Cucumovirus- and bromovirus-encoded movement functions potentiate cell-to-cell movement of tobamov- and potexviruses

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Abstract

Cucumber mosaic virus (CMV, a cucumovirus) and Brome mosaic virus (BMV, a bromovirus) require the coat protein (CP) in addition to the 3a movement protein (MP) for cell-to-cell movement, while Cowpea chlorotic mottle virus (CCMV, a bromovirus) does not. Using bombardment-mediated transcomplementation assays, we investigated whether the movement functions encoded by these viruses potentiate cell-to-cell movement of movement-defective Tomato mosaic virus (ToMV, a tobamovirus) and Potato virus X (PVX, a potexvirus) mutants in Nicotiana benthamiana. Coexpression of CMV 3a and CP, but neither protein alone, complemented the defective movement of ToMV and PVX. A C-terminal deletion in CMV 3a (3aΔC33) abolished the requirement of CP in transporting the ToMV genome. The action of 3aΔC33 was inhibited by coexpression of wild-type 3a. These findings were confirmed in tobacco with ToMV-CMV chimeric viruses. Either BMV 3a or CCMV 3a alone efficiently complemented the movement-defective phenotype of the ToMV mutant. Therefore, every 3a protein examined intrinsically possesses the activity required to act as MP. In transcomplementation of the PVX mutant, the activities of BMV 3a, CCMV 3a, and CMV 3aΔC33 were very low. The activities of the bromovirus 3a proteins were enhanced by coexpression of the cognate CP but the activity of CMV 3aΔC33 was not. Based on these results, possible roles of cucumo- and bromovirus CPs in cell-to-cell movement are discussed.

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Introduction

The ability of a virus to move from the site of infection to neighboring cells through plasmodesmata is a prerequisite for successful infection in a susceptible host plant. Consequently, many plant viruses encode one or more proteins, referred to as movement proteins (MPs), to facilitate this cell-to-cell movement. Tobamoviruses, including Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV), encode a single ~30-kDa MP (Deom et al., 1987; Meshi et al., 1987). TMV MP is among the best-characterized MPs and has the ability to target to plasmodesmata (Tomenius et al., 1987), to increase plasmodesmal permeability (Wolf et al., 1989), and to bind RNAs (Citovsky et al., 1990, 1992). These characteristics are thought to reflect the activity of MPs in transporting the genomic RNA from cell to cell (Carrington et al., 1996; Lazarowitz and Beachy, 1999).

Many viruses also need the coat protein (CP) for cell-to-cell movement (Callaway et al., 2001), in contrast to tobamoviruses, which do not (Takamatsu et al., 1987). Cucumber mosaic virus (CMV) and Brome mosaic virus...
(BMV), the type members of the *Cucumovirus* and *Bromovirus* genera, respectively, belonging to the family *Bromoviridae*, are this type of virus (Canto et al., 1997; Schmitz and Rao, 1996; Suzuki et al., 1991). However, whereas BMV needs encapsidation-competent CP for efficient cell-to-cell movement, as far as has been examined (Okinaka et al., 2001; Rao, 1997; Rao and Grantham, 1995, 1996; Schmitz and Rao, 1996), the ability to form virions is not required for CMV CP to facilitate cell-to-cell movement (Kaplan et al., 1998; Schmitz and Rao, 1998). Interestingly, C-terminal truncation of the CMV MP (3a protein) abolishes the CP requirement (Nagano et al., 2001). *Cuspea chlorotic mottle virus* (CCMV), a dicot-adapted species of the *Bromovirus* genus, can spread from cell to cell in the absence of CP (Rao, 1997). Similar to tobamoviral MPs, the 3a MPs of CMV, BMV, and/or CCMV are found in plasmodesmata and are capable of binding to RNA and modulating plasmodesmal permeability (Ding et al., 1995; Fujita et al., 1998, 1999; Jansen et al., 1998; Li and Palukaitis, 1996; Vaquero et al., 1994). It has yet to be determined how the CPs of these mutually related viruses function in cell-to-cell movement.

While tobamo-, cucumo-, and bromoviruses encode a single MP, the viruses of several genera, including *Potato virus X* (PVX), the type member of the *Potexvirus* genus, encode three MPs that are translated from partially overlapping open reading frames (ORFs) termed the triple gene block (TGB) (Morozov et al., 1989; Morozov and Solovyev, 1999). PVX requires the CP for cell-to-cell movement in addition to the TGB-encoded proteins, p25, p12, and p8 (Baulcombe et al., 1995; Chapman et al., 1992; Tamai and Meshi, 2001a).

There are numerous examples showing that movement functions are interchangeable between different virus families encoding dissimilar MPs and that movement-defective phenotypes of specific viruses are complemented by MPs encoded by taxonomically distinct viruses (for reviews, see Atabekov et al., 1999; Atabekov and Taliansky, 1990). These observations suggest that movement mechanisms may be shared among viruses, at least in some respects, and may be considered separate from replication. On the other hand, there are also examples indicating that factors other than MPs are also involved in the complementation of movement defects (Atabekov et al., 1999). However, in many instances of complementation and many analyses of cell-to-cell movement, the movement-defective phenotype of a given virus or mutant in a host was deduced from little or no accumulation of the genome or other viral products (usually CP) in the inoculated leaves; therefore, the possibility of subliminal movement remains, in which the given virus infects a few cells surrounding the initial site of infection. If this were the case, phenomenological complementation may be due to the inhibition of host resistance, including RNA silencing, by a factor supplied in *trans*, either virally or transgenically, and not due to true complementation in cell-to-cell movement processes involving the action of an MP.

