Movement of potexviruses requires species-specific interactions among the cognate triple gene block proteins, as revealed by a trans-complementation assay based on the bamboo mosaic virus satellite RNA-mediated expression system

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The intra- and intercellular transport of potexviruses require interactions among viral RNA, coat protein and elements of the triple gene block proteins (TGBps). In this study, the requirement of bamboo mosaic virus (BaMV) TGBps for movement functions and the compatibilities with those of two potexviruses, Potato virus X (PVX) and Foxtail mosaic virus (FoMV), were examined using a satellite RNA-mediated trans-complementation assay system. Single or multiple TGBps of BaMV, PVX and FoMV were expressed from BaMV satellite RNA (satBaMV RNA) vectors to complement the functions of green fluorescent protein-tagged, movement-defective BaMV with mutation(s) in the matching gene(s). It was found that individual BaMV TGBps expressed from the satellite vector could function normally in trans, whereas bi-gene BaMV TGBp constructs in which the expression of TGBp3 might be impaired and individual TGBp genes from PVX or FoMV could not complement the movement functions of the defective helper viruses. Furthermore, alterations of the ratio among TGBps by ectopic expression of individual components of TGBps from satBaMV RNA vectors did not affect the cell-to-cell movement capabilities of wild-type BaMV significantly. The results indicate that species-specific interactions among movement proteins are obligatory for the cell-to-cell movement of BaMV and possibly other potexviruses.

INTRODUCTION

Successful establishment of systemic infection by plant viruses requires that the viruses spread from the initially infected cells to adjacent cells and subsequently into and out of the vascular systems. Virus-encoded proteins are essential for cell-to-cell movement of most plant viruses through plasmodesmata. Based on the requirement of coat proteins (CPs), formation of virions and the involvement of tubule structures, at least three different models for cell-to-cell movement of plant viruses have been proposed (for recent reviews, see Morozov & Solovyev, 2003; Lucas & Lee, 2004; Verchot-Lubicz, 2005).

For viruses that do not require virion formation for cellular movement, many have been found to contain three overlapping open reading frames (ORFs) termed the triple gene block (TGB), which encode three movement proteins (MPs) designated TGBp1–3 (Mushegian & Koonin, 1993; Morozov & Solovyev, 2003). According to primary structure comparisons and involvement of the CP in cell-to-cell movement, the TGB-containing viruses have been classified further into hordei-like and potex-like classes (Solovyev et al., 1996; Wong et al., 1998; Morozov & Solovyev, 2003).

For hordei-like viruses, TGBp1 contains a stretch of positively charged amino acids in the N-terminal portion and the CP is not required for cell-to-cell movement (Petty & Jackson, 1990; Schmitt et al., 1992; Sit & Abouhaidar, 1993; Solovyev et al., 1996; Herzog et al., 1998; Erhardt et al., 1999; McGeachy & Barker, 2000). Cell-to-cell movement of hordei-like viruses requires highly specific interactions among the cognate TGBps. It has been shown that the accumulation level of TGBp1 of Beet necrotic yellow vein virus (BNYVV) is adversely affected by mutations in TGBp2 and TGBp3, indicating the existence of a highly
coordinated control for the interactions among the elements of the movement machineries (Lauber et al., 1998). Furthermore, TGBp2 and TGBp3 of *Potato mop-top virus* have been suggested to be required for the transport of TGBp1 to and through plasmodesmata (Zamyatin et al., 2004).

