Fig mosaic emaravirus p4 protein is involved in cell-to-cell movement

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Plant viruses spread from cell to cell through plasmodesmata (PDs), channels across the cell wall that facilitate symplastic transport. Cell-to-cell movement via PDs is essential for a plant virus to establish a systemic infection (Harries & Ding, 2011). Plant viruses encode a class of proteins, so-called movement proteins (MPs), which increase the size exclusion limit (SEL) of PDs and interact with virions or viral genomes to execute viral cell-to-cell movement (Wolf et al., 1989; Otulak & Garbaczewksa, 2011).

The newly formed genus Emaravirus contains segmented negative-strand RNA viruses; Fig mosaic virus (FMV), European mountain ash ringspot-associated virus (EMARaV), rose rosette virus (RRV) and raspberry leaf blotch virus (RLBV). These viruses are thought to consist of four to six genomic segments, depending on the species, and are transmitted by eriophyid mites (Mielke & Muehlbach, 2007; Elbeaino et al., 2009a; Walia et al., 2009b; Laney et al., 2011; McGavin et al., 2012). Emaraviruses are distantly related to tospoviruses and tenuiviruses, which are other negative-strand RNA viruses, common to all reported emaraviruses. Each of the genomic RNA segments contains one ORF; those of RNA1, RNA2 and RNA3 putatively encode RdRp, glycoprotein precursor and nucleocapsid protein, respectively (Mielke & Muehlbach, 2007; Elbeaino et al., 2009b; Laney et al., 2011; McGavin et al., 2012).

Emaraviruses are distantly related to tospoviruses and tenuiviruses, which are other negative-strand RNA viruses, based on RNA-dependent RNA polymerase (RdRp) sequence similarity (Benthack et al., 2005). However, little is known about the molecular characteristics of emaraviruses, owing to the lack of direct and indirect evidence for protein functions of these viruses.

FMV, which was identified in 2009 as a member of the genus Emaravirus (Elbeaino et al., 2009a; Walia et al., 2009), contains six genomic RNA segments (Elbeaino et al., 2009b; Ishikawa et al., 2012). RNA5 and RNA6 are unique to FMV; they share no significant similarity with any known viral sequences, including RNA5 of RLBV, a member of the same genus (Ishikawa et al., 2012). In contrast, RNA1–RNA4 are common to all reported emaraviruses. Each of the genomic RNA segments contains one ORF; those of RNA1, RNA2 and RNA3 putatively encode RdRp, glycoprotein precursor and nucleocapsid protein, respectively (Mielke & Muehlbach, 2007; Elbeaino et al., 2009b; Laney et al., 2011; McGavin et al., 2012). The RNA4 of FMV encodes a protein p4 that shares sequence similarity with those of RRV and RLBV, but not with that of EMARaV (Elbeaino et al., 2009b; Laney et al., 2011; McGavin et al., 2012). Previous work using confocal laser scanning microscopy showed that transiently expressed RLBV-p4 was localized to PDs in the leaves of Nicotiana benthamiana (McGavin et al., 2012), and RLBV-p4 was assumed to function as the MP. However, whether the p4s of emaraviruses actually function as MPs is unknown due to a lack of direct experimental evidence for their biological function.

In the present study, we found that transiently expressed FMV-p4 was localized to PDs and to the plasma membrane, and that it induced and assembled into tubule-like structures similar to those observed in MPs of other viruses. We also obtained functional evidence that FMV-p4 facilitates cell-to-cell trafficking of a movement-defective mutant of potato virus X (PVX). FMV-p4 also prompted GFP diffusion between cells, indicative of an increase in the SEL. In addition, we found that FMV-p4...
moved to neighbouring cells in the absence of other viral components, like the MPs of other viruses. Together, our results demonstrated that FMV-p4 is involved in cell-to-cell movement.

The function of FMV-p4 was predicted by various methods based on the amino acid sequence of a JS1 isolate (GenBank accession no. BAM13816; Fig. 1a). As is the case for RLBV-p4 (McGavin et al., 2012), a secretory signal peptide within the first 19 aa of the N terminus and a cleavage site between residues Gly19/Met20 were predicted (Signal P4.0; Petersen et al., 2011). The putative mature protein, the length of which is 342 aa, has a calculated molecular mass of 38.5 kDa. A glycoamylase (GA) domain was weakly conserved between residues Ser70 and Arg115 (Pfam; Finn et al., 2010). In its C-terminal half, the peptide-binding domain of DnaK (bacterial heat-shock protein 70; Hsp70) family was predicted between residues Asp242 and Lys339 [National Center for Biotechnology Information (NCBI) conserved domain search; Zhu et al., 1996; Sung et al., 2001; Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2009]. This finding is in accordance with the prediction from another report (Laney et al., 2011) of a DnaK domain in the C-terminal half of p4-RRV. Although the biological functions of the signal peptide and GA domain in FMV-p4 are uncertain, the Hsp70 homologue encoded by closterovirus was shown to facilitate viral cell-to-cell movement (Agranovsky et al., 1998). Thus, we speculated that FMV-p4 is involved in viral movement.

