in the RNA helicase domains (data not shown). The contribution of individual differences was not investigated.

There is a difference of 21 nt between 3' [Pol+TGB+CP] clones 1 and 7 (eight in RdRp-Pol; six in TGB1; one each in TGB3, the intergenic region and the 3' UTR and four in CP). There were only 5 aa differences between 1-[Pol+TGB+CP] and 7-[Pol+TGB+CP]: R(1110)P,
K(1121)R, R(1255)K in the ‘core’ Pol domain, S(1535)P in the ‘variable’ Pol C-terminal domain (pfam00978; Fig. 2a), and TGB1 P(88)L, in the helicase domain (pfam01443; Fig. 2b), with no differences in TGB2, TGB3 or CP. A TGB1 amino acid alignment of AltMV and closely related potexviruses showed that most had Leu, as for AltMV position 88 in clones 3-7 and 4-7, but PapMV has Ile and CCMV has Val; no other GenBank sequences examined had Pro at this position (Fig. 2c and data not shown).

AltMV-SP exists in infected N. benthamiana as a mixture of variants

To determine the prevalence of each cloned variant in AltMV-SP, we amplified a region containing changes distinguishing the 5’ and 3’ clones (nt 3112 and 3456), using primers PP3/PP20 (Supplementary Table S1). Analysis of 15 cloned RT-PCR products revealed four types of sequences (Fig. 3a); 11 clones identical to the 4-7 sequence and two identical to 3-1 were identified. Sequences identical to 3-7 (most severe) and 4-1 (mildest) were not found. Two additional clones had a silent A upstream from the *MluI* site where all infectious clones had a G; downstream one of these clones was identical to 4-7, the other to 3-1. These variants were not studied further.

We also inoculated AltMV-SP to two groups of five *N. benthamiana*, maintained at 25 and 15 °C. Q-RT-PCR was performed at 30 days p.i. with individual plants in order to distinguish 3-1 and 4-7 sequence types. Primer pairs RdRp-F(3-1)/RdRp-R(3-1) and RdRp-F(4-7)/RdRp-R(4-7) have 3’ nucleotides distinguishing these variants (positions 418 and 574; Supplementary Table S1). Q-RT-PCRs were adjusted to enhance specificity and results were normalized to actin. At 25 °C, *C*<sub>t</sub> values relative to actin of the (4-7)-like target were 8.61(±3.80) times higher than the (3-1)-like target, consistent with the ratio of cloned RT-PCR products. At 15 °C, *C*<sub>t</sub> values for the (4-7)-like target were only 1.74(±0.97) times higher than the (3-1)-like target (data not shown).

Mimicking AltMV-SP with infectious clones

Co-inoculation of 4-7 and 3-1 produced symptoms indistinguishable from AltMV-SP. Q-RT-PCR showed that RNA accumulation of [3-1 + 4-7] was indistinguishable from 3-1 alone or from AltMV-SP at either 15 or 25 °C.
Accumulation of 4-7 alone was significantly higher. In each case accumulation was fourfold higher at 15 than at 25°C (Fig. 3b). Plants infected with 4-7 at 15°C died before 30 days p.i., while plants infected with AltMV-SP, 3-1 or [3-1 + 4-7] survived past 30 days p.i. with mosaic symptoms. At 10 days p.i., accumulation of 4-7 RNA at 15°C was \( \frac{50}{\text{higher than for AltMV-SP, 3-1 or [3-1 + 4-7]}} \) (Fig. 3b).

**Mixed infections of fluorescently labelled clones reveal differential systemic distribution**

To compare cell-to-cell movement and distribution, 3-1 and 4-1 were modified to express the enhanced green fluorescent protein (eGFP), and 3-7 and 4-7 to express DsRed. Equal amounts of 3-1:eGFP and 4-7:DsRed were co-inoculated to *N. benthamiana*. At 25°C, 3-1:eGFP and 4-7:DsRed were equally distributed in systemically infected leaves at 10 days p.i. (data not shown). At 30 days p.i., 4-7:DsRed spread to an area about 10-fold larger than 3-1:eGFP in the uppermost leaves, with many co-infected cells (Fig. 4b). The same pattern occurred when 3-1:eGFP and 3-7:DsRed were co-inoculated, indicating correlation with the 3' rather than the 5' region.

