Intracellular distribution, cell-to-cell trafficking and tubule-inducing activity of the 50 kDa movement protein of *Apple chlorotic leaf spot virus* fused to green fluorescent protein

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The 50 kDa protein (50KP) encoded by ORF2 of *Apple chlorotic leaf spot virus* (ACLSV) fused to green fluorescent protein (GFP) was expressed transiently in cells of *Nicotiana occidentalis* and *Chenopodium quinoa* leaves. Its intracellular distribution, cell-to-cell trafficking in leaf epidermis and tubule formation on the surface of protoplasts were analysed. The 50KP–GFP fluorescence was distributed as small irregular spots or a fibrous network structure on the periphery of epidermal cells and protoplasts of both plant species. In leaf epidermis of *N. occidentalis*, the protein spread from the cells that produced it into neighbouring cells in both young and mature leaves and targeted plasmodesmata in these cells. In contrast, GFP was restricted to single cells in most cases in mature leaves. When 50KP and GFP were co-expressed in leaf epidermis of *N. occidentalis*, GFP spread more widely from the initial cells that produced it than when GFP was expressed alone, suggesting that 50KP facilitated the cell-to-cell trafficking of GFP. 50KP–GFP was able to complement local spread of 50KP-deficient virus when expressed transiently in leaf epidermis of *C. quinoa*. Expression of 50KP–GFP in protoplasts resulted in the production of tubular structures protruding from the surface. Mutational analyses showed that the C-terminal region (aa 287–457) was not essential for localization to plasmodesmata, cell-to-cell trafficking, complementation of movement of 50KP-deficient virus or tubule formation on protoplasts. In contrast, deletions in the N-terminal region resulted in the complete disruption of all these activities.

Introduction

Plant viruses move from cell to cell in plant tissues through plasmodesmata, which connect the cytoplasm of neighbouring cells (Carrington *et al.*, 1996; Lucas & Gilberston, 1994). Many plant viruses encode movement proteins (MPs) to assist their spread (Koonin & Dolja, 1993). At present, there are at least two distinct pathways for the cell-to-cell movement of plant viruses. One is exemplified best by *Tobacco mosaic virus* (TMV), in which the MP interacts with the plasmodesmata at the infection front, allowing the movement of an MP–virus RNA complex to neighbouring cells (McLean *et al.*, 1995; Oparka *et al.*, 1997; Padgett *et al.*, 1996; Reichel *et al.*, 1999). In another type, represented by *Cucumber mosaic virus* (CMV), virus particles move from cell to cell through tubular structures protruding from plasmodesmata (van Lent *et al.*, 1990, 1991). Plant virus MPs are reported to be multifunctional. Generally, the MP is localized to the plasmodesmata in infected and transgenic plant cells and induces a significant increase in plasmodesmatal permeability (Atkins *et al.*, 1991; Ding *et al.*, 1992; Tomenius *et al.*, 1987; Wolf *et al.*, 1989). The protein binds single-stranded nucleic acids and can traffic from cell to cell itself (Citovsky *et al.*, 1990; Fujiwara *et al.*, 1993; Waigmann & Zambryski, 1995). Recent studies with MP fused to green fluorescent protein (GFP) showed that MP is associated with microtubules and the endoplasmic reticulum (ER), suggesting the involvement of the cytoskeleton and ER in the intracellular trafficking of MP from the site of synthesis in the cytoplasm to the plasmodesmata (Heinlein *et al.*, 1995, 1998; McLean *et al.*, 1995; Huang & Zhang, 1999). MP–GFP fusions of *Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV) are reported to be capable of cell-to-cell trafficking in the leaf epidermis (Itya *et al.*, 1997; Huang & Zhang, 1999).