The bombardment-mediated transcomplementation method, originally described by Morozov et al. (1997) for PVX and then by Tamai and Meshi (2001b) for ToMV, is based on the observation that movement-defective reporter-tagged virus generated from the corresponding infectious plasmid DNA can spread from the bombarded cells to adjoining cells when a separately cloned MP gene is cobombarded. This method enables us to detect the activity of MPs explicitly and to compare the activity of different MPs in the same situation. Furthermore, it also allows us to express multiple proteins simultaneously.

In this work, we investigated the movement functions encoded by members of the family *Bromoviridae* by using this bombardment-mediated transcomplementation assay. Our results show that the movement functions of CMV, BMV, and CCMV rescue the movement-defective phenotypes of ToMV and PVX mutants, although the efficiency of transcomplementation and the requirement of CP were different between these three viruses and also depended on the genome (PVX or ToMV) that was transported.

## Results

### Experimental basis of the transcomplementation assay

An infectious clone of ToMV, piL.erG3, has the erG3GFP gene in place of the CP gene (Fig. 1A). The erG3GFP gene encodes a variant of green fluorescent protein (GFP) (Tamai and Meshi, 2001b), which is localized in the ER and does not diffuse between cells, as described below. When piL.erG3 was bombarded into mature leaves of *Nicotiana benthamiana*, the progeny virus, designated L.erG3, multiplied to produce erG3GFP replication-dependently under the control of the CP subgenomic RNA promoter. Fig. 2A shows an example of the foci of L.erG3 at 2 days postbombardment (dbp), which consists of tens of infected cells. When piL.erG3(SF3), which has a frameshift mutation in the MP gene (one-base deletion at the 10th codon) (Fig. 1A), was bombarded, single epidermal cells were infected (Fig. 2B), whereas when it was cobombarded with another plasmid expressing a tobamoviral MP, the movement-defective progeny virus, L.erG3(SF3), moved multicellularly, forming clusters of GFP-fluorescent cells (Tamai and Meshi, 2001b). Fig. 2C shows an example of the transcomplementation of L.erG3(SF3) achieved by ToMV MP at 2 dbp. The L.erG3(SF3)-induced foci reached near-maximum sizes at 2 dbp, at which time the infected cells exhibited similar levels of GFP fluorescence (Fig. 2C). This indicates the limitation in the effects of an MP that is expressed in *trans* in the bombarded cells.

It has been shown that cytosolic GFP moves between cells in source tissues, although the movement is highly restricted, compared with that in sink tissues (Oparka et al., 1999). When pBIG3 encoding G3GFP (a cytosolic GFP variant) (Fig. 1C) alone was bombarded into the mature leaves of *N. benthamiana*, GFP fluorescence was restricted...
to the bombarded cells in 94% of the sites at 1 dpb (Fig. 3A and Table 1). However, G3GFP diffused from the bombarded cells to their neighbors over time, forming halos (Fig. 3B). Under our assay conditions, halos were seen in 39 and 69% of the sites at 2 and 3 dpb, respectively (Table 1). Such diffusion of G3GFP is greatly enhanced when coexpressed with an MP, such as tobamovirus MPs and PVX p12 (Tamai and Meshi, 2001a,b). Therefore, the cytosolic type of GFP variants cannot be used as a reporter to explicitly identify the infected cells when an MP is expressed in the same epidermal cells. As exemplified in Fig. 3C and summarized in Table 1, the 3a MPs of CMV, BMV, and CCMV, which were used in this work, also exhibited the plasmodesmal permeability-modulating activity.

In contrast, when pBlG3 encoding erG3GFP (Fig. 1C) was bombarded alone or together with a plasmid or plasmids encoding movement function, erG3GFP signals were restricted to single cells in 97–98% of the sites either at 1 or 3 dpb (Fig. 3D and E, and Table 1) (Tamai and Meshi, 2001b). Two adjoining cells exhibited GFP signals at a frequency of 2–3% (Table 1). Because these two cells had almost the same fluorescence intensity, this frequency (2–3%) represents the background level at which two adjoining cells were fortuitously bombarded.