At 15°C, 4-7:DsRed and 3-1:eGFP infected similar areas of the leaf at 30 days p.i. with many co-infected cells (Fig. 4c, d).

The *MluI* junction region was amplified at 30 days p.i. from plants co-infected with 3-1:eGFP and 4-7:DsRed, maintained at 25 or 15°C. Analysis of RT-PCR-derived clones revealed a 4-7:DsRed to 3-1:eGFP ratio of 8 : 1 at 25°C and 6 : 4 at 15°C (Fig. 4e).

Mixed infection (at 25°C) of 3-7:DsRed and 4-1:eGFP resulted in minimal GFP expression at 10 days p.i.; GFP was not detectable systemically at 30 days p.i., while DsRed was detected throughout (data not shown).

**Substitution of RdRp Pol or TGB1 from 3-7 into 4-1 enhances replication and induces necrosis**

To determine which regions are important for severe symptoms, we performed gene exchanges between the mildest clone 4-1 and the most severe 3-7. We separately substituted the 3-7 ‘core’ Pol [P\(_{1110}\)R\(_{1121}\)R\(_{1255}\)] and [P\(_{1110}\)R\(_{1121}\)R\(_{1255}\) + TGB1\(_{1110}\)] domains into 4-1 to create 4-1PRK, 4-1R, and 4-1PRKL, respectively (Fig. 5a). We also replaced 3-7 Pol with Pol [R\(_{1110}\)K\(_{1121}\)R\(_{1255}\)] from 4-1 to create 3-7RKR, and TGB1\(_{1110}\) with TGB1\(_{1110}\)P\(_{1255}\) to create 3-7P (Fig. 5a). In all 4-1 background chimeric clones, RdRp residue 1535 was changed to S1535, while in the 3-7 background 1535 was maintained, in order to examine the effects of TGB1 residue 88 in a uniform background. Neither 4-1 nor 4-1R developed significant symptoms by
10 days p.i. However, 4-1L produced symptoms by 20 days p.i. and showed an approximately $10^4$-fold increase in virus replication (Fig. 5c).

4-1PRK showed severe symptoms at 10 days p.i., but symptoms were milder at 20 days p.i. in newly formed leaves. There was a similar increase in virus replication to that with 4-1L (Fig. 5b, c). Q-RT-PCR revealed no significant difference in RNA levels between 4-1PRK and 4-1L (Fig. 5c). Only 4-1PRKL induced necrosis indistinguishable from 4-7, showing a further threefold enhancement of virus replication (Fig. 5b, c) consistent with the role of TGB1 in suppression of silencing.

Whereas AltMV 3-7 induced severe symptoms and necrosis at 10 days p.i., substitution of 4-1 Pol (3-7RKR) or TGB1 (3-7p) produced milder symptoms without necrosis (data not shown).

**TGB1 P(88)L is critical for silencing suppression**

The TGB1 protein of PVX functions to suppress RNA silencing (Voinnet et al., 2000), and this activity is required for cell-to-cell movement (Bayne et al., 2005). We therefore investigated whether AltMV TGB1 functions to suppress host RNA silencing, using an *Agrobacterium*-mediated transient expression system in *N. benthamiana* (Bragg & Jackson, 2004). In leaf areas co-agroinfiltrated with soluble-modified GFP (smGFP) and empty pGD vector, minimal fluorescence was observed at 2 days p.i., whereas GFP fluorescence was prominent with smGFP in combination with HC-Pro (Lim et al., 2005) or p19 (Qiu et al., 2002) (Fig. 6a). TGB1L(88) and TGB1P(88) differed dramatically in the promotion of smGFP expression; only TGB1L(88) showed silencing suppression activity comparable to HC-Pro and p19 controls, while TGB1P(88) showed no obvious activity, comparable to the negative control (Fig. 6b). GFP fluorescence was not observed for smGFP co-expressed with AltMV TGB2, TGB3 or CP (Fig. 6a, b).

