INTRODUCTION

The movement of plant viruses from cell to cell and over long distances requires the assistance of virus-encoded movement proteins (MPs) (reviewed by Atabekov & Taliansky, 1990; Gilbertson & Lucas, 1996). Several mechanisms, including the *Tobacco mosaic virus* (TMV)-like and tubule-based mechanisms, have been reported for intercellular transport of plant viruses (Carrington *et al.*, 1996). For each mechanism, the MPs involved seem to possess different functions. In the TMV-like mechanism, the MP is able to increase the size-exclusion limit (SEL) of the plasmodesmata (Wolf *et al.*, 1990), to transfer virions through the tubules via specific MP–capsid protein (CP) complexes (Wellink & van Kammen, 1989).

The three proteins encoded by the triple gene block (TGBps) of potexviruses are essential for the cell-to-cell movement of viruses of this group (Angell *et al.*, 1996; Beck *et al.*, 1991). However, TGBps are structurally and functionally different from the MPs described above. Among the three TGB proteins, TGBp1 appears to exist in soluble and cytoplasmic inclusion forms. The soluble form of TGBp1 binds cooperatively to ssRNA, possesses ATPase (Rouleau *et al.*, 1994; Wung *et al.*, 1999) and RNA helicase activities (Kalinina *et al.*, 2002), increases the SEL of plasmodesmata (Angell *et al.*, 1996; Lough *et al.*, 1998) and forms a ribonucleoprotein complex (Lough *et al.*, 1998, 2000). TGBp1 is also a gene silencing suppressor, which prevents spread of the gene silencing signal in *Nicotiana benthamiana* (Voinnet *et al.*, 2000). TGBp1 can move from cell to cell (Lough *et al.*, 1998; Yang *et al.*, 2000) and mediates cell-to-cell movement of TGBp2 and TGB3 (Krishnamurthy *et al.*, 2002). Further analysis of the soluble...
TGbp1 of Bamboo mosaic virus (BaMV) has revealed that Arg-11, Arg-16 and Arg-21 in the N-terminal region of this protein are essential for its RNA-binding activity (Liou et al., 2000). Although BaMV TGbp1 belongs to the RNA helicase superfamily I (Kadare & Haenni, 1997; Morozov et al., 1999; Liou et al., 2000), Arg-16 and Arg-21, which are located outside the seven conserved motifs of the superfamily I RNA helicase, are also critical for the NTPase activity of TGbp1 (Liou et al., 2000). TGbp1s of potexviruses are in fact mainly associated with cytoplasmic inclusions in infected tissues (Chang et al., 1997; Davies et al., 1993; Rouleau et al., 1994). The TGbp1 obtained from the cytoplasmic inclusions also possesses NTP-binding and NTPase activities but lacks the RNA-binding activity (Liou et al., 2000).

TGbp2 and TGbp3 both contain stretches of hydrophobic amino acids and are associated with cell wall and membrane fractions both in vivo and in vitro (Donald et al., 1993; Hefferon et al., 1997; Morozov et al., 1990, 1991; Niesbach-Klosgen et al., 1990; Solovyev et al., 2000). Using a microinjection and transient expression strategy, TGbp2 was shown to assist the movement of the ribonucleoprotein complex of potexviruses (Lough et al., 1998) and to be capable of increasing the SEL of the plasmodesmata (Tamai & Meshi, 2001). However, contradictory results were obtained for TGbp3. It has been reported to be required for virus movement (Lough et al., 1998) and to be associated with the movement of virus particles. The satellite RNA (satBaMV) has been found to associate with the BaMV-V isolate (Lin & Hsu, 1994). The satBaMV is a satellite RNA (836 nucleotides in length, excluding the poly(A) tail, and encodes a 20 kDa non-structural protein (P20). The satBaMV can be used as a plant expression vector since P20 is not essential for satellite replication and can be replaced with other proteins such as chlor-amphenicol acetyltransferase (Lin et al., 1996).

In this study, we analysed the relationship between the RNA-binding and NTPase activities of TGbp1 and the capability of BaMV to move from cell to cell. With the aid of the infectious CDNA clones of BaMV-S and a satellite replicon based on satBaMV (Lin et al., 1996), we have demonstrated that Arg-16 and Arg-21 in the N-terminal region of the BaMV TGbp1, residues that are essential for the RNA-binding and NTPase activities of TGbp1 (Wung et al., 1999; Liou et al., 2000), are also crucial for cell-to-cell movement of BaMV in host plants.

