The N-terminal Region of the *Plantago asiatica* mosaic virus Coat Protein Is Required for Cell-to-Cell Movement but Is Dispensable for Virion Assembly

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Potexvirus cell-to-cell movement requires coat protein (CP) and movement proteins. In this study, mutations in two conserved in-frame AUG codons in the 5' region of the CP open reading frame of *Plantago asiatica* mosaic virus (PIAMV) were introduced, and virus accumulation of these mutants was analyzed in inoculated and upper noninoculated leaves. When CP was translated only from the second AUG codon, virus accumulation in inoculated leaves was lower than that of wild-type PIAMV, and the viral spread was impaired. *Trans*-complementation analysis showed that the leucine residue at the third position (Leu-3) of CP is important for cell-to-cell movement of PIAMV. The 14-amino-acid N-terminal region of CP was dispensable for virion formation. Immunoprecipitation assays conducted with an anti-TGBp1 antibody indicated that PIAMV CP interacts with TGBp1 *in vivo* and that this interaction is not affected by alanine substitution at Leu-3. These results support the concept that the N-terminal region of potexvirus CP can be separated into two distinct functional domains.

Successful systemic infection by plant viruses requires viral replication in the initially infected cells, spread of the virus to adjacent cells (i.e., cell-to-cell movement), and viral spread through the plant vascular system (i.e., long-distance movement). The cell-to-cell movement of plant viruses in host plants proceeds through plasmodesmata (PD), which are plasma membrane-lined intercellular connections, and this requires virus-encoded movement proteins (MP) and host proteins.

Potexvirus encode three MP, which are referred to as triple gene block protein 1 (TGBp1), TGBp2, and TGBp3. TGBp1, which contains an NTPase/helicase domain, increases the size exclusion limit (SEL) of PD and moves to adjacent cells through PD (Verchot-Lubicz 2005; Verchot-Lubicz et al. 2007). It has been reported that TGBp1 is a suppressor of RNA silencing and that suppression of RNA silencing is required for potexvirus cell-to-cell movement (Bayne et al. 2005; Voinnet et al. 2000). TGBp2 and TGBp3 act as accessory proteins that promote cell-to-cell movement (Samuels et al. 2007). Amino acid sequence analysis demonstrates that TGBp2 and TGBp3 have two and

one transmembrane domains, respectively, that are associated with the endoplasmic reticulum (Ju et al. 2005; Krishnamurthy et al. 2003).

In addition to TGBps, coat protein (CP) is also required for potexvirus cell-to-cell movement (Morozov and Solovyev 2003; Verchot-Lubicz et al. 2007). It was reported that CP of *Potato virus X* (PVX) is targeted to PD, but it has neither the ability to increase SEL nor the capacity to move from cell to cell by itself (Oparka et al. 1996). However, the role of CP in the cell-to-cell movement remains largely unknown. Furthermore, the actual form in which viral RNA is transported from cell to cell also remains unclear, and several models have been proposed to explain the requirement of CP; these include movement via virion or nonvirion ribonucleoprotein (RNP) complexes containing viral RNA, TGBp1, and CP or via filamentous virions (Santa Cruz et al. 1998; Karpova et al. 2006; Lough et al. 1998, 2000; Oparka et al. 1996).

The central region of potexvirus CP is highly conserved but the N- and C-terminal regions are quite variable and show low levels of similarity among potexvirus. According to a model of the tertiary structure of PVX CP molecules, the N-terminal region of PVX CP is located at the surface of filamentous virions. Several deletion mutants of PVX CP have shown that this region is dispensable for cell-to-cell movement and virion assembly (Chapman et al. 1992; Lico et al. 2006).

Plantago asiatica mosaic virus (PIAMV) was first isolated in the Russian Far East (PIAMV-Ru). PIAMV is classified as a species in the genus *Potexvirus* of the family *Flexiviridae* (Solovyev et al. 1994). Recently, a new strain of PIAMV, PIAMV-Li, was isolated from a lily in Japan, and its complete nucleotide sequence was determined (Komatsu et al. 2008). Another PIAMV strain, PIAMV-Na, was isolated from a nandina in California (Hughes et al. 2005). PIAMV has two in-frame AUG codons in the 5' region of the CP open reading frame (ORF), and this feature is conserved in all PIAMV isolates reported to date. A second in-frame AUG codon (AUG2) of CP ORF is located 42 nucleotides downstream of the first AUG codon (AUG1) (Fig. 1A). Although Solovyev and co-workers indicated that some potexvirus have two in-frame AUG codons in the 5' region of CP ORF (Solovyev et al. 1994), no experimental data are available on the significance of this feature in virus replication and movement. In this study, we analyzed the significance of two AUG codons that are located at (AUG1) or near (AUG2) the 5' terminus of the CP ORF in cell-to-cell movement. We

identified a specific residue of CP involved in virus cell-to-cell movement by *trans*-complementation analysis.