To evaluate the biological properties of FMV-p4 in plant cells, the subcellular distribution was examined by confocal laser scanning microscopy. Translational C-terminal fusions with yellow fluorescent protein (p4:YFP) were transiently expressed in N. benthamiana leaves using Agrobacterium tumefaciens. To construct p4:YFP, Gateway technology and the pEarleyGate 101 expression vector were used. Transformation and infiltration were conducted as described previously (Takahashi et al., 2006). In brief, transformed bacterial cells were harvested and resuspended in infiltration buffer to a final optical density of 1.0 at 600 nm, and leaves on a young plant were pressure infiltrated. Observation of the epidermal cells at 2.5 days post-infiltration (p.i.) revealed YFP fluorescence in punctate spots on the cell wall (Fig. 1b), similar to the distribution of viral MPs on PDs (Padgett et al., 1996; Oparka et al., 1996). Next, the infiltrated leaves were incubated in 4 % (w/v) NaCl for 15 min (Oomen et al., 2011) and the distribution during plasmolysis was assessed. Punctate p4:YFP fluorescence was noted at the cell wall during plasmolysis (Fig. 1c, d), confirming that p4:YFP was

![Fig. 1. (a) Schematic representation of FMV-p4. The predicted signal peptide (SP) was identified using SignalP 4.0. The predicted glycoamylase (GA) and DnaK domains were identified using a Pfam search and NCBI conserved domain search, respectively. (b–h) p4:YFP targets PDs and the plasma membrane with tubule-like structures. (b) p4:YFP (overlay on the bright-field image) was observed at PDs at 2.5 days p.i. (c, d) Plasmolysis analysis of p4:YFP. Black arrowheads indicate the retracted protoplast. (c) Bright-field image taken during plasmolysis. (d) Arrows indicate fluorescent spots remaining within the cell wall during plasmolysis (overlay on the bright-field image). (e, f) p4:YFP distribution at 3.5 days p.i. (e) Accumulation in the plasma membrane. White arrowheads indicate the tubule-like structures. (f) Detailed view of the tubule-like structures. (g) p4:YFP distribution in protoplasts isolated from agrobacterium-infiltrated N. benthamiana leaves at 3.5 days p.i. Bars, 25 μm (b–e) and 10 μm (f–h). (i) Immunoblot analysis using anti-GFP antibodies. The fractions correspond to the supernatant (S) and pellet (P) after centrifugation at 30 000 g.](http://vir.sgmjournals.org)
localized to PDs. At 3.5 days p.i., marked YFP fluorescence was noted at the plasma membrane, indicating the accumulation of p4:YFP. In addition, tubule-like structures extending from the puncta were observed (Fig. 1e, f). In studies of other virus MPs, tubule formation from PDs was observed in virus-infected or transiently expressing plant cells (Niehl & Heinlein, 2011). Our observation indicates that p4:YFP appeared to localize to the plasma membrane at 3.5 days p.i., but we could not eliminate the possibility of p4:YFP secretion into the intercellular space due to its N-terminal signal peptide. To confirm the localization of p4:YFP, protoplasts were isolated from p4:YFP-expressing and non-fused YFP-expressing N. benthamiana leaves at 3.5 days p.i. p4:YFP was localized to the plasma membrane, while non-fused YFP was observed in the cytoplasm and nucleus (Fig. 1g, h). In addition, total protein was extracted from p4:YFP-expressing leaves at 3.5 days p.i. and separated into cytosolic (S30) and membrane fractions (P30) by centrifugation at 30,000 g. Next, immunoblot analysis was performed using mouse monoclonal anti-GFP antibody (Roche Diagnostics), which can detect GFP. A signal corresponding to the size of p4:YFP (~65 kDa) was detected in the membrane fraction (P30), but not in the cytosolic fraction (S30) (Fig. 1i). Together, these data indicate that p4:YFP localizes to PDs and subsequently to the plasma membrane. The accumulation of p4:YFP at the plasma membrane probably obscured the YFP signal in the puncta on PDs at 3.5 days p.i. The distribution of FMV-p4 at PDs and the plasma membrane is in agreement with that of RLBV-p4 (McGavin et al., 2012), although the biological role of FMV-p4 at the plasma membrane was not implicated.