**METHODS**

**Infectious BaMV and satBaMV cDNA clones.** The plasmid pCB is an infectious clone of BaMV-S (EMBL/GenBank accession no. AF018156). Expression of the BaMV genome in this plasmid is under the transcriptional control of the 35S promoter of Cauliflower mosaic virus (CaMV). Plasmid pCBG is a derivative of pCB; a green fluorescent protein gene (gfp) (Sheen et al., 1995) has been inserted between the two open reading frames of TGbp3 and CP in the BaMV genome and is controlled by a duplicated subgenomic promoter for CP expression (J.-T. Liao & Y.-H. Hsu, unpublished data). The recombinant BaMV bearing the gfp gene in pCBG can be transcribed from the 35S promoter of CaMV. Plasmid pCBSF4, derived from pBSF4 (Lin et al., 1996), is an infectious cDNA clone of the satBaMV, in which expression of the cDNA sequence is driven by the 35S promoter as described for the pCass plasmid (Ding et al., 1995).

**Construction of mutant BaMV and satellite replicons.** Each of the mutant BaMV with Arg→Ala substitution(s) in TGbp1 was derived from pCBG by site-directed mutagenesis (Sambrook et al., 1989). Primers used for mutagenesis are shown in Table 1. PR11A, PR16A and PR21A are plasmids that contain a single Arg→Ala substitution at amino acid positions 11, 16 and 21 of TGbp1, respectively. Plasmids PR11/16A, PR11/21A and PR16/21A containing double Arg→Ala substitutions were derived from PR11A or PR16A.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Description</th>
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<tr>
<td>3572R</td>
<td>ATGAAAGCAAGCCACCATG</td>
<td>All mutations</td>
</tr>
<tr>
<td>508I</td>
<td>ATCCACCTGCTTAGTGTGGG</td>
<td>All mutations and RT</td>
</tr>
<tr>
<td>R11A</td>
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<td>R11A substitution</td>
</tr>
<tr>
<td>R16A</td>
<td>CTGATGTGCAGTACGGACATC</td>
<td>R16A substitution</td>
</tr>
<tr>
<td>R21A</td>
<td>CCGGCCGCTTCGTGGTCTGATG</td>
<td>R21A substitution</td>
</tr>
<tr>
<td>P28N</td>
<td>TATCCAAAGCAGTGGATGAAACCGGATAA</td>
<td>Amplification TGbp1</td>
</tr>
<tr>
<td>P28C</td>
<td>CAGCCTCTGGGGGAGGGTGTGCTGGCCAGATG</td>
<td>Amplification TGbp1</td>
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</tr>
<tr>
<td>4818</td>
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<td>Amplification nt 3572–4818</td>
</tr>
<tr>
<td>3759R</td>
<td>CGCCAACTGTCAGGTGAGGAG</td>
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The italicized bases indicate the BstXI or EcoNI restriction site. The site of Arg→Ala substitution is in bold.
To construct pSat28, the P20-coding sequence of the satBaMV in pCBSF4 was replaced with the TGBp1-coding sequence amplified from pCBG using primers P28N and P28C (Table 1). The plasmid pSat28f was a mutant clone of pSat28, in which a single-base deletion was observed at position 7 of the TGBp1-coding sequence, resulting in a shift of reading frame from the third amino acid of TGBp1.

Inoculation of plants with the cDNA clones of BaMV.
Methods used for inoculation of *N. benthamiana* or *Chenopodium quinoa* with clones of BaMV were as previously reported (Lin & Hsu, 1994; Lin et al., 1996), except that each leaf was inoculated with 1 μg plasmid DNA instead of RNA transcript. Methods for total protein extraction and Western blot analysis were as previously described (Lin et al., 1996). Western blot analyses of viral products.

Fluorescent images of leaves infected with *BaMV*-infected leaves. Fluorescent images of leaves infected with the wild-type BaMV or the TGBp1 mutants were obtained with a fluorescent microscope (Zeiss LSM410). Laser illumination at 488 nm (argon ion laser) was recorded through a confocal laser-scanning microscope (Zeiss LSM410). Laser illumination at 488 nm (argon ion laser) was recorded through a confocal laser-scanning microscope (Zeiss LSM410). Laser illumination at 488 nm (argon ion laser) was recorded through a confocal laser-scanning microscope (Zeiss LSM410).

FluorImage and confocal laser scanning microscopy of the *BaMV*-infected leaves. Fluorescent images of leaves infected with the wild-type BaMV or the TGBp1 mutants were obtained with a FluorImage (Molecular Dynamics, model 595) with an excitation filter of 488 nm and an emission filter using calibration files. Laser illumination at 488 nm (argon ion laser) was recorded through a bandpass filter normally used for the detection of fluorescence. Image analysis and display (adjustments in contrast, brightness, etc.) were all performed using Adobe Photoshop.