RESULTS

CP translated from the first AUG codon is required for systemic infection of PIAMV.

We previously cloned PIAMV-Li1 cDNA into a binary vector to construct pLi1, enabling efficient infection using agroinfiltration (Ozeki et al. 2006). To obtain insight into the role of the two AUG codons of CP, we constructed three PIAMV mutants in which AUG1, AUG2, or both AUG1 and AUG2 of pLi1 were mutated (Fig. 1A). In pLi1-m1, AUG2 was designed to be the translation initiation codon of CP and produce an N-terminal 14-amino-acid truncated CP product. In pLi1-m2, the full-length CP was designed to be translated from AUG1. In pLi1-m12, both AUG1 and AUG2 were modified to block expression of any CP product. These three PIAMV mutants as well as pLi1 were transformed into *Agrobacterium tumefaciens*. For simplicity, we refer to *Agrobacterium* strains containing a binary vector plasmid by the name of the plasmid they carried.

We previously reported that pLi1 induces systemic necrosis in *Nicotiana benthamiana* (Ozeki et al. 2006). To confirm pathogenicity of these three PIAMV CP mutants, each mutant was inoculated into *N. benthamiana*, and development of local

and systemic symptoms was monitored. pLi1-m2-inoculated plants developed necrosis in inoculated leaves at 5 days postinfiltration (dpi) and systemic necrosis at 13 dpi (Fig. 2A). Conversely, pLi1-m1- and pLi1-m12-inoculated plants developed little to no necrosis, although necrosis was occasionally observed in pLi1-m1-inoculated leaves. To confirm CP expression in PIAMV mutants, total proteins extracted from inoculated leaves were subjected to Western blot analysis with an anti-CP antibody. CP expressed in pLi1-m1 migrated slightly faster than that expressed in pLi1, indicating that CP was translated from AUG2. CP expressed in pLi1-m2 migrated in a manner similar to that expressed in pLi1 (Fig. 2B).

Next, we analyzed viral RNA accumulation in inoculated leaves and in upper noninoculated leaves. Northern blot analysis with a CP-specific probe showed that viral RNA of all PIAMV mutants, including those of pLi1-m1 and pLi1-m12, accumulated in inoculated leaves; however, the level of viral RNA accumulation varied depending on the viruses. pLi1-m2 accumulation in inoculated leaves was approximately equal to that of pLi1. The accumulations of both pLi1-m1 and pLi1-m12 in inoculated leaves were observed but were slightly less than that of pLi1 (Fig. 2C). We should note that it is not clear whether the variation in the accumulation of mutant viruses is due to the RNA replication, the cell-to-cell movement, or both, because we inoculated viruses by agroinfiltration, by which

Fig. 1. Expression vectors used in this study. **A**, Schematic representation of pLi1 (wild-type) and mutant virus vectors. The 5' terminal nucleotide sequences of open reading frame (ORF)5 (coat protein [CP]) of mutant viruses are represented. The position of AUG1 and AUG2 are boxed. Underlines show nucleotide substitutions. **B**, Schematic representation of pLi1GFPΔCP used in *trans*-complementation analysis. The green fluorescent protein (*gfp*) gene is inserted downstream of an ORF4 termination codon in-frame to a remaining CP ORF. **C**, Schematic representation of transient CP expression vectors. The N-terminal amino acid residues are represented. The gray shadings show AUG1 and AUG2. Underlines indicate alanine-substituted residues.

viruses can accumulate well without cell-to-cell movement. In the upper noninoculated leaves, pLi1-m2 accumulation was detected, whereas pLi1-m1 and pLi1-m12 accumulations were not observed (Fig. 2D).

CP translated from the first AUG codon is required for viral spread in inoculated leaves.

We hypothesized that deletion of the N-terminal region of CP might result in either a decrease in virus replication or deficiency of virus movement. We first analyzed the effect of mutations in the AUG codons of CP ORF on viral replication in initially infected cells. To discriminate the effect of viral replication from that of cell-to-cell movement after viral accumulation, we introduced a deletion mutation in the TGBp2 and TGBp3 ORF of pLi1, pLi1-m1, and pLi1-m2 to disrupt the function of MP in cell-to-cell movement. Northern blot analysis of total RNA extracted from inoculated leaves showed that the accumulations of pLi1ΔTGB23-m1 and -m2 were comparable with that of pLi1ΔTGB23 (Fig. 3A), demonstrating that mutations in AUG1 or AUG2 do not significantly affect viral replication in initially infected cells.