**CMV-encoded movement function potentiates cell-to-cell movement of ToMV**

By using the transcomplementation method described above, we examined whether the movement function of CMV can promote cell-to-cell movement of movement-defective ToMV. When piL.erG3(SF3) was cobombarded with p35YM encoding CMV 3a (Fig. 1C), almost all the infected sites (98.6%) were single isolated cells (Figs. 2D and 4A). The result indicates that CMV 3a alone was unable to transcomplement the movement-defective phenotype of L.erG3(SF3). Since CMV is known to require both 3a MP and CP for its own cell-to-cell movement (Canto et al., 2001b), Coexpression of the CMV 3a and p8 genes with the movement-defective ToMV clone piL.erG3(SF3) resulted in a significant enhancement of its cell-to-cell movement (Fig. 3C and Table 1). The result indicates that the movement function of CMV can promote cell-to-cell movement of movement-defective ToMV.
1997; Suzuki et al., 1991), we added p35YCP encoding CMV CP (Fig. 1C) to the inoculum. As shown in Fig. 2E and summarized in Fig. 4A, as many as 90% of the infection foci contained two or more GFP-expressing cells at 2 dpb. The efficiency was similar to that achieved with transcomplementation with the cognate ToMV MP (Fig. 4A). No such complementation was observed when CMV CP alone was expressed (Fig. 4A). The reporter erG3GFP did not move detectably from the bombarded cells to adjoining cells when three plasmids, p35YM, p35YCP, and pBIerG3, were cobombarded (Fig. 3E and Table 1). Therefore, movement-defective ToMV can spread from cell to cell when both CMV 3a and CP are expressed.

**CMV-encoded movement function potentiates cell-to-cell movement of PVX**

Using the same DNA infection system, we next investigated whether the CMV-encoded movement function can promote the cell-to-cell movement of PVX. As a movement-defective PVX construct, we used piX.erG3(TdCd), which has the erG3GFP gene downstream from a duplicated CP subgenomic promoter and multiple deletions in the genes required for cell-to-cell movement (Fig. 1B). Bombardment of piX.erG3(TdCd) alone resulted in single-cell infections but when all TGB-encoded proteins and CP were
expressed together, the resultant virus, designated PVX.erG3(TdCd), spread to neighboring cells (Fig. 2K; Tamai and Meshi, 2002a). Compared with ToMV, fewer GFP-positive cells were observed in the infection foci (Figs. 2C, 2K, and 4), because p8 functions intracellularly (Tamai and Meshi, 2002a). In the transcomplementation of PVX.erG3(TdCd), we collected data at 3 dpb, because GFP fluorescence was still weak at 2 dpb and because the sizes of foci reached near maximum at 3 dpb. Fig. 2J shows an example of the foci formed 3 days after bombardment of a movement-competent construct, piX.erG3 (Tamai and Meshi, 2001a), which produces PVX.erG3 (Fig. 1B).

When piX.erG3(TdCd) was bombarded with p35YM and p35YCP, fluorescent cell clusters were observed in about half (51%) the infection sites (Figs. 2M and 4B). No such clusters were detected when either p35YM or p35YCP was bombarded with piX.erG3(TdCd) (Figs. 2L and 4B). Therefore, coexpression of CMV 3a and CP rescued the movement-defective phenotype of the PVX mutant, although with a lower efficiency than that achieved by the PVX-encoded movement function.

Such a broad spectrum of CMV-encoded movement function is in contrast to the activities of PVX- and TMV/ToMV-encoded functions (Fig. 4). For example, coexpression of four proteins involved in PVX movement did not potentiate the cell-to-cell movement of the ToMV mutant (Fig. 4A). Conversely, tobamovirus-encoded MPs only inefficiently transcomplemented the PVX mutant under our assay conditions (Fig. 4B); the fraction of infection sites containing two or more GFP-fluorescent cells was only 2.7% for ToMV MP (all composed of two cells) and 6.9% for TMV MP. Such a low frequency of transcomplementation is far below the full activity achieved by the coexpression of PVX-encoded proteins. However, the level achieved by TMV MP is still significantly higher than background levels, because bombardment of piX.erG3(TdCd) alone resulted almost exclusively in single-cell infections (Tamai and Meshi, 2001a), and because we never observed the fortuitous bombardment of three adjoining cells with pBlErG3 (Table 1).

CMV 3a mutant with a C-terminal truncation (3aΔC33) efficiently transports movement-defective ToMV in the absence of CP

Some CMV 3a derivatives with a C-terminal truncation abolish the requirement of CP in cell-to-cell movement

<table>
<thead>
<tr>
<th>Expressed proteins</th>
<th>dpb</th>
<th>Frequency of GFP signals in two or more adjoining cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3GFP</td>
<td>1</td>
<td>11/196 (5.6)</td>
</tr>
<tr>
<td>G3GFP</td>
<td>2</td>
<td>54/139 (38.8)</td>
</tr>
<tr>
<td>G3GFP</td>
<td>3</td>
<td>74/108 (68.5)</td>
</tr>
<tr>
<td>G3GFP + CMV 3a</td>
<td>1</td>
<td>73/116 (62.9)</td>
</tr>
<tr>
<td>G3GFP + CMV CP</td>
<td>1</td>
<td>7/145 (4.8)</td>
</tr>
<tr>
<td>G3GFP + BMV 3a</td>
<td>1</td>
<td>105/156 (67.3)</td>
</tr>
<tr>
<td>G3GFP + CCMV 3a</td>
<td>1</td>
<td>76/156 (66.1)</td>
</tr>
<tr>
<td>erG3GFP</td>
<td>1</td>
<td>3/133 (2.3)</td>
</tr>
<tr>
<td>erG3GFP + CMV 3a + CMV CP</td>
<td>1</td>
<td>14/433 (3.2)</td>
</tr>
<tr>
<td>erG3GFP + CMV 3a + CMV CP</td>
<td>3</td>
<td>2/182 (1.1)</td>
</tr>
<tr>
<td>erG3GFP + BMV 3a + BMV CP</td>
<td>1</td>
<td>6/243 (2.5)</td>
</tr>
<tr>
<td>erG3GFP + BMV 3a + BMV CP</td>
<td>3</td>
<td>7/263 (2.7)</td>
</tr>
<tr>
<td>erG3GFP + CCMV 3a + CCMV CP</td>
<td>1</td>
<td>4/200 (2.0)</td>
</tr>
<tr>
<td>erG3GFP + CCMV 3a + CCMV CP</td>
<td>3</td>
<td>10/330 (3.0)</td>
</tr>
</tbody>
</table>