RT-PCR and cDNA sequencing. Total RNAs were extracted from the inoculated leaves of *C. quinoa* and *N. benthamiana* as previously described (Lin et al., 1996). Reverse transcription was then carried out to synthesize the first strand cDNA of TGBp1 using primer 5081 (Table 1) complementary to the 3’ end of the genomic or sub-genomic RNA for TGBp1. Following that, 30 PCR cycles were performed using the forward primer (3572R) and the reverse primer (4818), which are complementary to the cDNA sequence of TGBp1. The PCR products were finally sequenced using primer 3759R with (4818), which are complementary to the cDNA sequence of TGBp1.

RESULTS

Replacement of Arg-16, Arg-21 or both Arg-16 and Arg-21 of TGBp1 with alanine affects cell-to-cell movement of BaMV

To determine whether there is any relationship between the RNA-binding and NTPase activities of TGBp1 and the capability of BaMV to move from cell to cell, six plasmid clones of mutant BaMV, each containing a single- or a double-alanine substitution at Arg-11, Arg-16 and Arg-21 of TGBp1, were constructed (see Methods) and designated pR11A, pR16A, pR21A, pR11/16A, pR16/21A and pR11/21A, according to the arginine residue(s) replaced (Fig. 1). Inoculation of *C. quinoa* and *N. benthamiana* with each of the mutant TGBp1 BaMV plasmids or the wild-type TGBp1-containing plasmid, pCBG, was then performed. Since an expressible *gfp* gene had been inserted in between the coding sequences of TGBp3 and CP in the BaMV genome (see Methods), we expected to see spread of green fluorescence if the mutant BaMV was able to replicate and move from cell to cell. As shown in Fig. 2, spread of green fluorescence was only observed in leaves of *N. benthamiana* 10 days (Fig. 2A) and *C. quinoa* 5 days (Fig. 2B) post-inoculation (p.i.) with pCBG or pR11A. No fluorescence spread was detected when plant leaves were inoculated with the other mutant BaMVs. These results indicated that only the R11A mutant is able to move from cell to cell and that the movement function is eliminated for the other mutant BaMVs. Since the alanine codon (GCA) remained unchanged in the progeny of the R11A mutant present in the inoculated leaves of *C. quinoa* (Fig. 3) and in both the inoculated (data not shown) and systemic leaves of *N. benthamiana* (Fig. 3), we concluded that R11A-TGBp1 is functional for cell-to-cell movement.

The inability of the R16A, R21A, R11/16A, R11/21A or R16/21A mutants to move from cell to cell was also evidenced by the lack of fluorescence spread between adjacent cells monitored by a confocal laser scanning microscope (Fig. 4). Cell-to-cell spread of fluorescence was undetectable, even in epidermal cells infected with the five BaMV mutants; it was restricted to a single cell (Fig. 4E and F). In contrast, the spread of green fluorescence between adjacent cells...

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<th>16</th>
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<tr>
<td><em>Wt</em></td>
<td>N8- MDNR1TDLLTRSGYLRTSEPAGQQQLVH VAGAGKT</td>
<td>C'</td>
<td>C'</td>
</tr>
<tr>
<td>R11A</td>
<td>N8- ----------A-</td>
<td>C'</td>
<td></td>
</tr>
<tr>
<td>R16A</td>
<td>N8- ----------A-</td>
<td>C'</td>
<td></td>
</tr>
<tr>
<td>R21A</td>
<td>N8- ----------A-</td>
<td>C'</td>
<td></td>
</tr>
<tr>
<td>R11/16A</td>
<td>N8- ----------</td>
<td>A-</td>
<td>C'</td>
</tr>
<tr>
<td>R15/21A</td>
<td>N8- ----------</td>
<td>A-</td>
<td>C'</td>
</tr>
<tr>
<td>R11/21A</td>
<td>N8- ----------</td>
<td>A-</td>
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Fig. 1. The N-terminal amino acid sequences of TGBp1 of the wild-type (*Wt*) and mutant BaMVs. The positions of arginine (R) being replaced with alanine (A) are indicated above the amino acid sequence of the wild-type TGBp1.
was easily detected in the epidermal (data not shown) and mesophyll cells of *C. quinoa* infected with pCBG (Fig. 4C) or pR11A (Fig. 4D). The restriction of movement of the mutant BaMVs containing R16A, R21A, R11/16A, R11/21A or R16/21A in TGBp1 was further corroborated by the absence of viral CP in leaves of both *C. quinoa* and *N. benthamiana* 10 days p.i. (Fig. 5A and B).