When active suppressor TGB1L(88) was co-infiltrated with inactive TGB1P(88), the interaction effectively negated the suppression activity of TGB1L(88) (Fig. 6c), suggesting a specific deleterious interaction abolishing functionality. No function-compromising interaction was observed between TGB1P(88) and tomato bushy stunt virus (TBSV) p19 (Fig. 6d).

A yeast two-hybrid assay confirmed the TGB1 P(88) and TGB1L(88) interaction (Table 1).

**DISCUSSION**

Four infectious clones each showed biological activity distinct from the parental isolate AltMV-SP, with symptoms in *N. benthamiana* ranging from almost symptomless (4-1) to more severe than AltMV-SP (3-7), following a population cloning strategy similar to that employed with Cymbidium mosaic virus (Yu & Wong, 1998).
There were significant differences in RNA accumulation between infectious clones when inoculated separately at 25 °C, with levels of 3-7 >4-7 >3-1 >4-1. The 5′ clone 3 resulted in higher replication than clone 4, and the 3′ clone 7 to higher replication than clone 1, indicating that both 5′ and 3′ regions are separately involved in pathogenicity, as RNA accumulation was strongly correlated with symptom severity (Fig. 1).

Because of the many differences between the 5′ clones, the contribution of individual differences was not investigated further, but amino acid differences in the RdRp Pol domain (residues 1110, 1121 and 1255) in the Pol ‘core’ domain (pfam00978) that is conserved among many flexiviruses were examined. The S(1535)P difference in the variable Pol C-terminal domain was not further investigated. The TGB1 P(88)L change is in the relatively conserved helicase domain (pfam01443), and was interesting as the most similar potexvirus TGB1 sequences have Leu (most common), Ile or Val at that position. Pro was not observed at the equivalent position in any other potexvirus TGB1 sequence.
A single amino acid substitution in the Pol domain of PVX and PIAVMV RdRps contributes to necrotic symptoms in N. benthamiana (Kagiwada et al., 2005; Ozeki et al., 2006). Amino acid substitutions between the helicase and methylase regions of TMV and Pepper mild mottle virus RdRps also affect viral accumulation and symptom expression (Hagiwara et al., 2002; Lewandowski & Dawson, 1993). For AltMV, not only the Pol domain but also multiple differences in the upstream RdRp regions contribute to necrosis. A single residue in TGB1 also has major effects on viral accumulation and necrosis. Although PVX TGB1 has been identified as the elicitor of Nb-mediated HR (Malcuit et al., 1999), we believe this is the first identification of TGB1 as a pathogenicity determinant affecting the severity of disease. The potexvirus TGB1 protein has several functions: cell-to-cell movement, RNA helicase, silencing suppression and increasing PD size exclusion limit (Verchot-Lubicz et al., 2007). Bayne et al. (2005) obtained several random TGB1 mutants in which abrogation of silencing suppressor activity was linked to loss of cell-to-cell movement activity. In contrast, we have identified a TGB1 mutant which significantly affects replication and silencing suppression, but maintains full movement functions.

We have demonstrated that 4-7 (severe) and 3-1 (mild) variants are both maintained in AltMV-SP and in co-inoculated plants and that their prevalence differs with temperature (Fig. 3). Co-inoculation mimics AltMV-SP infection in both symptoms and virus replication levels (Fig. 3). Coexistence was visualized in vivo in systemic infections of eGFP- and DsRed-expressing clones (Fig. 4). 3-1:eGFP infected far fewer cells than 4-7:DsRed at 25 °C, but at 15 °C infection by 3-1:eGFP increased from about 10 to 50 % of the leaf area, whereas 4-7:DsRed infected almost all cells and co-infection of many cells was evident. Co-infections of 3-1:eGFP with 3-7:DsRed yielded similar data, suggesting that the difference in TGB1 rather than between the 5’ regions of 3-1 and 4-7 is most important.