* Proteins were expressed under the control of the 35S promoter.
* dpb, days post bombardment.
* Data are presented as the number of bombarded sites containing two or more GFP-positive cells/the total number of bombarded sites examined. The frequency (%) is shown in parentheses. G3GFP trafficked between trichome cells; therefore, they were not counted.
* Bomarded sites exhibiting halos.
* Bomarded sites composed of two adjoining cells with nearly the same fluorescence intensity. No bombarded sites composed of three or more GFP-positive cells were found.

Table 1

Cell-to-cell trafficking of GFP variants and the activity of cucumovirus and bromovirus 3a proteins

Fig. 4. Summary of the transcomplementation of movement-defective ToMV and PVX. (A) piL.erG3(SF3) was bombarded with a plasmid or plasmids from which a protein or proteins (indicated on the left) were expressed in trans. The numbers of GFP-positive cells (epidermal cells only) at each infection site were counted at 2 dpb, categorized as indicated at the bottom, and represented graphically as a percentage. The total numbers of foci counted are indicated on the right. (B) piX.erG3(TdCd) was bombarded with a plasmid or plasmids from which a protein or proteins (indicated on the left) were expressed in trans. Data were collected at 3 dpb and summarized as in (A).
(Nagano et al., 2001). Of these, the 3a mutant lacking the 33 C-terminal amino acids (3aΔC33) showed the highest activity in promoting cell-to-cell movement of CMV and chimeric BMV genomes in the absence of CP (Nagano et al., 2001). Here, we explored whether such a C-terminal deletion of CMV 3a potentiates cell-to-cell movement of ToMV and PVX mutants CP-independently.

When piL.erd3(SF3) was bombarded with p35YMd expressing 3aΔC33 (Fig. 1C), a cluster of GFP-fluorescent cells was observed (not shown). To compare the activity of 3aΔC33 alone with the activity of wild-type (wt) 3a plus CP more precisely, we added the plasmid pBI-NDr, which encodes nuclear-localized DsRed, so that each inoculum contained a fixed amount of plasmids. The inclusion of pBI-NDr affected the efficiency of transcomplementation by 3aΔC33 little; ~90% of the foci contained two or more GFP-positive cells (Fig. 4A). Instead, red fluorescent nuclei made it possible to identify the bombarded cells in the infection foci that consisted of multiple green fluorescent cells (Figs. 2F and G). In this way, we found that 3aΔC33 was able to transport L.erd3(SF3) through more than one cell boundary beyond the bombarded cells. Therefore, the truncation of the C-terminal portion of 3a also abolished the CP requirement in transporting the ToMV genome. The activity of 3aΔC33 was nearly the same as that of ToMV MP and slightly higher than that of wt 3a plus CP, as judged from the efficiency of transcomplementation and the sizes of the foci (Figs. 2C, 2F, and 4A).

When piX.erd3(TdCd) was bombarded with p35YMd, only ~10% of foci contained two or more fluorescent cells, which was similar to the case of TMV MP and much lower than the complementation achieved by coexpression of wt 3a and CP (Fig. 4B). The coexpression of CP did not enhance the activity of 3aΔC33 (Fig. 4B). Therefore, the activity of 3aΔC33 resembles that of tobamoviral MPs more than the activity exhibited by wt 3a plus CP.

It has been reported that when 3aΔC33 and wt 3a are coexpressed, 3aΔC33-mediated CP-independent cell-to-cell movement of CMV or chimeric BMV is antagonistically inhibited (Nagano et al., 2001). When piL.erd3(SF3) was bombarded with p35YM and p35YMd, single-cell infections apparently increased, whereas multiple cell infections decreased (Fig. 4A; compare CMV 3a + 3aΔC33 with CMV 3a + CP or 3aΔC33 + DsRed), indicating that the expression of wt 3a inhibited the activity of 3aΔC33 in transporting the ToMV genome.

**BMV- and CCMV-encoded movement functions can transport ToMV and PVX genomes**

BMV and CCMV are related viruses, belonging to the *Bromovirus* genus; however, they differ in the requirement of CP in cell-to-cell movement (see Introduction). This phenomenon is analogous to the relationship between the activity of wt 3a plus CP and the activity of 3aΔC33 of CMV. Here, we examined whether these bromovirus-encoded 3a proteins can transport ToMV and PVX genomes and whether the expression of CP is required for their function.