*Apple chlorotic leaf spot virus* (ACLSV), the type species of the genus *Trichovirus*, has very flexuous filamentous particles,
approximately 600–700 nm in length, and contains a polyadenylated, plus-sense ssRNA with a molecular mass of 2.48 × 10^6 Da and a single coat protein of 22 kDa (Yoshikawa & Takahashi, 1988). The genome of an apple isolate of ACLSV (P-209) consists of 7552 nt and contains three open reading frames (ORFs 1, 2 and 3) (Sato et al., 1993). The 216 kDa protein (KP) encoded by ORF1 is a replication-associated protein and a coat protein is encoded by ORF3. The 50 kDa protein (50KP) encoded by ORF2 is thought to be an MP, based on the following evidence. (i) The amino acid sequence of 50KP has some similarity to MPs of other plant viruses and the protein was detected in the cell wall fraction from infected tissues (Sato et al., 1993, 1995). (ii) Immunelectron microscopy with an antisera against 50KP showed that the protein is localized to plasmodesmata in infected Chenopodium quinoa cells (Yoshikawa et al., 1999). (iii) In transgenic plants expressing 50KP fused to GFP, the fluorescence was associated with plasmodesmata and accumulated in sieve elements (Yoshikawa et al., 1999). (iv) Transgenic Nicotiana occidentalis plants producing 50KP can complement the systemic spread of movement-defective ACLSV (Yoshikawa et al., 2000).

In this study, we transiently expressed ACLSV 50KP fused to GFP in leaf epidermal cells and in leaf mesophyll protoplasts of N. occidentalis and C. quinoa and analysed its subcellular distribution, intercellular trafficking in epidermal cells and tubule formation on the surface of protoplasts. The results indicate that 50KP–GFP is associated with a network, thought to be cortical ER, on the periphery of epidermal cells and protoplasts. 50KP–GFP moved into adjacent cells from the cells that produced it in the leaf epidermis. The protein also induced formation of tubular structures on the surface of plasmodesmata. The protein was also detected in the cell wall fraction from infected tissues (Sato et al., 1993, 1995). Immunelectron microscopy with an antisera against 50KP showed that the protein is localized to plasmodesmata in infected Chenopodium quinoa cells (Yoshikawa et al., 1999). In transgenic plants expressing 50KP fused to GFP, the fluorescence was associated with plasmodesmata and accumulated in sieve elements (Yoshikawa et al., 1999). Transgenic Nicotiana occidentalis plants producing 50KP can complement the systemic spread of movement-defective ACLSV (Yoshikawa et al., 2000).

**Methods**

- **Construction of plasmids.** For construction of transient-expression vectors, the fragment between the HindIII and EcoRI sites of pBE2113-GUS (Mitsuhara et al., 1996) was ligated to the same restriction sites of pUC18 and the resulting plasmid was designated pUC2113-GUS. Three plasmids, p35S50KP, p35S50KP–GFP and p35SGFP (Fig. 1), were constructed by replacing the GUS gene of pUC2113-GUS with each gene, as described previously (Yoshikawa et al., 2000).

- **Particle bombardment.** In transient GFP expression experiments, leaves were detached from N. occidentalis (12 true-leaf stage) or C. quinoa (8 true-leaf stage) plants and placed in a Petri dish containing wet filter paper. The lower epidermis was bombarded with microparticles coated with DNA constructs by using the PDS-1000/He particle delivery system (Bio-Rad) as described before (Satoh et al., 1999). Leaves were kept under moist conditions at 25 °C until used for observation.

To examine the complementation of cell-to-cell movement of 50KP-deficient virus by 50KP–GFP, the fifth true leaf of a plant of C. quinoa (7 true-leaf stage) was bombarded with a mixture (1:1) of p35S50Nhe and p35S50KP–GFP. The plants were maintained in a glass chamber for 5 days. Total RNA was extracted from bombarded leaves and then subjected to Northern hybridization analysis with an RNA probe, as described previously (Yoshikawa et al., 2000).