Next, we investigated whether the N-terminal deletion of CP could result in a deficiency of viral spread. To analyze viral spread of each mutant in inoculated leaves, we inoculated *N. benthamiana* leaves with pLi1, pLi1-m1, pLi1-m2, or pLi1ΔTGB23 using a needle instead of agroinfiltration. pLi1ΔTGB23, which cannot move beyond the initially infected cells, was used as a negative control. Dark-colored spots in pLi1- or pLi1-m2-inoculated leaves were much larger than those detected in pLi1ΔTGB23-inoculated leaves, as detected by an anti-CP antibody in hammer blot analysis. In contrast, spots detected in pLi1-m1-inoculated leaves were much smaller than

those in pLi1-inoculated leaves (Fig. 3B). To compare the spread of mutant viruses, we analyzed the size of colored spots detected by an anti-CP antibody using Image J software. The size of infected foci in pLi1-m2-inoculated leaves was nearly the same as that in pLi1-inoculated leaves. In contrast, the size of infected foci in pLi1-m1-inoculated leaves was significantly lower and nearly the same as that in the negative control (Fig. 3C). These results suggest that the N-terminal region of CP is required for spread of PIAMV.

The N-terminal region of PIAMV CP is required for cell-to-cell movement.

Because the hammer blot analysis in Figure 3B does not present a high-resolution image adequate for discrimination of the cell-to-cell movement, we next performed *trans*-complementation analysis using agroinfiltration to investigate the role of the N-terminal region of PIAMV CP in cell-to-cell movement. A PIAMV derivative named pLi1GFPΔCP, which expresses green fluorescent protein (GFP) instead of CP, was constructed (Fig. 1B). When pLi1GFPΔCP was introduced alone into *N. benthamiana*, GFP fluorescence was observed only in single cells and the fluorescence did not move to adjacent cells. Several PIAMV CP expression vectors (pCPfull, pCP14Δ, and pCP5Δ) which express CP or its mutants from the 35S promoter were constructed to analyze whether they could complement the cell-to-cell movement of pLi1GFPΔCP. pCPfull was expected to express a full-length CP and pCP14Δ was expected to express a 14-amino-acid truncated CP, which corresponds to a CP expressed from pLi1-m1. pCP5Δ was expected to express a 5-amino-acid truncated CP (Fig. 1C). Indeed, when CP derivatives were expressed in *N. benthamiana* by agroinfiltration, Western blot analysis showed that the molecular weights of CP detected

Fig. 2. Pathogenicity and accumulation of *Plantago asiatica* mosaic virus (PIAMV) coat protein (CP) mutants. **A**, Systemic symptoms of *Nicotiana benthamiana* inoculated with pLi1, pLi1-m1, pLi1-m2, and pLi1-m12. *N. benthamiana* plants were inoculated by agroinfiltration and photographs were taken at 13 days postinfiltration (dpi). **B**, CP accumulation of PIAMV CP mutants. Total protein from infiltrated leaves at 3 dpi was separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blot analysis using an anti-CP antibody. **C** and **D**, Viral RNA accumulation of PIAMV CP mutants. Total RNA was prepared from **C**, infiltrated leaves at 3 dpi or **D**, upper noninfiltrated leaves at 7 dpi and detected by Northern blot analysis with a CP-specific RNA probe. Equal loading of each lane was confirmed by ethidium bromide staining of rRNA.

in pCP5Δ or pCP14Δ were slightly smaller than that of pCPfull, indicating that pCP5Δ and pCP14Δ expressed the N-terminal truncated CP (Fig. 4A).

We performed *trans*-complementation analysis by co-infiltration with pLi1GFPΔCP and pCPfull, pCP14Δ, pCP5Δ, or an

Fig. 3. Viral RNA replication and the spread of *Plantago asiatica* mosaic virus (PIAMV) coat protein (CP) mutants. **A**, Viral RNA accumulation of movement-deficient CP mutants. *Nicotiana benthamiana* plants were inoculated by pLi1ΔTGB23, pLi1ΔTGB23-m1, and pLi1ΔTGB23-m2 which contain a deletion mutation in the triple gene block protein (TGBp)2 and TGBp3 open reading frame to disrupt the cell-to-cell movement. Total RNA was prepared from the infiltrated leaves of each mutant virus at 5 days postinfiltration (dpi), and Northern blot analysis was performed using a CP-specific RNA probe. The lower panel shows rRNA as a loading control. **B**, Spread of PIAMV CP mutant in inoculated leaves. *N. benthamiana* leaves were inoculated with pLi1, pLi1-m1, pLi1-m2, and pLi1ΔTGB23 using a needle and the viral spread in the inoculated leaves was detected immunologically by hammer-blot analysis at 4 dpi using an anti-CP antibody. **C**, Size of colored spots detected by hammer-blot analysis. Images of colored spots in B were analyzed by Image J software. Error bars represent the standard deviations.

empty vector. Using a handheld UV lamp, we observed GFP fluorescence when pLi1GFPΔCP was co-infiltrated with pCPfull but did not detect fluorescence when it was co-infiltrated with pCP14Δ, pCP5Δ, or an empty vector (Fig. 4B). When these leaves were monitored by confocal laser scanning microscopy, approximately 90% of GFP foci in pLi1GFPΔCP- and pCPfull-infiltrated leaves consisted of multiple cells (usually more than nine cells). In contrast, approximately 80% of GFP foci consisted of single cells in leaves co-infiltrated with pLi1GFPΔCP and an empty vector. In leaves co-infiltrated with pLi1GFPΔCP and pCP14Δ or pCP5Δ, most of the GFP foci (80 and 82%, respectively) consisted of single cells (Fig. 4B; Table 1). These data suggest that pCP14Δ and pCP5Δ could not complement the cell-to-cell movement of pLi1GFPΔCP.