Unexpectedly, BMV 3a as well as CCMV 3a efficiently transcomplemented the movement-defective phenotype of L.erd3(SF3) even in the absence of CP (Figs. 2H, 2I, and 4A). The efficiency (the fraction of foci containing two or more GFP-fluorescent cells) was comparable to that of tobamoviral MPs, indicating that both the BMV and the CCMV 3a proteins, by themselves, are competent in the transport function.

On the other hand, neither of the 3a proteins alone was highly active in transporting the PVX mutant PVX.erd3(TdCd) (Fig. 4B). When the cognate CP was coexpressed, the fraction of multicellular infections increased ~4- to ~6-fold (from 11 to 39% and from 2.7 to 17% for CCMV and BMV proteins, respectively) (Figs. 2N, 2O, and 4B). No diffusion of er3GFP was observed in the coexpression of 3a and CP of these bromoviruses (Table 1). Therefore, the expression of the CPs enhanced the activity of the corresponding bromovirus 3a proteins in transporting PVX.erd3(TdCd), although the efficiency of transcomplementation did not reach the level achieved by the coexpression of CMV 3a and CP.

**Hybrid ToMV expressing 3aΔC33 spreads in tobacco**

To confirm the activity of 3aΔC33 to transport the ToMV genome, as well as the inhibitory effect of wt 3a on 3aΔC33-mediated ToMV movement, we prepared several recombinant ToMV (infectious transcripts) harboring the wt 3a and/or 3aΔC33 genes and examined their ability to move from cell to cell in tobacco (Fig. 5B). Progeny viruses derived from each pTL series plasmid are indicated by removing the prefix p from the name of the progenitor plasmid. A slash (/) and a dot (.) denote the replacement of the ToMV MP and CP genes, respectively. SF3 in parentheses denotes the frame-shift mutation in the ToMV MP gene as in the case of piL.erd3(SF3).

Because the replicate and MP genes overlap, we first modified the sequence around the initiation codon of the MP gene and the termination codon of the replicate gene to facilitate the swapping of MP genes (pTLMR, Fig. 5A). The resulting virus, TLMR, spread normally, forming local lesions on Xanthi nc tobacco plants, indicating that the modification did not affect the activity of the MP gene (not shown). TL/YMd and TL.YMd(SF3) (Fig. 5B), which had the 3aΔC33 gene in place of the ToMV MP and CP genes, respectively, also formed local lesions on Xanthi nc leaves (Fig. 6A). Thus, 3aΔC33, expressed under the control of either the MP or the CP subgenomic promoter, was competent in promoting cell-to-cell movement of the ToMV genome. On the other hand, TL/YM, which had the wt 3a gene in place of the MP gene, did not induce necrotic lesions on Xanthi nc leaves (Fig. 6B).

We investigated the multiplication of these hybrid vi-
ruses in tobacco protoplasts (Fig. 5C). In this experiment, we added a coatless mutant, TLΔC1, in which the initiation codon for ToMV CP had been changed to AGA, to eliminate the effects of virion formation on the accumulation of genomic RNA. As shown in Fig. 5C (lanes 5–7), all the ToMV derivatives containing the CMV 3a-derived sequence exhibited a decreased level of multiplication. The effect was more pronounced when the ToMV CP gene was replaced with the 3aΔC33-coding sequence (e.g., compare TL/YMd(SF3) and TL/YMd). Remarkably and unexpectedly, the accumulation of the subgenomic mRNAs was greatly impaired in TL/YMd(SF3) (Fig. 5C, lane 7). Western blot analysis, however, revealed that 3a and 3aΔC33 were expressed (Fig. 5D, lanes 2–4). A slightly greater amount of 3aΔC33 was expressed from TL/YMd(SF3) than from TL/YMd (Fig. 5D, lanes 3 and 4). TL/YM replicated and produced wt 3a (Fig. 5C lane 5 and 5D, lane 2). TL/YM formed necrotic lesions on a transgenic Xanthi nc line expressing TMV MP (not shown). Considering these results together, we concluded that TL/YM is replication-competent and movement-defective.

To address the antagonistic effects of wild-type 3a against 3aΔC33, we constructed TL/YM/YMd, in which 3a and 3aΔC33 were expressed under the control of the MP and CP subgenomic promoters, respectively (Fig. 5B). TL/YM/YMd induced only a few small lesions on Xanthi nc leaves (Fig. 6C, arrowheads), whereas many lesions were formed on Xanthi nc expressing TMV MP (Fig. 6D). On the
other hand, TL/YMf.YMd, a structural control with a 4-bp insertion at the 42nd codon of the wt 3a gene, formed many lesions on Xanthi nc (Fig. 6C). In protoplasts, TL/YMf.YMd and TL/YMf.YMd multiplied similarly (Fig. 5C, lanes 8 and 9), producing similar levels of 3aΔC33 (Fig. 5D, lanes 5 and 6). TL/YM.YMd expressed wt 3a additionally (Fig. 5D, lane 5). Therefore, the impairment of the lesion-forming activity of TL/YM.YMd was concluded to be due to defective movement, resulting from the inhibitory action of wt 3a against 3aΔC33.

These observations with in vitro synthesized infectious transcripts are consistent with the results of the bombardment experiments; i.e., 3aΔC33 is functional in transporting the ToMV genome and wt 3a is nonfunctional by itself and inhibits the functioning of 3aΔC33.