Similar to the result observed in inoculated leaves, spread of fluorescence was observed in systemic leaves of *N. benthamiana* inoculated with pCBG or pR11A (Fig. 5E and F), but not in systemic leaves of *N. benthamiana* inoculated with any of the remaining five BaMV mutants (data not shown). Since a similar level of green fluorescence or CP was observed in both the inoculated (Figs 2, 4 and 5A and B) and systemic leaves (Fig. 5C–F) infected with pCBG or pR11A, we believe that the Arg-11→Ala substitution of TGBp1 had, at most, only a slight effect on the capability of cell-to-cell and long-distance movement of BaMV.

**BaMV mutants defective in cell-to-cell movement are able to replicate and synthesize TGBp1**

Several explanations could account for the failure of mutant BaMVs with TGBp1 Arg→Ala substitutions at either position 16, 21 or both 16 and 21 to move from cell to cell. One possibility is that the Arg→Ala substitution(s)
Fig. 4. Fluorescence of the inoculated leaves of *C. quinoa* as viewed using a confocal laser-scanning microscope. Fluorescence in epidermal cells inoculated with water (A), pCB (B), pR16A (E), pR21A (F) or fluorescence in mesophyll cells inoculated with pCBG (C) or pR11A (D) 5 days p.i. Bar, 50 μm.

Fig. 5. Western blot analyses of CP and fluorescence spread in leaves inoculated with the plasmid clones of wild-type or mutant BaMVs. (A, B) CP in the inoculated leaves of *C. quinoa* (A) and *N. benthamiana* (B) 10 days p.i. (C, D) CP in the systemic leaves of *N. benthamiana* infected with the wild-type or mutant BaMVs 15 (C) and 30 (D) days p.i. The total proteins extracted from the inoculated or systemic leaves harvested at the indicated time points were separated by SDS-PAGE, blotted onto PVDF membrane and probed with polyclonal antiserum against CP (Lin & Chen, 1991). The plasmid clone of BaMV used to infect the leaves is shown above each panel. M, CP from purified BaMV. (E, F) Fluorescence images of the systemic leaves of *N. benthamiana* infected with the wild-type pCBG or pR11A 15 (E) and 30 (F) days p.i.
directly renders the mutant TGBp1 defective in movement function. Alternatively, it could be that these TGBp1 mutations interfere with virus replication. Finally, it is possible that the synthesis and accumulation of TGBp1 in leaves infected with the mutant BaMVs are blocked for as yet unknown reasons. To rule out the latter two possibilities, the replication of viral RNA and the synthesis of TGBp1 were analysed in protoplasts of *N. benthamiana* infected with each of the movement-defective BaMVs. As shown in Fig. 6(A), similar patterns of genomic and subgenomic RNAs of BaMV were observed in protoplasts inoculated with the wild-type or mutant BaMVs, indicating that the wild-type and mutant BaMVs possess similar replication activities. In addition, similar amounts of TGBp1 were also detected in protoplasts infected with the wild-type and mutant BaMVs (Fig. 6B). These results indicated that the movement-defective BaMVs are able to replicate and to synthesize TGBp1 in the plant cells. Taken together, these results demonstrate that the inability of the mutant BaMVs with Arg→Ala substitutions at position 16, 21 or both 16 and 21 of TGBp1 to move from cell to cell must be attributed to the loss of certain important TGBp1 functions.

**Complementation of the movement defect of the mutant BaMVs by a satellite replicon harbouring the wild-type TGBp1**

To illustrate further that the movement defect of the mutant BaMVs containing R16A and/or R21A in TGBp1 was indeed due to the loss of TGBp1 function, a satellite replicon, pSat28, capable of expressing the wild-type TGBp1, was co-inoculated with equal amounts of pR16A or pR21A into *N. benthamiana* leaves. In parallel, co-inoculation of pR16A or pR21A with a mutant satellite replicon, pSat28f, harbouring a frameshifted TGBp1, was also conducted. The latter treatment served as a negative control for the complementation assay since no intact and functional TGBp1 could be expressed. As expected, no spread of fluorescence was detected in leaves co-inoculated with pR16A and pSat28f or with pR21A and pSat28f (Fig. 7A). In contrast, fluorescence spread was easily detected when pR16A or pR21A was co-inoculated with pSat28 (Fig. 7A). To verify that the complementation of the movement defect by pSat28 was due to the synthesis and accumulation of wild-type TGBp1, the TGBp1 content of the inoculated leaves was analysed. TGBp1 was absent from leaves inoculated with pR16A or pR21A alone (Fig. 7B, lanes 2 and 3) or further co-inoculated with pSat28f (Fig. 7B, lanes 4 and 5). However, TGBp1 was clearly observed in leaves that were co-inoculated with pR16A and pSat28 or with pR21A and pSat28 (Fig. 7B, lanes 6 and 7). Taken together, these results indicated that introduction of a wild-type TGBp1 allele to the leaves rescues the mutant TGBp1 defect and enables the mutant BaMV to move from cell to cell. Since the mutant TGBp1s are devoid of the RNA-binding and NTPase activities and can be complemented by the wild-type TGBp1, it is assumed that the RNA-binding and NTPase activities are required for the movement of BaMV.