Because the deletion mutations in both pCP14Δ and pCP5Δ might have an impact on overall protein folding, we next performed alanine substitution mutagenesis on four amino acid residues that were absent in pCP5Δ and constructed four CP expression vectors (pCPL3A, pCPN4A, pCPQ5A, and pCPP7A) (Fig. 1C). CP expression from these vectors was confirmed by Western blot analysis (Fig. 4A). We performed *trans*-complementation analysis by co-infiltration with pLi1GFPΔCP and pCPL3A, pCPN4A, pCPQ5A, pCPP7A, or an empty vector. When pLi1GFPΔCP was co-infiltrated with pCPN4A, pCPQ5A, or pCPP7A, GFP fluorescence in the infiltrated leaves was detected with a handheld UV lamp (Fig. 4C). In those leaves, 69 to 80% of GFP foci consisted of more than nine cells (Table 1). In contrast, GFP fluorescence was not detected with a handheld UV lamp in pLi1GFPΔCP- and pCPL3A-infiltrated leaves, where 86% of GFP foci consisted of a single cell. This result demonstrates that the N-terminal region of PIAMV CP, especially the leucine residue at the third position (Leu-3), is important for PIAMV cell-to-cell movement.

In vivo interaction between CP and TGBp1 is not affected by alanine substitution at Leu-3.

The above results demonstrate that Leu-3 of PIAMV CP is an important amino acid residue involved in cell-to-cell movement. Several studies suggest a specific interaction between potexvirus CP and its cognate TGBp1 (Zayakina et al. 2007). Therefore, we investigated the interaction of PIAMV CP with TGBp1 by immunoprecipitation-Western blot assay to determine whether the alanine substitution at Leu-3 would affect the interaction. We constructed pLi1-L3A, a PIAMV mutant that contains an alanine substitution at Leu-3 (Fig. 1A), and inoculated pLi1-L3A or pLi1ΔTGB23 into *N. benthamiana*. TGBp1 was then immunoprecipitated with an anti-TGBp1 antibody, and the immunoprecipitate was analyzed by Western blotting with an anti-CP antibody. More CP was detected in the anti-TGBp1 antibody precipitate in pLi1ΔTGB23-inoculated leaves than that detected in preimmune antibody, indicating that PIAMV TGBp1 interacts with CP in vivo (Fig. 5A). Using pLi1-L3A-inoculated leaves, the amount of CP detected in the anti-TGBp1 antibody precipitate was also more than that in preimmune antibody (Fig. 5B). Therefore, these data demonstrate that PIAMV CP interacts with TGBp1 in vivo and that this interaction is not affected by an alanine substitution at Leu-3 of PIAMV CP.

The N-terminal region of CP is dispensable for virion assembly.

We next investigated whether the N-terminal region of PIAMV CP is required for virion assembly. *N. benthamiana* was co-infiltrated with pLi1GFPΔCP and pCPfull or pCP14Δ, followed by virus purification. pLi1 was used as a positive control. Filamentous viral particles were detected by transmission electron microscopy in purified suspensions from leaves in which pLi1GFPΔCP was co-infiltrated with pCPfull or pCP14Δ (Fig.

6). These results demonstrate that at least the N-terminal 14-amino-acid region of PIAMV CP is dispensable for virion assembly.

DISCUSSION

Potexvirus CP is a bifunctional protein that is required for both virion formation and cell-to-cell movement. However, the relationship between these two functions remains unclear. In

previous studies, a PVX CP mutant, lacking the C-terminal 18 amino acid residues of CP, could form virus particles in *N. tabacum* protoplasts but could not move cell to cell in *N. benthamiana* (Fedorkin et al. 2000, 2001). Deletion of the C-terminal 31 amino acid residues of *White clover mosaic virus* (WCIMV) CP did not affect virus particle formation (Forster et al. 1992) but the deletion of the C-terminal 5 amino acid residues resulted in cell-to-cell movement deficiency (Lough et al. 2000). These results suggest that the C-terminal region of potexvirus CP may