To further understand the involvement of Pol and TGB1 differences in viral replication, we made substitutions between the phenotypic extremes of 3-7 and 4-1, neither of which was detected in the AltMV-SP population. Variants bearing the Pol (4-1PRK) and TGB1 (4-1L) from 3-7 had replication levels increased by approximately 1000-fold with respect to 4-1, and similar effects on symptom severity. Synergism was observed when Pol and TGB1 were inserted together (4-1PRKL), resulting in approximately...
TGB1 P(88) indicates strong growth by 48 h; ‘+’ indicates no growth.

Table 1. Yeast two-hybrid interactions of TGB1 L(88) and TGB1 P(88)

TGB1 sequences were fused to the activation domain (AD) or binding domain (BD) and interactions assessed by growth at 48 h. ‘+’ indicates strong growth by 48 h; ‘−’ indicates no growth.

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Threefold higher RNA levels, together with early necrosis and sustained symptom severity (Fig. 5). These effects were confirmed by reciprocal exchanges in the 3-7 background.

Low temperature (15 °C) dramatically increased accumulation of each variant inoculated separately, with symptoms following the same trend (Fig. 1). This was most apparent with 3-1 and 4-1. The explanation may be differences in plant RNA silencing activity; at lower temperature the host defence silencing mechanism appears to be less effective (Chellappan et al., 2005; Jovel et al., 2007; Qu et al., 2005; Siddiqui et al., 2008), allowing a proportionately greater increase in virus replication and/or number of cells infected by the challenging virus.

Hypothesizing the involvement of RNA silencing suppression, we tested AltMV CP, TGB3, TGB2 as well as TGB1 P(88) and TGB1 L(88) variants in a silencing suppressor assay. Only TGB1 L(88) had appreciable silencing suppressor activity and a single point mutation in TGB1 P(88) – not observed in any previously characterized potexvirus isolate – is able to dramatically reduce TGB1 silencing suppressor ability, while maintaining viral movement functions. Interactions between TGB1 P(88) and TGB1 L(88) significantly diminished functionality of TGB1 L(88) (Fig. 6), and may decrease the proportion of severe variant in a mixed infection.

In conclusion, differences in virus replication and symptom production are likely to be due to interactions between RdRp and TGB1 determinants. The previously undescribed TGB1 P(88) mutation can explain how 3-1 has lower relative fitness at 25 °C and increased fitness at 15 °C due to reduced effectiveness of host silencing activity. The reduction of silencing activity demonstrated when TGB1 P(88) and TGB1 L(88) interact may explain how 3-1 is able to reduce replication of 4-7 at both high and low temperature in mixed infections in which many cells are co-infected, and maintenance of both variants in AltMV-SP. This is consistent with the work of Simón et al. (2005), who have shown that minority genotypes can have an important influence on the overall pathogenicity of viral populations.

METHODS

Plant material. N. benthamiana were grown in 10 cm pots, and 3- to 4-week-old plants were used for virus infection. Inoculated plants were grown in an air-conditioned greenhouse maintained at 15 °C, or a greenhouse at 25 °C, under a 14 h light regime. Mechanical inoculations were performed using 1% K2HPO4 with celite added as an abrasive.