Discussion

*N. benthamiana* is a common susceptible host for the six viruses (four genera) used in this work; therefore, all the proteins encoded by these viruses can interact productively with *N. benthamiana* factors in normal infection processes, including cell-to-cell movement. The movement-defective constructs of ToMV and PVX used in this work expressed only the components required for replication and to monitor their infection (erG3GFP). Thus, whether or not the mutant ToMV or PVX moved primarily reflected the affinity between the replicated ToMV or PVX (or replication-associated and induced factors) and the proteins expressed in trans from the cobombarded plasmids. We focused on cell-to-cell movement from primary infected epidermal cells (i.e., the bombarded cells) to their immediate neighbors, to minimize the possible effects of host resistance.

Our results show that the movement functions encoded by a cucumovirus (CMV) and two bromoviruses (BMV and CCMV) are capable of transporting movement-defective ToMV (a tobamovirus) and PVX (a potexvirus) in mature leaves of *N. benthamiana* (Fig. 4). However, the requirement of CP in the transcomplementation was different between cucumov- and bromoviruses and, in addition, it depended on the viral genome that was transported (ToMV or PVX) (Fig. 4).

CCMV can spread in the absence of CP in cowpea, *Chenopodium quinoa*, and *N. benthamiana* (Rao, 1997), while BMV requires the expression of encapsidation-competent CP for cell-to-cell movement in *C. quinoa*, barley and *N. benthamiana* (Okinaka et al., 2001; Rao, 1997; Rao and Grantham, 1995, 1996; Schmitz and Rao, 1996), although limited movement in the absence of CP has exceptionally been reported in *C. hybridum* (Flasinski et al., 1995). In this context, it is remarkable that the 3a proteins of both viruses were highly active in transporting the ToMV genome in *N. benthamiana* (Figs. 2H, 2I, and 4A). This indicates that BMV and CCMV 3a proteins have the basic activity required for a movement protein to transport a genomic RNA from cell to cell.

On the other hand, in transporting the PVX genome, these 3a proteins on their own exhibited only low-level activity, whereas coexpression of the cognate CP resulted in four to sixfold enhancement of transcomplementation (Fig. 4B). Considering the size of the recombinant PVX genome (>6.0 kb), which is longer than the RNA molecules that bromovirus CPs can encapsidate (e.g. Choi and Rao, 2000), it seems unlikely that expression of the CPs resulted in their assembly into normal virions. In other words, if the CPs interacted with the PVX genome to form a movement-competent ribonucleoprotein complex, it would be different in structure from the icosahedral virus particle. Alternatively, the CPs might not participate in the assembly into the putative movement-competent complex of PVX and may simply enhance the activity of the 3a proteins through direct or indirect interactions or inhibit a host resistance response (see later for further discussion).

In contrast to BMV 3a and CCMV 3a proteins, CMV 3a alone did not support the transport of the ToMV or PVX genomes (Fig. 4). This finding itself is consistent with the previous observations: (1) CMV CP-deficient mutants did not spread from cell to cell in cowpea or tobacco (Canto et al., 1997; Suzuki et al., 1991); (2) transgenically expressed CMV 3a could not rescue the movement-defective phenotype of TMV or ToMV mutants in tobacco (Cooper et al., 1996; Kaplan et al., 1995); and (3) a coatless BMV chimera expressing CMV 3a instead of BMV 3a did not detectably move from cell to cell in *C. quinoa* (Nagano et al., 1999). On the other hand, when both CMV 3a and CP were expressed, movement-defective ToMV and PVX moved from cell to cell in *N. benthamiana* (this work). Furthermore, chimeric BMV in which the 3a and CP genes were exactly replaced with the corresponding CMV genes was competent in cell-to-cell movement in *C. quinoa* (Nagano et al., 1999). Interestingly, this CP requirement was abolished by the deletion of the C-terminal region of CMV 3a. The resulting 3a mutant 3aΔC33 was highly active in transporting ToMV (this work) as well as CMV and BMV (Nagano et al., 2001). Thus, the CMV 3a protein intrinsically possesses the activity required to act as MP. Coexpression of wt 3a and 3aΔC33 resulted in impaired cell-to-cell movement of these viruses (Nagano et al., 2001; this work). Therefore, it seems likely that 3aΔC33 potentiated the cell-to-cell movement of ToMV, CMV, and BMV by the same or a similar mechanism.