Fig. 4. Trans-complementation of pLi1GFPΔCP with various coat proteins (CP). **A**, CP accumulation from CP expression vectors. *Nicotiana benthamiana* leaves were infiltrated by *Agrobacterium* cultures containing each binary vector. Total protein from infiltrated leaves at 3 days postinfiltration (dpi) was separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blot analysis using an anti-CP antibody. **B**, Trans-complementation analysis using CP deletion mutants. *Agrobacterium* cultures transformed with pLi1GFPΔCP and cultures transformed with pCambia1301 vector, pCPfull, pCP5Δ, or pCP14Δ mixed at the ratio of 1:5000. These mixtures were infiltrated into *N. benthamiana* leaves, and green fluorescent protein (GFP) fluorescence was assessed at 6 dpi with a handheld UV lamp and confocal laser scanning microscopy (CLSM). Photographs in the upper panels were taken under a handheld UV lamp, and images in the lower panels were taken by CLSM. A bar corresponds to 100 μm. **C**, Trans-complementation analysis using CP point mutants. *Agrobacterium* cultures were transformed with pLi1GFPΔCP and cultures transformed with pCambia1301 vector, pCPL3A, pCPN4A, pCPQ5A, or pCPP7A were mixed at the ratio of 1:5000. Conditions of agroinfiltration, detection, and figure layout are the same as in B.

be required for cell-to-cell movement but is dispensable for virion formation. In contrast, deletion of at least 30 amino acids of the N-terminal region of PVX CP did not affect cell-to-cell movement significantly (Chapman et al. 1992; Lico et al. 2006). Studies on the N-terminal regions of other potexvirus CP have not been conducted, and the importance and generality of these findings remain unclear. In this report, we evaluated the pathogenicity of PIAMV in plants by introducing a point mutation at AUG codons of CP and analyzed the importance of the N-terminal region of CP during cell-to-cell movement and virion assembly. Furthermore, we investigated whether the interaction between CP and TGBp1 is interrupted by an alanine substitution at Leu-3 of CP.

Although several potexvirus have two in-frame AUG codons at or near the 5'-terminus of CP ORF (Abouhaidar and Lai 1989; Jelkmann et al. 1990; Yamaji et al. 2001), there are no experimental data concerning whether this feature could be involved in virus pathogenicity or cell-to-cell movement. To analyze the role of two in-frame AUG codons of CP, we constructed pLi1-m1 and pLi1-m2, which contain mutations in AUG1 and AUG2, respectively. pLi1-m1 did not systemically infect *N. benthamiana*, though viral RNA accumulation was

clearly detected in inoculated leaves (Fig. 2). This result suggests that CP translated from AUG2 does not act as an alternative to CP translated from AUG1. This speculation agrees with a previous in vitro translation assay, which suggested that AUG1 was the major initiator for CP translation of PIAMV-Ru (Solovyev et al. 1994).

pLi1-m1 could accumulate in inoculated leaves but the levels of its accumulation were lower than that of pLi1 (Fig. 2C). In contrast, RNA accumulation of pLi1ΔTGB23-m1 was similar to that of pLi1ΔTGB23-m2 (Fig. 3A). Moreover, hammer blot analysis demonstrated that pLi1-m1 could not spread in inoculated leaves (Fig. 3B and C). Therefore, it is likely that the mutation in AUG1 did not reduce virus replication and that the observed differences between pLi1 and pLi1-m1 accumulation in infiltrated leaves were due to the cell-to-cell movement deficiency of pLi1-m1.

Although the nature of potexvirus cell-to-cell movement remains unknown, the most recent model is that viruses move cell to cell because RNP complex, which is composed of viral RNA, CP, and TGBp1, would interact with host proteins (Lucas 2006; Verchot-Lubicz et al. 2007). According to this model, RNP complex might be delivered to PD with support of TGBp2, TGBp3, and host factors. Previous studies revealed that TGBp1 is a multifunctional protein which increases PD SEL, has RNA helicase activity, promotes translation of virion RNA, and suppresses RNA silencing (Verchot-Lubicz 2005). On the other hand, we have limited knowledge about the role of CP, except for virion formation and cell-to-cell movement. In this study, we showed that pCPL3A could not complement the cell-to-cell movement of pLi1GFPΔCP (Fig. 4C). We speculate that pCPL3A could form virus particles, because the N-terminal 14 residues of CP are dispensable for virion formation (Fig. 6). This result suggests that a specific N-terminal residue of potexvirus CP is required for viral cell-to-cell movement and that CP might interact with viral or host proteins during the cell-to-cell movement.