In contrast, cell-to-cell movement of potex-like viruses requires the presence of CP in addition to TGBps (Chapman et al., 1992; Forster et al., 1992) and involves the suppression of gene silencing (Voinnet et al., 2000; Bayne et al., 2005), as well as reorganization of the endoplasmic reticulum (ER) into vesicles, which may be involved in gating through plasmodesmata (Ju et al., 2005). The TGBps of potex-like viruses have been studied extensively (Rouleau et al., 1994; Angell et al., 1996; Hefferon et al., 1997; Lough et al., 1998, 2000; Wung et al., 1999; Liou et al., 2000; Solovyev et al., 2000; Yang et al., 2000; Tamai & Meshi, 2001; Kalinina et al., 2002; Krishnamurthy et al., 2002, 2003; Mitra et al., 2003; Morozov & Solovyev, 2003; Haupt et al., 2005; Ju et al., 2005; Schepetilnikov et al., 2005). However, despite the wealth of data on the biological functions, cellular localizations and trafficking of TGBps, a full understanding of the specific requirement of TGBp3 and the functional interchangeability of TGBps among different potexviruses for intercellular movement remains elusive. TGBp3 is essential for the movement for *White clover mosaic virus* (WClMV, Lough et al., 1998), but may or may not be absolutely required for *Potato virus X* (PVX) (Lough et al., 2000; Tamai & Meshi, 2001). Schepetilnikov et al. (2005) demonstrated that the hydrophobic segment of PVX TGBp3 is a major determinant of the protein intracellular movement. Thus, further investigations are needed to clarify the requirement of TGBp3 for BaMV, as different potexviruses seem to employ somewhat different mechanisms for cell-to-cell movement functions.

The functional interchangeability of viral MPs among various viral species has been demonstrated with chimeric viruses (Giesman-Cookmeyer et al., 1995; Solovyev et al., 1996, 1999; Agranovsky et al., 1998; Tamai et al., 2003; Ajikuttira et al., 2005). Previous studies have shown that the movement functions of several defective potexviruses could be complemented *in trans* by one or more cognate TGBps expressed from plasmids or transgenic plants (Morozov et al., 1997; Lough et al., 2001; Schepetilnikov et al., 2005). However, little is known concerning the functional interchangeability or compatibility of the TGBps from different species of potexviruses and the influence of expression levels of individual TGBps.

In this study, three closely related potexviruses (Lin et al., 1994), *Bamboo mosaic virus* (BaMV), PVX and *Foxtail mosaic virus* (FoMV), were used to address these questions. A transient expression vector system based on satellite BaMV (satBaMV) RNA (Lin et al., 1996) was adopted to express individual TGBps or combinations of TGBps of the three potexviruses. By co-inoculating *Chenopodium quinoa* and *Nicotiana benthamiana* with green fluorescent protein (GFP)-expressing BaMV mutants carrying defects in the corresponding TGBps, we assessed the functional interchangeability among TGBps of different potexviruses (Lin et al., 1994). The results of such trans-complementation experiments indicated that (i) all three TGBps are required for the cell-to-cell movement of BaMV; (ii) there is a strict functional requirement for the interactions of cognate TGBps from the same species and (iii) ectopic expression of TGBp from satBaMV RNA vector does not affect the cell-to-cell movement of BaMV significantly.

**METHODS**

**Construction of BaMV mutants and chimeric satBaMV.** To examine the requirements of TGBps in BaMV cell-to-cell movement and the compatibility of TGBps of BaMV with those of PVX and FoMV, various movement-defective BaMV TGB mutants (Fig. 1a) were constructed based on pCBG (Lin et al., 2004), an infectious clone of BaMV-S in which the GFP reporter gene, controlled by a duplicated promoter of the subgenomic RNA for the CP, has been inserted between the ORFs encoding TGBp3 and CP. The TGBp1-defective mutant pR21A, which has an Arg-to-Ala substitution at aa 21 of TGBp1, was generated by site-directed mutagenesis (Lin et al., 2004). The plasmids pd13, pd6, pd136 and pdT are TGBp-defective deletion mutants generated by deletions in TGBp2, TGBp3, TGBp2 plus TGBp3 and all three TGBps, respectively. In pd13, 29 nt (nt 5025–5053) of the TGBp2 gene were deleted. In pd6, 4 nt (nt 5317–5320) of the TGBp3 gene were deleted. In pd136, 253 nt (nt 5070–5322) of the TGBp2 and TGBp3 genes were deleted. In pdT, 1084 nt (nt 4311–5394) of the triple gene block were deleted. Deletion mutants were constructed according to Sambrook et al. (1989).