To explain how CMV CP enables wt 3a to transport these viral genomes, Nagano et al. (2001) postulated that some activity of CMV 3a may be masked by its C-terminal region in the absence of CP, while CP can cancel such masking. However, this idea alone does not seem to explain the large difference between the activity of 3aΔC33 and that of wt 3a plus CP in transporting the PVX mutant. Another possibility is that 3aΔC33 and 3a plus CP act differently in transporting the PVX genome. This possibility can be dis-
discussed in relation to the function of the CPs in the cell-to-cell movement of cucumo- and bromoviruses. That is, CP-dependent and CP-independent movement might differ mechanistically in some respects. The possibility that a given virus can move from cell to cell in multiple ways is intriguing, insofar as it potentially broadens the host ranges given virus can move from cell to cell in multiple ways is mechanistically in some respects. The possibility that a dependent and CP-independent movement might differ cell movement of cucumo- and bromoviruses. That is, CP-
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In the transcomplementation of ToMV and PVX by cucumo- and bromovirus wt 3a proteins, coexpression of CP resulted in nonselective transport of the genomes, at least phenomenologically. In this regard, the function of the CPs might be involved in the selection of, or accessibility to, the target that is transported by the corresponding 3a proteins. For example, cucumo- and bromovirus CPs might facilitate the 3a MPs reaching the site of PVX replication, whereas bromovirus 3a and CMV 3aΔC33, but not CMV 3a, may have access to the site of ToMV replication without the help of an additional factor. This possibility would be compromised by data implying that movement and replication processes have some affinity for, or interact directly or indirectly with, each other (Gal-On et al., 1994; Hirashima and Watanabe, 2001; Tamai and Meshi, 2001b; Traynor et al., 1991).

It is also possible that coexpression of CP and 3a might induce nonselective movement of mRNAs. Such an event would occur infrequently, if at all, because we did not detect any diffusion of ER-localized GFP from the bombarded cells when erG3GFP was coexpressed with 3a and CP (Fig. 3E and Table 1). However, a self-amplifiable RNA, such as a virus (PVX in this work), would be detected, if even only minute amounts of the RNA were transported or diffused in a passive fashion.

Previously, Fedorkin et al. (2001) and Morozov et al. (1997) showed by bombardment-mediated transcomplementation experiments that MPs of some tobamoviruses, including TMV and ToMV, potentiated cell-to-cell movement of p25- or CP-deficient PVX mutants in small leaves of N. benthamiana, p24-defective PVX (CP strain), however, did not move in transgenic tobacco plants expressing TMV MP (Ares et al., 1998). We confirmed that TMV MP transcomplements the defective movement of the PVX mutant in the mature leaves of N. benthamiana. However, since the efficiency was low (Fig. 4B), this level of transcomplementation might be classified as subliminal infection when these proteins are virally or transgenically expressed. Therefore, our observation does not necessarily conflict with that of Ares et al. (1998).

The bombardment-mediated transcomplementation assay is a powerful tool to reveal the potential activity of movement proteins, especially when cell-to-cell movement is the result of the combinatorial actions of multiple proteins. As exemplified by the ToMV hybrids, a modification of the genomic sequence often impairs replicability or other functions. The cobombardment method has the advantage of enabling us to compare the transport activities of different proteins, using a target genome with the same organization. However, there are still limitations in controlling the amounts of proteins produced in each bombarded cell, as well as the time of their expression relative to the stages of viral replication. This is not necessarily overcome by using recombinant viruses and, therefore, other novel approaches are required.

Materials and methods

Construction of plasmids

An infectious plasmid for GFP-tagged movement-defective ToMV, piL.erG3(SF3), and plasmids for transient expression of G3GFP (pBIG3), erG3GFP (pBlerG3), ToMV MP (p35LM), TMV MP (p35OM), and CMV 3a (p35YM) were described previously (Tamai and Meshi, 2001b). An infectious clone of movement-competent ToMV, piL.erG3, was constructed by replacing the G3GFP gene of piL.G3 with the erG3GFP gene as previously described for piL.erG3(SF3) (Tamai and Meshi, 2001b). The infectious PVX clones, piX.erG3 and piX.erG3(TdCd), and plasmids for expression of PVX p25 (p35X25), p8 (p35X8), and CP (p35XCP) were also previously described (Tamai and Meshi, 2001a). The plasmid p35X12(8m), differing from p35X12 (Tamai and Meshi, 2001b) at one base in the initiation codon of p8 (AUG to ACG), was used to express p12. p35X12(8m) has the same characters as p35X12 in all the aspects we examined. The ORFs for CMV CP and 3a-C33 were amplified from pT7CKY3 and pB3C3a247T (Nagano et al., 1997), respectively, by polymerase chain reaction (PCR) with primer sets. 5'-AGTGGATCCATGG-AACAAATCTGAATCA-3' plus 5'-TCTGAGCTAGACT-GGGACACCTC-3' and 5'-GGGATCCATATTG-CCGAAGCTTGATT-3', respectively (BamHI and SacI sites are underlined). Each PCR fragment was cut with BamHI and SacI and then inserted between the corresponding sites of pBI221 (Clontech) to create p35YCP and p35YMd. A DNA fragment encoding BMV 3a protein was inserted from pT7CKY3 and pB3C3a247T (Nagano et al., 1997), respectively, by polymerase chain reaction (PCR) with primer sets. 5'-TGAACCCGGCTACCTATAAACCC-5' (SacI sites are underlined).