To assess whether FMV-p4 facilitates viral cell-to-cell movement, a viral movement complementation assay was performed. In this assay, we utilized a GFP-tagged movement-defective mutant of PVX (PVXΔTGBp1–GFP; Senshu et al., 2011), which lacks most of the triple gene block protein 1 gene (TGBp1). The pEarleyGate 100 was used for the expression of GUS, TGBp1 and FMV-p4. An agrobacterium culture carrying the PVXΔTGBp1–GFP binary plasmid, which was resuspended in infiltration buffer as described above, was diluted 5000-fold and mixed 1:1 with three agrobacterium cultures carrying the binary plasmid of GUS, TGBp1 and FMV-p4, respectively. Almost all of the fluorescent spots were retained in the initially infected single cells in those leaves expressing PVXΔTGBp1–GFP and GUS, while GFP fluorescence spread to multiple cells in those leaves expressing PVXΔTGBp1–GFP and TGBp1 or FMV-p4 (Fig. 2a). When the fluorescent areas of each spot were measured and

**Fig. 2.** Functional analysis of FMV-p4 in cell-to-cell trafficking using fluorescent proteins. (a, c, e) Leaves on a young plant were pressure infiltrated with an agrobacterium culture carrying various plasmids. (a) Viral complementation assay with PVXΔTGBp1–GFP in leaves expressing FMV-p4. (c) FMV-p4 facilitated the diffusion of sGFP. (e) Cell-to-cell spread of p4:YFP in the absence of other viral components. Bars, 100 μm. (b, d, f) Quantification of the size of the fluorescent spots. Twenty fluorescent spots were selected at random from two leaves, and the areas were measured using ImageJ software v1.40 (NIH). The relative sizes normalized to GUS or trGUS:YFP are shown. Error bars represent SD. Asterisks denote significant differences compared with the results of GUS or trGUS:YFP (P<0.01). Cells were visualized using fluorescence microscopy, and photographs were taken at 4 days p.i.
The cell-to-cell movement function of FMV p4 protein

compared at 4 days p.i., the mean size of the spots in the leaves expressing PVXΔTGBp1-GFP and FMV-p4 or TGBp1 was more than threefold greater than that in the leaves expressing PVXΔTGBp1-GFP and GUS (Fig. 2b).

A distinct feature of MPs is their ability to increase the SEL and to enhance cell-to-cell trafficking. The function of FMV-p4 in cell-to-cell trafficking was investigated by observing the diffusion capacity of GFP (Crawford & Zambrský, 2000). An agrobacterium culture carrying the synthetic GFP (sGFP) binary plasmid, which was resuspended in infiltration buffer as described above, was diluted 5000-fold and mixed 1:1 with two agrobacterium cultures carrying the binary plasmid of GUS or FMV-p4. The sGFP fluorescence was restricted to single cells when co-expressed with GUS, while the diffusion of sGFP was observed when it was co-expressed with FMV-p4 (Fig. 2c). The mean area of the fluorescent spots was significantly larger in those leaves expressing FMV-p4 than that in the leaves expressing GUS (Fig. 2d).

Previous studies have shown that the cell-to-cell spread of MPs occurs in the absence of other viral components (Lazarowitz & Beachy, 1999). To accurately measure the intercellular trafficking capacity of FMV-p4, a YFP fusion with a truncated version of GUS, having the same molecular mass as p4:YFP (trGUS:YFP), was constructed. Agrobacterium cultures carrying the binary plasmid of p4:YFP or trGUS:YFP, which was resuspended in infiltration buffer as described above, were diluted 5000-fold. The trGUS:YFP was strictly retained in individual epidermal cells, whereas p4:YFP spread to neighboring cells (Fig. 2e). The mean size of the spots in p4:YFP-expressing leaves was significantly greater than that of trGUS:YFP (Fig. 2f).

In this study, we obtained direct evidence showing that FMV-p4 plays an important role in cell-to-cell movement, and this is the first such demonstration, to the best of our knowledge, for a member of the genus Emaravirus. A close investigation of the subcellular distribution of p4:YFP showed that the protein localized to PDs, frequently accompanied by tubule-like structures. Furthermore, FMV-p4 facilitated cell-to-cell trafficking of a movement-defective mutant of PVX and the diffusion of sGFP. The cell-to-cell spread of p4:YFP occurred in the absence of other viral components. The FMV-p4 shares significant sequence similarities with RRV-p4 and RLBV-p4, suggesting that they share similar properties. However, the p4 of EMaRaV, another member of the genus Emaravirus, did not show sequence similarity with FMV-p4. Our findings in this study are in accordance with those obtained for other negative-strand RNA plant viruses, including Tomato spotted tospovirus, Rice stripe tenuivirus and Rice grassy stunt tenuivirus (Lewandowski & Adkins, 2005; Xiong et al., 2008; Hiraguri et al., 2011). Our results provide new information that will further our understanding of the genetics of cell-to-cell movement. Further study will be needed to elucidate the function of emaravirus p4s.

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References