Chimeric satBaMV vectors expressing TGBps were generated by replacing the P20-coding sequence of pCBSF4 (a cDNA clone of satBaMV; Lin et al., 2004) with a TGBp-coding sequence amplified from pCBG (Fig. 1b), pUF4 (a cDNA clone of FoMV; C.-Y. Chang & Y.-H. Hsu, unpublished) (Fig. 1c) or pTXSGFP (a cDNA clone of PVX carrying GFP, kindly provided by Dr David Baulcombe, The Sainsbury Laboratory, John Innes Centre, Norwich, UK) (Fig. 1d) using primers that contained an EcoNI and BstXI site at their 5′ extremities.

**Protoplast preparation, inoculation assays and detection of viral products.** The procedures for preparation of *N. benthamiana* protoplasts, inoculation of cDNA of BaMV, Northern and Western blot analyses of viral products and observations of GFP by fluorescence microscopy have been described previously (Lin et al., 2004). Polyclonal antiserum against BaMV CP (Lin & Chen, 1991) was used to detect the presence of BaMV. TGBp1 of BaMV (Chang et al., 1997) was included as a control in Western blots. Procedures for bacterial overexpression and purification of recombinant TGBp1 of PVX and FoMV and preparation of the respective rabbit polyclonal antiserum were as described for BaMV TGBp1 (Chang et al., 1997).

**RESULTS**

**Movement-defective BaMV mutants and chimeric satBaMV RNAs carrying MP gene(s) are replication-competent**

Various movement-defective BaMV TGB mutants (Fig. 1a) were constructed to examine the requirements of TGBps in BaMV cell-to-cell movement and the interchangeability of
BaMV TGBps and those of PVX and FoMV. To demonstrate the replication competencies of BaMV TGB mutants and chimeric satBaMV RNAs, protoplasts of N. benthamiana were inoculated with BaMV TGB mutants alone or co-inoculated with wild-type BaMV (pCBG) plus each of the satBaMV RNA vectors bearing the various TGBp cistrons. Total RNAs were extracted from protoplasts and assayed by Northern blot hybridization. It was found that accumulation levels of genomic and subgenomic RNAs of BaMV in protoplasts inoculated with TGBp-defective (pR21A, pd13, pd6, pd136 and pdT) or wild-type BaMV were similar, indicating that BaMV mutants retained a wild-type BaMV replication activity (Fig. 2a). The chimeric satBaMV RNA could also be detected, although at a lower level than the wild-type satBaMV RNA (Fig. 2b and c), indicating that the chimeras maintained their ability to replicate in N. benthamiana protoplasts. To verify protein expression from the chimeric satBaMV RNA constructs in plant cells, total protein extracts from N. benthamiana protoplasts co-inoculated with TGB-deleted BaMV (pdT) and chimeric satBaMV were analysed by Western blotting using antisera specific for BaMV CP and BaMV TGBp1, FoMV TGBp1 and PVX TGBp1. The results showed that similar accumulation levels of CP were detected in protoplasts infected with the wild-type and mutant BaMV (Fig. 2d) and the corresponding proteins of the chimeric satBaMV clone could be detected in N. benthamiana protoplasts (Fig. 2e and f). However, the expression of BaMV TGBp2 and TGBp3 could not be confirmed by Western blotting, due to the lack of specific antisera. To assess the in planta replication and movement of the chimeric satBaMV expressing the TGBp(s) in C. quinoa with the support of pCBG, total RNAs were extracted from inoculated leaves at 7 days post-inoculation (p.i.). All chimeric satBaMV RNAs could be detected by Northern blot analysis, indicating that the chimeric satBaMV RNAs could indeed be replicated and move from cell-to-cell in the inoculated leaves, although Sat13 and Sat6 accumulation was lower than that of the wild-type or chimeric Sat28 (Fig. 3). Furthermore, similar accumulation levels of genomic RNA were detected in inoculated leaves when pCBG was inoculated either with or without chimeric satBaMV RNA (Fig. 3), indicating that replication of the helper virus was not affected by the presence of chimeric satBaMV RNAs.