Virus isolates and construction of AltMV clones infectious as transcripts. AltMV-SP was isolated from Phlox stolonifera 'Sherwood Purple' by mechanical transmission (Hammond et al., 2006), and serially passaged in N. benthamiana over several years. Infected N. benthamiana tissue stored at −70 °C from various time points (serial passages) was used separately to reinstate AltMV-SP, and to examine the presence of distinct sequence types over time. Total RNA was isolated from N. benthamiana leaves using the RNasy Mini kit (Qiagen); 2 µg total RNA was used to generate cDNA using SuperScript III RNase H− Reverse Transcriptase (Invitrogen) separately with an oligo(dT)20 primer and internal reverse primer M13-R, containing an M13 site that is unique in the sequence of AltMV-PA (Hammond et al., 2006) at nt 3125. Two cDNAs were amplified by (i) a 5′ non-coding region primer including a T7
promoter sequence upstream of AltMV nt 1 (PsrI-F) paired with MluI-R, and (ii) MluI-F and XbaI-R1, respectively (Supplementary Table S1) using Pfu polymerase (Stratagene). The two PCR products were separately cloned into a TOPO vector (Invitrogen), yielding four clones containing the 5’ portion of the genome (nt 1–3143), and seven clones containing the 3’ region [nt 3117 to the poly(A) tail]; each contained the nt 3125 MluI site. The seven 3’ region clones were digested individually with MluI and XbaI, and each insert individually ligated into each of the 5’ region clones similarly cleaved with MluI and XbaI to create 28 distinct full-length AltMV clones. Each full-length clone was linearized with XbaI, and transcribed in vitro (see below); three young N. benthamiana plants per clone were inoculated with the transcripts. Inoculated plants were assayed by RT-PCR using AltMV-specific primers PP12 and PP15 (Supplementary Table S1) and Western blotting. The infectious clones obtained with the putative silencing suppressor; pGD:smGFP was co-infiltrated withalkaline phosphatase (Supplementary Table S1) using an Ms3005P QPCR System and Brilliant SYBR Green QPCR Master Mix (Stratagene) as previously described (Bae et al., 2006). Each 25 μl reaction contained 12.5 μl 2× Brilliant SYBR Green QPCR Master Mix, 5 μl 10-fold diluted cDNA, 2.5 μM each gene-specific primer and reference dye (final concentration=300 nM). Reaction conditions were: 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s. Transcript levels (AltMV CP or RdRp) were normalized to actin (copy number relative to actin), a constitutively expressed gene. Mean values were calculated from four biological replications. After identification of the dominant variants present in isolate AltMV-SP (see below), primer pairs RdRp-F(4-7)/RdRp-R(4-7) and RdRp-F(3-1)/RdRp-R(3-1) (Supplementary Table S1) were used in differential Q-RT-PCR from infected tissues.

Statistical analysis (ANOVA) of Q-RT-PCR results was carried out using Microsoft Excel to separate reactions into classes that were significantly different at P=0.05.

Identification of sequence variants in AltMV-SP-infected N. benthamiana. RNA was extracted and cDNA produced as previously described, from an infection established from tissue stored at −70 °C for approximately 3 years and multiple transfers before production of the infectious clones. A PCR product of about 800 bp, spanning the MluI site junction of the 5’ and 3’ clones, was amplified with primers PP3/PP20 (Supplementary Table S1) and cloned; 15 clones were sequenced using primers M13F and M13R (Invitrogen).

**Q-RT-PCR.** Total RNA was isolated from young systemically infected leaves of N. benthamiana (as above), and treated with DNase I per the manufacturer’s recommendation; 2 μg total RNA was used to generate cDNA in a 20 μl reaction with 0.5 μg of an oligo(dT)20 primer (as above). N. benthamiana actin (GenBank accession no. AY179605) was utilized as an internal control. Q-RT-PCR was performed with primer sets targeting AltMV RdRp and CP, and actin mRNA (Supplementary Table S1) using an Ms3005P QPCR System and Brilliant SYBR Green QPCR Master Mix (Stratagene) as previously described (Bae et al., 2006). Each 25 μl reaction contained 12.5 μl 2× Brilliant SYBR Green QPCR Master Mix, 5 μl 10-fold diluted cDNA, 2.5 μM each gene-specific primer and reference dye (final concentration=300 nM). Reaction conditions were: 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s. Transcript levels (AltMV CP or RdRp) were normalized to actin (copy number relative to actin), a constitutively expressed gene. Mean values were calculated from four biological replications. After identification of the dominant variants present in isolate AltMV-SP (see below), primer pairs RdRp-F(4-7)/RdRp-R(4-7) and RdRp-F(3-1)/RdRp-R(3-1) (Supplementary Table S1) were used in differential Q-RT-PCR from infected tissues.