Although high-resolution X-ray diffraction data for potexvirus virions are not available, it was proposed that the N-terminal region of PVX CP is exposed at the virion surface and that the

Table 1. Trans-complementation of movement-deficient pLi1GFPΔCP in *Nicotiana benthamiana* leaves by coat protein (CP) vectors

Plasmids used ^b	GFP fluorescence foci (%) ^a		
	1 cell	2–8 cells	9+ cells
pLi1GFPΔCP + Vector	158 (84)	31 (16)	0 (0)
pLi1GFPΔCP + pCPfull	8 (4)	12 (5)	201 (91)
pLi1GFPΔCP + pCP5Δ	169 (82)	36 (18)	0 (0)
pLi1GFPΔCP + pCP14Δ	175 (80)	43 (20)	0 (0)
pLi1GFPΔCP + pCPL3A	181 (86)	30 (14)	0 (0)
pLi1GFPΔCP + pCPN4A	49 (15)	40 (12)	242 (73)
pLi1GFPΔCP + pCPQ5A	64 (18)	45 (13)	250 (69)
pLi1GFPΔCP + pCPP7A	76 (15)	25 (5)	394 (80)

^a Number of green fluorescent protein (GFP) foci each having one, two to eight, or more than nine cells were counted at 6 days postinfiltration. Frequency (%) is shown in parentheses.

^b Plasmids used in trans-complementation tests. pLi1GFPΔCP could replicate in inoculated cells and express GFP but it lacked *Plantago asiatica* mosaic virus (PIAMV) CP. pCP series plasmids expressed wild-type and mutant CP under the control of the 35S promoter.

Fig. 5. Immunoprecipitation and immunoblotting analysis. *Nicotiana benthamiana* leaves were inoculated with pLi1ΔTGB23 or pLi1-L3A, and protein extracts were prepared from inoculated leaves using RIPA buffer (5 mM Tris-HCl, pH 8.0; 15 mM NaCl; 0.1% Nonidet P-40; 0.05% sodium deoxycholate; 0.01% sodium dodecyl sulfate; and 0.1 mM phenylmethanesulfonyl fluoride). Protein immunoprecipitation was carried out using an anti-TGBp1 antibody or pre-immune antibody and protein G agarose beads. Proteins that immunoprecipitated with the beads were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blot analysis with an anti-coat protein (CP) antibody (bottom panel). The input protein was analyzed by immunoblot analysis with an anti-CP antibody (top panel).

Fig. 6. Electron microscopy analysis of pLi1GFPΔCP complemented by coat protein (CP) or a CP mutant. *Nicotiana benthamiana* plants were inoculated with pLi1, or co-infiltrated with pLi1GFPΔCP and pCPfull or pCP14Δ. pLi1 was used as a positive control. *Plantago asiatica* mosaic virus virions in these inoculated leaves were purified at 3 days postinfiltration and samples were negatively stained, followed by transmission electron microscopy observation. A bar corresponds to 100 nm.

C-terminal region is buried in the virus particle (Baratova et al. 1992a,b). Potexvirus CP could interact with other virus or host proteins, including CP, TGBp1, and viral RNA. For example, RNA from the PVX virion is completely nontranslatable in vitro; however, selective binding of TGBp1 to one end of the virion converted the viral RNA to a translatable form (Atabekov et al. 2000). Recently, it was suggested that the C-terminal region of PVX CP is involved in the interaction between CP and TGBp1 (Zayakina et al. 2007). CP was shown to associate with the 5' untranslated region (UTR) of potexvirus RNA (Kwon et al. 2005). The 5' UTR of PVX RNA contains *cis*-acting motifs required for virus replication and cell-to-cell movement (Kwon and Kim 2006; Lough et al. 2006). In this study, we showed that certain alanine-substituted CP could complement cell-to-cell movement of pLi1GFPΔCP but that alanine-substituted CP at Leu-3 could not complement it (Fig. 4C). It is possible that the interaction between the N-terminal region of CP and other factors is required for cell-to-cell movement, which might be affected by a single amino acid substitution. However, immunoprecipitation-Western assay suggested that the in vivo interaction between CP and TGBp1 is not interrupted by alanine substitution at Leu-3 (Fig. 5). Thus, we suggest several scenarios to explain the role of Leu-3 in cell-to-cell movement. One possibility is that the N-terminal region of CP might interact with the 5' UTR of PIAMV RNA. Alternatively, Leu-3 of CP might be an important residue that interacts with unknown host proteins required for cell-to-cell movement.

When multiple alignments of potexvirus CP are formed, a central region, including the core region formerly used as a criterion for phylogenetic analysis (Van Regenmortel et al. 2000), is highly conserved among potexviruses; the N- and C-terminal regions, however, are less conserved. The multiple alignment of potexvirus CP is shown in Figure 7. The relatively

short N-terminal region of PIAMV CP indicates that PIAMV CP may have a minimum functional length. This notion is corroborated by our *trans*-complementation analysis and the dispensability of an N-terminal region of PVX CP for cell-to-cell movement (Chapman et al. 1992; Lico et al. 2006). Although the N-terminal region of PVX CP contains a serine/threonine-rich region that is both glycosylated and phosphorylated, these modifications do not appear to have relevance in cell-to-cell movement (Atabekov et al. 2001; Baratova et al. 2004; Tozzini et al. 1994). We conjecture that the N-terminal region of potexvirus CP can be separated into two distinct functional domains: an N-extra (Nex) region and an N-core (Nc) region. The former region is dispensable for both cell-to-cell movement and virion assembly, although this region might have a role in a specific host plant. The latter region is required for cell-to-cell movement but is dispensable for virion assembly. This proposal is in accordance with other studies using several potexvirus mutants. Defining these domains would facilitate functional analysis of potexvirus CP and help explain their potential roles in cell-to-cell movement and virion assembly.