All three TGBps are necessary for cell-to-cell movement of BaMV

Since contradictory results have been presented for the requirement of TGBp3 in the movement of WClMV (Lough et al., 1998) compared with PVX (Tamai & Meshi, 2001), we examined whether all three BaMV TGBps are required for its cell-to-cell movement. All of the movement-defective BaMV mutants (Fig. 1a) were inoculated into C. quinoa leaves and virus movement was analysed by monitoring the spread of GFP in the inoculated leaves using a fluorescence microscope. As reported previously, none of the C. quinoa leaves inoculated with BaMV TGBp1 mutants showed cell-to-cell spread of green fluorescence (Lin et al., 2004). Similarly, none of the leaves of C. quinoa inoculated with the BaMV TGBp(s) deletion mutants (pd13, pd6, pd136 and pdT) supported the spread of green fluorescence (Fig. 4a, pd6, and g. Only the data for pd6 are shown, since those obtained for pd13, pd136 and pdT were similar). Previous work has demonstrated that lack of cell-to-cell spread of GFP on the inoculated leaves corresponds to lack of virus movement in these leaves (Lin et al., 2004). Therefore, our results indicated that all
three TGBps are required for cell-to-cell movement of BaMV.

**Complementation of cell-to-cell movement of TGBp-defective BaMV mutants by a chimeric satBaMV RNA expressing the corresponding gene(s)**

To test for trans-complementation of BaMV TGBp function, C. quinoa was co-inoculated with BaMV mutants carrying the corresponding defective gene(s) plus various satBaMV vectors expressing TGBp(s) individually or in combination (Table 1). At 7 days p.i., chlorotic lesions were observed on the inoculated leaves infected with pCBG and with pR21A + pSat28, but not on the inoculated leaves infected with other combinations (data not shown). At 12 days p.i., chlorotic lesions were observed on the inoculated leaves infected with pd13 + pSat13, pd6 + pSat6, pd13 + pSat136, pR21A + pSatT and pd13 + pSatT (Table 1; Fig. 4a). With fluorescence microscopy, clusters of green fluorescent cells could be observed on leaves inoculated with pCBG alone or co-inoculated with the single-gene-defective BaMV plus the satBaMV vector encoding the corresponding protein (Table 1; Fig. 4c–f and h–k). The movement function of the mutant with defective TGBp2 and TGBp3, pd136, could also be restored by co-inoculation of pSat13 + pSat6 (Table 1; Fig. 4l). However, no fluorescence was visualized in other complementation tests with overlapping ORFs of TGBp2 and TGBp3 (pSat136) or all three TGBps (pSatT) (Table 1). The ability of the rescued BaMV to move from cell to cell was...
FIG. 3. Accumulation of chimeric satBaMVs carrying one, two or three TGB genes in the leaves of C. quinoa analysed at 7 days p.i. by Northern blot analysis. Total RNAs were extracted from leaves inoculated with pCBG alone or together with pCBSF4, pSat28, pSat13, pSat6, pSat136 or pSatT. Northern blot analyses for genomic RNA and chimeric satBaMV RNAs were as described in Fig. 2. The RNA species are indicated to the right. The lower panel shows 18S rRNA, which was used as an internal control.

poorer than that of wild-type virus (with infection foci of 1–0–1.6 mm in diameter, 36 % of which showed necrotic symptoms at 7 days p.i.; Fig. 4c and h; Table 2). The rescued TGBp1-defective virus could induce infection foci of 0.4–1.0 mm in diameter, with 64 % being necrotic (Fig. 4d and i; Table 2), whereas rescued TGBp2- and TGBp3-defective viruses could spread to adjacent cells to form non-necrotic infection foci with different ratios in two size ranges of 0.6–1.0 and 0.2–0.5 mm in diameter at 7 days p.i., respectively (Table 2; Fig. 4e, f, j and k). The results demonstrated that the satBaMV vectors could express individual, functional TGBp, which could complement the functions of cell-to-cell movement of the corresponding TGBp-defective BaMV mutants, albeit at a lower efficiency.