**Construction of AltMV 3-1:eGFP, 4-1:eGFP, 3-7:DsRed and 4-7:DsRed.** A multiple cloning site (MCS) including NcoI, BamHI, MluI, BglII and Nhel restriction sites was inserted upstream of the CP gene in a 3'-terminal subclone of AltMV-3-1 by overlap PCR (Wurch et al., 1998). The TGB2/TGB3 region was amplified using forward primer TGB2/3-F and reverse primer TGB2/3-R1 (Supplementary Table S1) adding 20 μl transcription reaction and 10 μl inoculated to each of two leaves per N. benthamiana plant (Petty et al., 1989).

**Agrobacterium infiltration, AltMV constructs and silencing suppression assays.** All binary vectors were derived from pGD as previously described (Goodin et al., 2002). PCR primers for amplification of each gene are shown in Supplementary Table S1. TGB1 of AltMV 3-1 and 3-7, and TGB2, TGB3, and CP of 3-7 were separately amplified and inserted between the Xhol and BamHI sites of pGD. Constructs pGD:smGFP and pGDP:p19 (TBSV p19) was a gift of Andy Jackson (Department of Plant and Microbial Biology, University of California, Berkeley, USA; Bragg & Jackson, 2004), pGD:HC-Pro (Soybean mosaic virus HC-Pro) was amplified using T7 RNA polymerase in parallel reactions to obtain equivalent amounts of transcripts (Pettys et al., 1989). Transcripts were ethanol precipitated, resuspended in 20 μl GKP buffer (50 mM glycine, 30 mM KHPO₄, pH 9.2, 1% bmontenite, 1% celite) per 50 μl transcription reaction and 10 μl inoculated to each of two leaves per N. benthamiana plant (Petty et al., 1989).

An smGFP silencing suppression assay was used as previously described (Bragg & Jackson, 2004). Because 355 promoter-controlled GFP induces silencing of GFP expression (Shiboleth et al., 2007; Sihavy & Burgán, 2004), we used only pGD:smGFP co-infiltrated with the putative silencing suppressor; pGD:smGFP was co-infiltrated with pGD:TGB1L88 (3-7), and pGD:TGB1P(88) (3-1), pGD:TGB2, pGD:TGB3, pGD:CP, pGD:HC-Pro and pGD:p19, respectively.

**Substitution of Pol and TGB1 in chimeric AltMV clones.** The 3’ region clones 1 and 7 were used to prepare chimeric constructs. The Pol domain of clone 3’ clone 1 was digested with MluI (at nt 3125) and BamHI (at nt 4484) and exchanged with MluI/BamHI Pol fragment of 3’ clone 7. To replace [Pol+TGB1], clone 1 was digested with MluI (nt 3125) and Xmal (nt 5480) and this fragment replaced with the clone 7 MluI/Xmal [Pol+TGB1] fragment. The clone 1 TGB1 region was substituted with the BamHI/Xmal TGB1 fragment of clone 7, and TGB1 of clone 7 substituted by the BamHI/Xmal fragment of clone 1. Because the BamHI/Xmal fragment also included the Pol P(1555)/S difference between clones 1 and 7, P1555 was altered to S1555 in the clone 1 background so that differences due to TGB1 residue 88 could be evaluated independently. Overlap PCR (Wurch et al., 1998) was used to substitute S1555 into clone 1 using primers Pol-F/(P to S)-R and (P to S)-F/POL-R (Supplementary Table S1). Clone 7 Pol and TGB1 domains were similarly substituted into clone 1, and S1555 was changed to P1555 in the clone 7 background by overlap PCR using primers Pol-F/S(1555)P-F and S(1555)P-R/POL-R (Supplementary Table S1) such that the only amino acid change was TGB1 P(88). Structures of chimeric clones were confirmed by sequence analysis. The chimeric 3’ subclones were then digested with MluI and XbaI and combined with appropriate MluI/XbaI cleaved 5’ region clones to create chimeric full-length clones 4-1PRKL (Pol from clone 7); 4-1L (TGB1 from clone 7); 4-1PRKL (Pol+TGB1 from clone 7); 3-7PRKL (Pol from clone 1) and 3-7T (TGB1 from clone 1).