MATERIALS AND METHODS

Construction of PIAMV mutants and binary vectors for PIAMV CP expression.

The construction of pLi1 was described previously (Ozeki et al. 2006). All PIAMV mutants used in this study were constructed by recombinant polymerase chain reaction (PCR) and basic molecular biological methods. For pLi1-m1, a cDNA fragment containing the 3' terminus of ORF1 (encoding RdRp) to the 5' terminus of ORF5 (encoding CP) was amplified from pLi1 using the 2606F primer (5'-CAA AGT CTC CAC ATA TGC CG-3') that corresponds to nucleotides 2,606 to 2,625

Fig. 7. Amino acid alignment and predicted functional domains of potexvirus coat proteins (CP). The multiple alignment of amino acid sequences of various *Potexvirus* CP, including *Plantago asiatica mosaic virus* (PIAMV), *White clover mosaic virus* (WCIMV), *Cymbidium mosaic virus* (CymMV), *Bamboo mosaic virus* (BaMV), and *Potato aucuba mosaic virus* (PAMV), is shown. Asterisks indicate full conservation, colons represent highly conservative substitutions, and periods represent weakly conservative substitutions. Predicted functional domains in potexvirus CP are shown below. The N-extra (Nex) region is dispensable for both viral cell-to-cell movement and virion formation, and the N-core (Nc) region is required for viral cell-to-cell movement but is dispensable for virion assembly. Core region is highly conserved among *Potexvirus* spp. The C region is required for virus cell-to-cell movement but is dispensable for virion formation.

and the Plmut-5372R primer (5'-TTG AGT GCC gTG GTG GAG A-3'; the position of AUG of CP ORF is underlined and lowercase letters denote a mutated nucleotide) corresponding to nucleotides 5,354 to 5,372. Similarly, a cDNA fragment containing CP ORF and the 3' UTR was amplified with the following primers: Plmut-5354F (5'-TCT CCA CCA cGG CAC TCA A -3') corresponding to nucleotides 5,354 to 5,372 and 6095R (5'-CAG ACT TTC ACT GGT TGT AG-3') corresponding to nucleotides 6,076 to 6,095. The two consequent sets of PCR products were ligated using primers 2606F and 6095R and cloned into pLi1 between the *Bg*III (nucleotide 3,212) and *Spe*I (nucleotide 5,981) sites. For construction of pLi1-m2, the procedures were repeated using different primers: Plmut-5414R (5'-CGA AGG TCG cCG CGT TGA-3') was used instead of Plmut-5372R, and Plmut-5396F (5'-TCA ACG CGG cGA CCT TCG-3') was used instead of Plmut-5354F. pLi1-m12 was constructed using the same primers used for pLi1-m2 construction, except that pLi1-m1 was used as a polymerase chain reaction PCR template instead of pLi1. For construction of pLi1-L3A, the same procedures were repeated using different primers: LiCP-L3A-F (5'-ACA AAC TCT CCA CCA TGG CAG CCA ACC AAG CTC CGA CCT CTG-3') and LiCP-R (5'-CAG AGG TCG GAG CTT GGT TGG CTG CCA TGG TGG AGA GTT TGT-3').

For construction of pCPfull, a binary vector for CP expression which consisted of a cDNA fragment of PIAMV CP was amplified from pLi1 using primers PI-CPF-BaNc (5'-CGG GAT CCA TGG CAC TCA ACC AAG CTC CGA C-3') and PI-CPR-XbPma (5'-TCC CAC GTG TCT AGA CTA GTC GGA GGG GGA GGG GAG GAA TT-3'). The PCR products were digested by *Nco*I and cloned into pCambia-1301 at *Nco*I and *Pma*CI sites. For construction of other CP expression vectors, including pCP5Δ, pCP14Δ, pCPL3A, pCPN4A, pCPQ5A, and pCPP7A, the same procedures were repeated as described above, but different primers were used. The forward primers plcp5del-f (5'-TTT CCA TGG CGA CCT CTG ACG CC-3'), plCP2-F (5'-TTT CCA TGG CCT TCG CCG TCT CAT-3'), PICP-L3A-f (5'-TTT CCA TGG CAG CCA ACC AAG CTC-3'), PICP-N4A-f (5'-TTT CCA TGG CAC TCG CCC AAG CTC-3'), PICP-Q5A-f (5'-TTT CCA TGG CAC TCA ACG CAG CTC-3'), and PICP-P7A-f (5'-TTT CCA TGG CAC TCA ACC AAG CTG CGA CC-3') were used in the described order, and PI-CPR-XbPma was used as a common reverse primer.

pLi1ΔTGB23 was constructed from pLi1 by digesting the *Not*I (nucleotide 5,173) site, followed by blunting and religation. pLi1ΔTGB23-m1 and -m2 were constructed by the same procedures as pLi1-m1 and -m2, respectively.