Chimeric satBaMV RNA constructs expressing individual TGBps were also used in various combinations to complement the movement function of mutant BaMV with defects in two or three TGBps, i.e. pd136 and pdT, respectively. The constructs pSat13 + pSat6, pSat28 + pSat136 and pSat28 + pSat13 + pSat6 were co-inoculated with pd136 and pdT into C. quinoa, followed by fluorescence microscopy examination. It was found that clustered green fluorescent cells could only be observed on leaves co-inoculated with pd136 + pSat13 + pSat6 (Table 1). To examine the accumulation levels of the rescued mutants, CP in the inoculated leaves was analysed further by Western blotting. It was found that CP accumulation was similar in leaves inoculated with pCBG alone or with pR21A + pSat28. This accumulation level was ~12-fold more than from leaves co-inoculated with pd13 + pSat13, pd6 + pSat6 and pd136 + pSat13 + pSat6 and was ~250-fold more than from leaves co-inoculated with pd13 + pSat136 and pR21A + pSatT, respectively (Fig. 4l). Although the TGBp2-defective BaMV could be complemented by pSatT, which harbours the ORFs for all three TGBps, as revealed by microscopic observation of GFP dispersion (Table 1), the CP accumulation level of the pSatT-rescued TGBp2-defective BaMV was below the level of detection (data not shown). In all of the above in trans complementation tests, the expression of TGBp3 appeared to play a key role in determining whether the movement-defective mutants could be rescued.

In order to rule out the possibility of reversion by homologous recombination in the co-inoculations, the genomes of progeny of the rescued TGBp-defective BaMV mutants were cloned and confirmed by sequencing after RT-PCR. The results showed that the mutations in each defective genes were stably maintained (data not shown). To determine whether the complementation was functional only in C. quinoa, another host, N. benthamiana, was co-inoculated with pR21A + pSat28, pd13 + pSat13 or pd6 + pSat6. The results of fluorescence microscopy showed that clustered green fluorescent cells could be observed in the inoculated leaves (data not shown), showing that trans-complementation worked in at least two plant species. Interestingly, in C. quinoa not all of the infection foci developed into necrotic local lesions at 7 days p.i. For instance, 64 and 36 % of total infection foci examined did not develop necrosis on leaves inoculated with the wild-type construct (pCBG) and the TGBp1-defective mutant plus pSat28, respectively (Table 2; Fig. 4h and l). Moreover, the mean diameter of the necrotic centres induced by co-inoculation with pR21A and pSat28 in C. quinoa (0.25 ± 0.09 mm; represented by the dark yellow regions in the centre of Fig. 4d) was larger than that induced by pCBG (0.1 ± 0.03 mm; corresponding regions in Fig. 4c), suggesting a reverse correlation between cell-to-cell movement capability and necrotic symptom induction.

Cell-to-cell movement of MP-defective BaMV cannot be restored by counterparts from PVX or FoMV

The above results demonstrated that cell-to-cell movement of defective BaMV could be rescued by a satBaMV vector expressing the individual corresponding gene from the same species, indicating that the satBaMV vector can express an active MP that can function in trans. However, the functional interchangeability among the TGBps from different potexviruses has not been studied. The TGBps of two potexviruses, PVX and FoMV, with high overall sequence similarity to BaMV were also expressed by satBaMV vector (Fig. 1c and d) and used to complement the movement function of the BaMV mutants carrying the corresponding defects. The replication ability of each chimeric satBaMV construct was confirmed by inoculation of N. benthamiana protoplasts as above. Results of Northern and Western blot assays showed that these chimeric satBaMVs could be replicated and the corresponding proteins could be expressed in N. benthamiana protoplasts (Fig. 2e and f). By fluorescence microscopy, no clusters of green fluorescent cells could be observed on leaves of C. quinoa co-inoculated with various paired
combinations of TGBp-defective BaMV mutants and the chimeric satBaMV construct expressing the TGBp counterparts from PVX or FoMV (data not shown). The results thus indicated that cell-to-cell movement of potexviruses requires specific interactions among TGBps from the same viral species. Our data provide the first demonstration of the strict requirement of species-specific interactions among the TGBps of potexviruses.