**DISCUSSION**

It has been shown by deletion analyses that the TGBp1 homologues of potexviruses play important roles in assisting virus movement between plant cells (Beck et al., 1994; Angell et al., 1996; Lough et al., 1998; Verchot et al., 1998). With the aid of infectious plasmid clones of BaMV, we have shown that a single or double Arg→Ala substitution at positions 16 and 21, which diminishes the RNA-binding and ATP-utilizing activities of TGBp1 (Wung et al., 1999; Liou et al., 2000), also eliminates the ability of BaMV to move from cell to cell. Furthermore, our ability to trans-complement the mutant TGBp1 with its wild-type counterpart supports the idea that the RNA-binding activity and the NTPase activity of TGBp1 are necessary for the movement of potexviruses from cell to cell.

Complementation has long been used as a strategy to confirm the function of a specific gene. Strategies for complementation of a defective gene in a plant virus include co-bombardment of plants with the defective virus together with a plasmid expressing the target gene (Lough et al., 2001; Morozov et al., 1997; Tamai & Meshi, 2001),...
mixed infection of plants with the defective virus and a viral RNA replicon (Bleykasten-Grosshans et al., 1997), and microinjection of the target protein into a transgenic plant lacking the corresponding gene (Lough et al., 1998). The new strategy adopted in the present study involves the use of a novel satellite replicon, pSat28. The presence of TGBp1 and the spread of BaMV in N. benthamiana leaves co-infected with the mutant BaMVs and pSat28 (Fig. 7A) demonstrated that the satellite replicon strategy works as well as other strategies for complementation analyses.

There are several possible explanations for how the loss of ATPase and RNA-binding activities of TGBp1 could interfere with the movement of the mutant BaMVs. It is highly possible that the loss of the ATPase activity of TGBp1 leads to inactivation of its helicase activity, which is essential for unwinding the duplex region of the viral RNA (Kalinina et al., 2002). In addition, loss of RNA-binding activity of a TGBp1 mutant would probably negatively affect the ability of the mutant TGBp1 to form a streamlined nucleoprotein complex with the viral RNA suitable for movement (Lough et al., 1998, 2000). Thus, the movement process of the virus, at least in the initial stage of transport, is probably hampered by the loss of each of these two activities. However, it is also possible that the defective TGBp1 is unable to carry out steps in the movement process subsequent to the streaming of viral RNA. For example, the TGBp1 mutations could prevent the viral ribonucleoprotein complex from interacting with TGBp2, TGBp3 or some other host protein so that the movement of viral nucleic acid along the cytoskeleton and toward the plasmodesmata is blocked. Furthermore, loss of the ATPase activity may inhibit the ability of TGBp1 to increase the SEL of plasmodesmata (Lough et al., 1998) and thus decrease the efficiency of viral transport across the cell wall. Further studies are required to clarify these possibilities.

Since the R11A TGBp1, while retaining its NTPase activity, has significantly reduced RNA-binding activity (Liou et al., 2000; Wung et al., 1999), we originally predicted that this mutant would be unable to move from cell to cell. However, the R11A mutant was shown to be able to move in the present study (Figs 2, 4 and 5). Because no reversion was detected in the progenies of the R11A mutant (Fig. 3), we conclude that the R11A TGBp1 mutant is able to fulfill movement function. Probably, the residual RNA-binding activity of the R11A TGBp1 is enough to assist virus movement. Alternatively, there may be some other factors that are able to enhance the RNA-binding activity of the mutant TGBp1 in vivo. The ability of the TGBp1 protein of WCIMV to form ribonucleoprotein complexes with the viral CP (Lough et al., 1998, 2000) raises the possibility that CP may be one such factor. Other possibilities are interactions with TGBp2 and TGBp3 (Krishnamurthy et al., 2002).

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REFERENCES