- **Isolation and transfection of protoplasts.** Leaf mesophyll protoplasts were isolated from C. quinoa and N. occidentalis leaves as follows. Leaves were sliced into strips and then soaked for 3 h in an enzyme solution containing 2% cellulase ‘Onozuka’ R-10 (Yakult Pharmaceutical), 0.1% pectolyase Y-23 (Seishin Pharmaceutical), 10 mM CaCl_2 and 0.5 M mannitol, pH 5.6. The resulting protoplast suspension was washed twice with MC solution (0.5 M mannitol, 10 mM CaCl_2) and centrifuged in MC solution containing 20% sucrose for 3 min at 700 r.p.m. Protoplasts were recovered from the middle layer and washed in MC solution.

To the protoplasts (about 3 × 10^2 cells), 20 µg plasmid DNA and 500 µl inoculation buffer (10 mM MES, 40 mM CaCl_2, 0.5 M mannitol, pH 5.8) were added and then the suspension was mixed gently. Next, 900 µl PEG solution (40% PEG 4000, 40 mM CaCl_2, 0.5 M mannitol) was added, followed by incubation on ice for 30 min. After washing with 50 mM glycine, 50 mM CaCl_2, 0.5 M mannitol, pH 8.5, 10 ml inoculation buffer was added to the protoplasts, which were then incubated on ice for 30 min. The protoplasts were then suspended in a medium.
containing 0·5 M mannitol, 0·2 mM KH₂PO₄, 1 mM KNO₃, 1 mM MgSO₄, 10 mM CaCl₂, 1 mM KI, 0·01 mM CuSO₄, pH 6·5, and incubated at 25 °C.

Fluorescence and confocal laser scanning microscopy (CLSM). Observation of GFP fluorescence in epidermal cells and protoplasts was conducted by using a Leica DMLB fluorescence microscope as described previously (Yoshikawa et al., 1999). A laser scanning microscope (Leica DMIRB equipped with Yokogawa CLSM unit CSU10) was also used with excitation at 488 nm and emission at 516–700 nm. Digital images were acquired with a Yokogawa DNS10 CCD camera and processed by IPLab (Scananalytics).

Results

Intracellular distribution of 50KP–GFP in leaf epidermis

Plasmids p35SGFP and p35S50KP–GFP, which express GFP or 50KP–GFP transiently under the control of the 35S promoter, were constructed, as well as a series of p35S50KP deletion mutants (ΔA to ΔG) (Fig. 1; Table 1). The plasmids were delivered into leaf epidermis of N. occidentalis and C. quinoa by particle bombardment. Fluorescence became visible under a fluorescence microscope in the epidermal cells of both plant species 2 h after bombardment. Observation by CLSM showed that the 50KP–GFP fluorescence was observed mainly on the cell periphery and on the cell wall of the epidermal cells 12–24 h after bombardment. This was clear when the focus was brought to the upper, centre and lower planes of the cells (Fig. 2A, B). 50KP–GFP was present as small, irregular spots (Fig. 2B) or as a fibrous, structure-forming network (Fig. 2A, C) in the outermost periphery of the cells. This was in contrast with the cells expressing GFP only, in which bright fluorescence was found in the nucleus and cytoplasm (Fig. 2F). There was no difference between N. occidentalis and C. quinoa in the fluorescence-distribution patterns in epidermal cells.

Table 1. Functional activities of proteins expressed transiently in epidermal cells and protoplasts

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein expressed</th>
<th>Distribution on cell periphery, localization to plasmodesmata</th>
<th>Cell-to-cell trafficking</th>
<th>Complementation of local movement of 50KP-deficient virus</th>
<th>Tubule formation on surface of protoplasts</th>
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<tbody>
<tr>
<td>p35S50KP</td>
<td>50KP</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>p35S50KP–GFP</td>
<td>50KP–GFP</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
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<tr>
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<td>–</td>
<td>( + )</td>
<td>–</td>
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</table>

Targeting to plasmodesmata and cell-to-cell trafficking of 50KP–GFP in leaf epidermis