Table 1. Rescue of movement-defective BaMV in *C. quinoa* leaves with co-inoculation of the chimeric satBaMV RNA carrying one or more of the TGBp genes

<table>
<thead>
<tr>
<th>BaMV mutant</th>
<th>Defective gene(s)</th>
<th>Functional chimeric satBaMV RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pR21A</td>
<td>TGBp1 (p28)</td>
<td>pSat28, pSatT</td>
</tr>
<tr>
<td>pd13</td>
<td>TGBp2 (p13)</td>
<td>pSat13, pSat136, pSatT</td>
</tr>
<tr>
<td>pd6</td>
<td>TGBp3 (p6)</td>
<td>pSat6</td>
</tr>
<tr>
<td>pd136</td>
<td>TGBp2 + TGBp3</td>
<td>pSat13 + pSat6</td>
</tr>
<tr>
<td>pdT</td>
<td>TGBp1 + TGBp2 + TGBp3</td>
<td>None</td>
</tr>
</tbody>
</table>

*Chimeric satBaMV RNA that could restore the movement of defective BaMV.
Table 2. Restoration of cell-to-cell movement function of defective BaMV by different TGBps in leaves of inoculated C. quinoa at 7 days p.i.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Diameter of infection foci (mm)*</th>
<th>Ratio (%)†</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N (+)‡</td>
<td>N (−)§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCBG</td>
<td>1–0–1–6</td>
<td>64/176 (36)</td>
<td>112/176 (64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pR21A + pSat28</td>
<td>0–4–1–0</td>
<td>96/149 (64)</td>
<td>53/149 (36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pd13 + pSat13</td>
<td>0–2–0–5</td>
<td>0/140 (0)</td>
<td>32/140 (23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–6–1–0</td>
<td>0/140 (0)</td>
<td>108/140 (77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pd6 + pSat6</td>
<td>0–2–0–5</td>
<td>0/164 (0)</td>
<td>148/164 (90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0–6–1–0</td>
<td>0/160 (0)</td>
<td>16/164 (10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Diameter of the clustered green fluorescent cells of infection foci.
†Number of foci with indicated diameter/total number of foci examined. Percentages are shown in parentheses.
‡Clustered green fluorescent cells showing necrotic symptoms.
§Clustered green fluorescent cells without necrosis.

Cell-to-cell movement ability of BaMV is not affected significantly by ectopic TGBp expression

To investigate the effect on cell-to-cell movement of wild-type BaMV by ectopic expression of TGBps, the chimeric satBaMV constructs expressing individual TGBp were co-inoculated with pCBG into C. quinoa leaves. To increase the probability that the chimeric satBaMV and pCBG would replicate in the same host cell, the amount of chimeric satBaMV plasmid was doubled relative to that of the pCBG plasmid. At 3, 5, and 7 days p.i., the effect of ectopically expressed BaMV TGBps on cell-to-cell movement of pCBG was evaluated by measuring the diameter of green fluorescent cell clusters. By this assay, pCBG alone, pCBG + pSat28, pCBG + pSat13 and pCBG + pSat6 all formed infection foci, which were generally 0–2–0, 1–0–1–2 and 1–2–1–4 mm in diameter at 3, 5, and 7 days p.i., respectively (Table 3; Fig. 5b–e). These results indicated that cell-to-cell movement of BaMV was not affected significantly when different BaMV TGBps were ectopically expressed. To confirm that the chimeric satBaMV was stably maintained together with the BaMV genomic RNA, total RNAs were analysed by Northern blot hybridization at 7 days p.i. The result showed that all of the chimeric satBaMV RNAs could be detected, indicating that the chimeric satBaMV RNAs indeed could be replicated and move along with genomic RNA in these inoculated leaves. However, the accumulation levels of Sat13 and Sat6 were lower than those of Sat28 or wild-type satBaMV, with that of Sat6 being the lowest (Fig. 5f).

DISCUSSION

Cell-to-cell and systemic movement of viruses expressing TGBps have been subjects of extensive studies. For potexviruses, little is known concerning the requirement for each of the TGBps, the influence of alterations of the ratios of TGBp accumulation and the functional interchangeabilities among different potexviruses. Our study has presented evidence in support of the hypothesis that (i) all three TGBps are required for the cell-to-cell movement of BaMV; (ii) species-specific interactions among MPs are obligatory.