The EcoRV–Ndel fragment of pCC3TP4 (Allison et al., 1989) encoding the CCMV 3a protein was inserted between the EcoRV and Ndel sites of pBI221 to yield p35B3a. The EcoRV–Ndel fragment of pCC3TP4 (Allison et al., 1989) encoding the CCMV 3a protein was inserted between the EcoRV and Ndel sites of pBI221 to yield p35B3a.
TGA-3’, respectively (XhoI and SmaI sites in the primer are underlined). These fragments were cut with XhoI and SmaI and inserted between the XhoI and SmaI sites of p35LM in place of the ToMV MP ORF to yield p35BCP and p35CCCP. PCR-amplified regions and ligation junctions were confirmed by sequencing.

pTLW3 is a cDNA clone containing full-length ToMV cDNA, which was derived from pLFW3 (Meshi et al., 1986), with a T7 promoter for in vitro transcription. The initiation codon of the CP ORF was changed from ATG to AGA by a PCR-mediated method to create pTL∆C1. As a result, pTL∆C1 had a BglII site. To make pTLMR, two PCR fragments were prepared: one from nucleotides 4395 to 4922 (the KpnI site to the termination codon of the replicase gene) with a PCR primer having a SacI–EagI tag and a point mutation in the initiation codon of the MP gene (from ATG to ACG, Fig. 5A); and the other from nucleotides 4906 to 5462 (the initiation codon of the MP gene to the NcoI site) with a primer having an EagI tag. These two fragments were ligated at the EagI site and replaced for the corresponding KpnI–NcoI fragment of pTLW3. The BamHI fragment of p35YM carrying the CMV 3a ORF was inserted between the EagI and NcoI sites of pTLMR after the fragments were blunt-ended to yield pTL/YM, where the NcoI site was regenerated. pTL/YM was digested with AvrII (located at the 42nd codon of the CMV 3a ORF), filled in, and self-ligated to yield pTL/YMf. The AvrII–filled-in NcoI fragment of pTL/YM was exchanged with the AvrII–filled-in SacI fragment of p35YMd to make pTL/YMd. pTL-SB(SF3) was a subclone containing the sequence from the KpnI site to the termination codon of the MP gene derived from pLQSF3 (Meshi et al., 1987) plus a synthetic sequence TAAATCGAGCTCGGTAACC (underlined are the termination codon of the MP gene and the SacI site) between the KpnI and blunt SacI sites of pBluescriptKS(+). The BamHI–SacI fragment (just encoding 3aΔC33) of p35YMd was blunt-ended and inserted into the filled-in BstEII site of pTL−SB(SF3) to create pTL−YMd(SF3), where the BstEII site was regenerated. The insert (KpnI-BstEII fragment) in pTL−YMd(SF3) was exchanged for the corresponding fragment of pTLW3 to create pTL−YMd(SF3). The NcoI−BstEII fragment of pTL−YMd(SF3) was inserted into the corresponding sites of pTL/YM and pTL/YMf to create pTL/YM(YMd and pTL/YMf(YMd. pBI-NDr was made by inserting a sequence encoding the nuclear localization signal derived from SV40 large T antigen (Tamai and Meshi, 2001a) fused to the N-terminus of DsRED1-1 (Clontech) between the BamHI and filled-in SacI sites of pBII221.

Microprojectile bombardment

Bombardment was performed with PDS1000 (Bio-Rad Laboratories) as described previously (Tamai and Meshi, 2001a,b). Briefly, mature leaves of N. benthamiana were bombarded with rupture disks of 1350 lb/in.2 for piL.erG3(SF3) and 1100 lb/in.2 for piX.erG3(TdCd) and then incubated in the dark for 2 and 3 days, respectively. Diffusion of cytosolic G3GFP was assayed as described previously (Tamai and Meshi, 2001b). Fluorescence was observed under an Axioskop epifluorescence microscope (Carl Zeiss) equipped with a color-chilled 3CCD camera, C5810 (Hamamatsu Photonics). Filter sets 41014 (Chroma Technologies) and No. 15 (Carl Zeiss) were used to detect GFP and DsRed signals, respectively.

Plant and protoplast inoculation

pTL series plasmids were linearized with MluI and subjected to in vitro transcription with T7 RNA polymerase. N. tabacum cv. Xanthi nc and a transgenic Xanthi nc expressing TMV MP (line 2005, Deom et al., 1991) were used for local lesion assays. Plants were grown under greenhouse conditions (26°C on average, 16 h light). In some experiments, inoculated plants were kept at 32°C for 24 h from 1 day after inoculation, to enlarge local lesions. Protoplasts were prepared from suspension-cultured cells of tobacco (BY2), inoculated with in vitro transcripts by electroporation with Gene Pulser (Bio-Rad), and cultured essentially as described previously (Watanabe et al., 1987), except that actinomycin D was not included in the medium. RNAs were extracted with TRIzol (Invitrogen) according to the manufacturer’s instructions. The extracted RNAs were glyoxylated, fractionated on a 1.0% agarose gel, and blotted onto Hybond N membrane (Amersham-Pharmacia). Hybridization was performed with a riboprobe complementary to the 3’ noncoding region of ToMV RNA as described by Ishikawa et al. (1991). The signals on the membrane were visualized with a digital imaging analyzer (Typhoon 8600, Amersham Biosciences). Total proteins from infected protoplasts were separated by SDS–12% PAGE and transferred onto a PVDF membrane (Millipore). CMV 3a and 3aΔC33 were immunodetected with affinity-purified rabbit polyclonal antibody raised against full-sized CMV 3a as primary antibody and horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Pierce) as secondary antibody, followed by ECL (Amersham Biosciences).

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