For construction of pLi1GFPΔCP, a cDNA fragment was amplified from pLi1 using primer 2606F and GFP-LiCP-R (5'-TCC TCG CCC TTG CTC ACC ATC GGA TCC TCA GAG GTC GGA GCT TGG TT-3') corresponding to nucleotides 5,371 to 5,390 of PIAMV and nucleotides 1 to 20 of the GFP gene, respectively. The cDNA fragment was amplified from cDNA using LiCP-GFP-F (5'-AAC CAA GCT CCG ACC TCT GAG GAT CCG ATG GTG AGC AAG GGC GAG GA-3'), which is the reverse complement of GFP-LiCP-R, and GFP-R (5'-TTT ACT AGT TTA CTT GTA CAG CTC GTC CA-3'), corresponding to nucleotides 701 to 720 of the GFP gene. These PCR products were then ligated using recombinant PCR with 2606F and GFP-R and cloned into pLi1 at the *Bg*III and *Spe*I sites.

Plant material and agroinfiltration.

N. benthamiana were grown in a growth chamber at 25°C. Binary plasmids were transformed into *A. tumefaciens* strain EHA105 and cultured at 28°C in Luria-Bertani medium. The preparation for agroinfiltration was performed as described

previously (Ozeki et al. 2006). For *trans*-complementation analysis, *Agrobacterium* cultures carrying pLi1GFPΔCP and each CP expression vector were mixed in a 1:5000 ratio.

RNA isolation and Northern blot analysis.

RNA isolation and Northern blot analysis methods were described previously (Ozeki et al. 2006). A digoxigenin (DIG)-labeled RNA probe corresponding to nucleotides 5,362 to 5,985 of pLi1 was synthesized using a DIG northern Starter Kit (Roche Diagnostics, Basel, Switzerland). Membranes were hybridized and washed using this kit following the manufacturer's instructions and analyzed using LAS 1000 (FujiFilm, Tokyo) and Image Gauge software version 3.45 (FujiFilm).

Western blot analysis and hammer blot analysis.

N. benthamiana leaves infiltrated with *Agrobacterium* cultures were collected 5 dpi and homogenized in 100 volumes of phosphate-buffered saline buffer, and proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After blotting on a polyvinylidene fluoride membrane, PIAMV CP was detected with an anti-CP antibody following the previously described method (Ozeki et al. 2006). For needle-mediated inoculation, expanded *N. benthamiana* leaves were impaled with needles immersed in *Agrobacterium* cultures. Hammer blot analysis was performed as described (Kobayashi et al. 2002) with an anti-CP antibody. Colored spots detected by an anti-CP antibody were analyzed by Image J software 1.34.

GFP fluorescence analysis.

For *trans*-complementation analysis using pLi1GFPΔCP and each CP expression vector, infiltrated leaves were monitored using a B-100 BLACK-RAY long-wave ultraviolet (UV) lamp (Ultraviolet Products, Upland, CA, U.S.A.). GFP images were recorded with a Canon digital still camera (model EOS Kiss Digital). GFP fluorescence was also observed using a confocal laser scanning microscope (Zeiss LSM 5 PASCAL). All images were edited using Adobe Photoshop version 7.0 software.

Immunoprecipitation.

Immunoprecipitation was conducted as described previously (Yamaji et al. 2006), and Western blot analysis was conducted using an anti-CP antibody. *N. benthamiana* leaves infiltrated with appropriate *Agrobacterium* cultures were collected 3 dpi and homogenized in 1 volume of 1× RIPA buffer (5 mM Tris-HCl, pH 8.0; 15 mM NaCl; 0.1% Nonidet P-40; 0.05% sodium deoxycholate; 0.01% SDS; and 0.1 mM phenylmethanesulfonyl fluoride). For preparation of an anti-TGBp1 antibody, the TGBp1 fragment of pLi1 was amplified using primers PI-TG1F (5'-ATT TGA ATT CCA TAT GGA CAT AGT CAT CTC AGC-3') and PI-TG1R (5'-ATT TGT CGA CGT AGG TGG GGT GAG GTG G-3') and introduced into a pET-30a vector (Novagen, Madison, WI, U.S.A.). Expression and purification of recombinant proteins were carried out following a previously described method (Kakizawa et al. 2001).

Virion purification and electron microscopy.

Virus purification was conducted by previously reported methods (Kagiwada et al. 2002), and samples were negatively stained with 2% phosphotungstic acid (pH 7.0) on carbon-coated copper grids and observed under a JEM-1010 transmission electron microscope (JEOL, Tokyo).

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