Table 3. Effect of ectopic expression of BaMV TGBps on cell-to-cell movement of BaMV at different intervals after inoculation

The number of foci with the indicated diameter/total number of foci examined is shown in parentheses.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>3 days p.i.</th>
<th>5 days p.i.</th>
<th>7 days p.i.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0–2–0–4 mm</td>
<td>&gt;0–4 mm</td>
<td>0–8–1–0 mm</td>
</tr>
<tr>
<td>pCBG</td>
<td>97 (68/70)</td>
<td>3 (2/70)</td>
<td>5 (4/78)</td>
</tr>
<tr>
<td>pCBG + pSat28</td>
<td>100 (88/88)</td>
<td>0 (0)</td>
<td>53 (51/97)</td>
</tr>
<tr>
<td>pCBG + pSat13</td>
<td>94 (138/147)</td>
<td>6 (9/147)</td>
<td>43 (30/70)</td>
</tr>
<tr>
<td>pCBG + pSat6</td>
<td>81 (146/180)</td>
<td>19 (34/180)</td>
<td>43 (57/133)</td>
</tr>
</tbody>
</table>
for cell-to-cell movement of potexviruses and (iii) alterations of the ratio among TGBps by additional expression of individual components of TGBps from satBaMV RNA vectors do not significantly affect the cell-to-cell movement capabilities of the wild-type helper virus. We also demonstrated the value of a simple and useful tool based on a satellite RNA-mediated trans-complementation assay to study the potential role of MPs in cell-to-cell movement.

Functional complementation assays have been commonly used as a strategy for the characterization of viral MPs. Previously applied methods included co-inoculation, microinjection and bombardment of 35S promoter-driven or chimeric viral transient expression vector systems and transgenic approaches. For instance, using microprojectile bombardment to deliver constructs, it has been shown that cell-to-cell movement of TGBp1-defective PVX was restored by 35S promoter-driven transient expression vectors harbouring the TGBp1 gene of PVX or the MP gene of Tomato mosaic virus, Crucifer tohamovirus or Red clover necrotic mosaic virus (Morozov et al., 1997). A BNYVV RNA3-derived viral replicon has also been used to express MPs of BNYVV and other viruses in a trans-complementation assay to study the movement function of TGB-defective BNYVV (Lauber et al., 1998). Movement-defective WClMV mutants in which the TGB ORFs had been mutated were rescued in transgenic plants expressing corresponding TGBps of WClMV (Lough et al., 1998). Movement-deficient mutants of PVX could be rescued for cell-to-cell movement following co-bombardment and transient expression of the corresponding wild-type MP introduced on a separate transcriptional plasmid (Lough et al., 2000). Brome mosaic virus 3a, as well as Cowpea chlorotic mottle virus 3a, efficiently complemented the movement-defective PVX in trans (Tamai et al., 2003). Our study has provided another useful system based on satBaMV RNA vectors that ensures the co-expression of the
*trans*-complementing proteins in the right cells and mimics the natural expression strategies of the viruses.

In co-inoculation experiments, the chimeric satBaMV could be detected in inoculated leaves, although at lower levels than the wild-type satBaMV (Figs 3 and 5). In addition, Sat6 always displayed the lowest accumulation level among these chimeras. However, the accumulation level of the chimeric Sat6 was similar to that of other chimeras in *N. benthamiana* protoplasts, suggesting that the cell-to-cell movement ability of Sat6 could be defective. Although these satellite chimeric clones were constructed using the same restriction enzyme site, the RNA conformation of Sat6 critical for cell-to-cell movement functions could be severely interfered with by the short insert, whereas the constructs with longer inserts provided flexibility to allow proper folding.