**In vitro transcription of AltMV clones.** Twenty micrograms of XbaI-linearized plasmid DNA were used per 50 μl transcription reaction for each of the full-length clones, AltMV 3-1, 4-1, 3-7, and 4-7 and transcript RNA generated using T7 RNA polymerase in parallel reactions to obtain equivalent amounts of transcripts (Pettys et al., 1989). Transcripts were ethanol precipitated, resuspended in 20 μl GKP buffer (50 mM glycine, 30 mM KHPO₄, pH 9.2, 1% bentonite, 1% celite) per 50 μl transcription reaction and 10 μl inoculated to each of two leaves per N. benthamiana plant (Petty et al., 1989).

**Determinants of AltMV pathogenicity**
substituted into clones 3-1 and 4-1 to form 3-1:eGFP and 4-1:eGFP, and the DsRed Xmal/Xhol fragment substituted into clones 3-7 and 4-7, yielding 3-7:DsRed and 4-7:DsRed.

**Western blot analysis.** AltMV-CP was detected by Western blot analysis using a 1:2000 dilution of AltMV-specific antibody [a gift from Andrew Geering (Department of Primary Industries, Queensland Horticultural Institute, Queensland, Australia; Geering & Thomas, 1999)], alkaline phosphatase-conjugated goat anti-rabbit antibody (Kirkegaard & Perry), and nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) substrate (Hammond et al., 2006).

**Detection of fluorescence in epidermal cells of *N. benthamiana.* Expression of smGFP in whole plants was detected using a Fujifilm LAS-1000 imaging system (Fujifilm). Fluorescence in epidermal cells of *N. benthamiana* was visualized by laser scanning confocal microscopy using a Zeiss LSM 410 microscope (Carl Zeiss MicroImaging). An Argon laser was used to excite eGFP at 488 nm with the emission band pass detection at 500–520 nm. DsRed was excited at 543 nm and the emission passed to the PMT detector through 500–520 nm and LP570 nm emission filters.

**Yeast two-hybrid assays.** Yeast two-hybrid vectors (James et al., 1996) were modified to express fusions of the Gal4 activation (AD) or binding (BD) domains to the N terminus of the TGB1/2 (88) and TGB1/2 (88) to test for interactions. Each TGB1 was amplified using the primers indicated (Supplementary Table S1), and introduced as BamHI/PstI fragments to the AD (pGAD-TGB1) and the BD (pGBD-TGB1) plasmids.

Yeast strain Pf69-4A was transformed with LEU2 selected AD and URA3 selected BD constructs as previously described (Becker et al., 1991). Transformants containing both AD and BD plasmids were selected on SD-glucose medium containing 20 mg Ade, Met, Trp and His l−1, and 30 mg Lys l−1, and grown for 3 days at 28 °C. Yeast colonies were tested for interactions through expression of reporter genes ADE2 and HIS3 by streaking on SD-glucose medium containing 20 mg Met and Trp l−1, 30 mg Lys l−1 and grown for 3–5 days at 28 °C or at room temperature.

**REFERENCES**