The results of our *trans*-complementation assays also provide the first evidence indicating that species-specific interactions among the TGBps are critical for cell-to-cell movement of potexviruses, since the TGBps from PVX and FoMV could not complement the movement functions of BaMV individually. Our results are in accordance with findings from *trans*-complementation studies conducted on a hordei-like virus, BNYVV. *Trans*-complementation was successful with the MPs of BNYVV and TMV, but not with the tubule-forming MPs of *Alfalfa mosaic virus* and *Grapevine fanleaf virus*. In addition, *Peanut clump virus* TGBps supported BNYVV movement when supplied together, but not when substituted for the corresponding BNYVV TGBps individually (Bleykasten-Grosshans et al., 1997; Lauber et al., 1998). These data also indicate that there is a strict requirement for the three TGBps from the same species of virus to constitute the functional movement complexes. The very low degree of conservation in the amino acid sequences of TGBps (data not shown), especially TGBp3, provided further support for this notion. It has been suggested that specific interactions among the TGBps or other virally encoded proteins are required for potexvirus movement (Lauber et al., 1998; Morozov et al., 1999; Yang et al., 2000). Our findings also indicate that the MPs of PVX and FoMV do not interact with MP(s) or other viral protein(s) of BaMV that are required for cell-to-cell movement.

Interestingly, the chimeric satBaMV carrying TGBp2 + TGBp3 or all three genes of TGB could complement the TGBp2- or TGBp1-defective BaMV, respectively, but none of them could complement the TGBp3-defective BaMV. Furthermore, the chimeric satBaMV carrying three genes of TGB could complement the TGBp2-defective BaMV, whereas the chimeric satBaMV carrying TGB2 + TGB3 or all three TGBps could not complement the movement of BaMV mutants with defects in TGBp2 and TGBp3 or in all three TGBps, respectively (Table 1). These results are in contrast to the findings of others that plants expressing both TGBp2 and TGBp3 from a single mRNA are able to complement TGBp2- and TGBp3-defective mutants of BNYVV or PVX (Bleykasten-Grosshans et al., 1997; Lough et al., 1998). We hypothesize that the expression of TGBp2 and TGBp3 as overlapping ORFs requires additional regulatory sequences on the viral genome, which are not included in the satBaMV constructs, so that TGBp3 might not be expressed from these constructs for *trans*-complementation tests. The *trans*-complementation of movement of pd6 by pSat6 indicates that TGBp3 is indeed expressed from the chimeric satBaMV RNA construct carrying a single TGBp3 ORF. However, the translation level of TGBp3 in such systems could not be verified by immunological methods, since no antiserum of sufficient sensitivity to detect the TGBp3 protein of BaMV is available. For potexviruses, hordeiviruses and furoviruses, it has been shown that TGBp2 and TGBp3 are expressed from a single bi-cistronic mRNA (Morozov et al., 1991; Zhou & Jackson, 1996; Bleykasten-Grosshans et al., 1997; Verchot et al., 1998). However, the upstream regulatory sequences for the expression of TGBp2 and TGBp3 have not been defined clearly. Thus, it is possible that TGBp3 was not expressed or was expressed at a very low level from the satBaMV constructs pSat136 and pSatT used for the complementation experiments.

The cell-to-cell movement of PVX is thought to require precise regulation of the appropriate relative amounts of the three TGBps produced (Yang et al., 2000). An *in vitro* translation experiment of a subgenomic RNA with overlapping TGBp2 and TGBp3 ORFs yielded the two proteins at a 10 : 1 ratio (Zhou & Jackson, 1996). It has been shown that ectopic expression of TGBp3 of BNYVV leads to the inhibition of cell-to-cell movement (Bleykasten-Grosshans et al., 1997). However, the capability of BaMV cell-to-cell movement was not affected significantly with the additional expression of TGBp1, TGBp2 or TGBp3 from the satBaMV RNA vectors. The results suggest that either the amount of ectopic expression was not sufficiently high or that there is less need for a tight control of the relative amounts of the three TGBps for cell-to-cell movement of BaMV.

Although several models for the cell-to-cell movement of viruses have been proposed (Verchot-Lubicz, 2005), accumulating data indicate that different viruses may implement different mechanisms involving various combinations of viral or host proteins for their movement, even for viruses belonging to the same genus. Our study presents a convenient tool and provides further insights into the elucidation of viral movement mechanism(s) of BaMV and potexviruses.

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**REFERENCES**