In N. occidentalis leaves 24–48 h after bombardment with p35S50KP–GFP, fluorescence was observed on the cell wall of the cells neighbouring most of the cells that originally produced 50KP–GFP (Fig. 2G), suggesting that 50KP–GFP moves from the cells that produce it into the neighbouring cells. To demonstrate cell-to-cell trafficking of 50KP–GFP further, DNA of the plasmids p35S50KP–GFP or p35SGFP was bombarded into each half of split N. occidentalis leaves and the number of single cells and cell clusters showing fluorescence was counted 24 h after bombardment. 50KP–GFP spread from single cells that produced it into neighbouring cells in both young and mature leaves (Fig. 3), in contrast to GFP, which was restricted to single cells in more than 80% of cases in mature (3rd and 5th) leaves (Figs 2F and 3). At higher magnification, fluorescence in cells that adjoined cells initially bombarded with p35S50KP–GFP was detected as spots or strands passing through the cell wall (Fig. 2E), indicating that fluorescence was located at plasmodesmata, as reported for transgenic N. occidentalis plants expressing 50KP–GFP (Yoshikawa et al., 1999). Small, irregular spots or thick, fibrous structures, as described above, were not observed in these neighbouring cells.

When the p35S50KP deletion mutants (Fig. 1) were bombarded into leaf epidermis of N. occidentalis plants, the fluorescence derived from ΔA, ΔB or ΔC was observed as spots on the cell wall of the neighbouring cells, suggesting that these deleted proteins retained the same cell-to-cell trafficking ability as wild-type 50KP–GFP (Table 1). In contrast, the fluorescence from ΔD, ΔE, ΔF and ΔG was present as aggregates in the cytoplasm and was restricted to single cells in both young and mature leaves (Fig. 2D, H), indicating that these proteins had lost their ability to move from cell to cell.
Fig. 2. Detection by CLSM of fluorescence of 50KP–GFP expressed transiently in epidermal cells and protoplasts. (A) 50KP–GFP fluorescence of upper, middle and lower planes of an epidermal cell, showing fibrous structure on the cell periphery. (B)–(C) 50KP–GFP detected as small spots (right in B) and a fibrous structure forming a network (C) on the periphery of epidermal cells. (D) Fluorescence of ∆D, showing large aggregates in an epidermal cell. (E) Fluorescent spots on the cell wall of an epidermal cell expressing 50KP–GFP. (F)–(H) Fluorescence of GFP (F), 50KP–GFP (G) and ∆G (H) expressed in leaf epidermis of mature leaves following bombardment. (I) GFP distribution in a protoplast showing strong fluorescence in the nucleus. (J)–(L) 50KP–GFP fluorescence showing spots (J) and fibrous structures (K, L) on the periphery of protoplasts.
In observations of leaf epidermis of C. quinoa plants bombarded with p35SGFP, non-specific trafficking of GFP was found in young, developing leaves, similar to the situation in N. occidentalis. However, in epidermal cells of both young and mature leaves of C. quinoa bombarded with p35S50KP–GFP, fluorescent spots in cell walls were detected in only two or three cells around an originally transfected cell, rather fewer than in the case of N. occidentalis (data not shown).

Increased cell-to-cell trafficking of GFP co-expressed with 50KP in leaf epidermis

Plant virus MPs are reported to modify the plasmodesmata and to increase their size-exclusion limit (Derrick et al., 1992; Vaquero et al., 1994; Poisson et al., 1993; Wolf et al., 1989). As mentioned above, GFP was restricted to single cells in most cases in mature leaves (Fig. 3). In order to investigate whether GFP can spread from cell to cell in mature leaves when 50KP is co-expressed in the same cells, p35SGFP was bombarded into one half-leaf and p35S50KP plus p35SGFP were bombarded into the other half-leaf of N. occidentalis. When GFP was co-expressed with 50KP, the fluorescence spread more widely from the cells that initially produced it than when GFP was expressed alone (Table 2; Fig. 2M, N). The result suggests that the 50KP expressed in cells may modify the plasmodesmata and facilitate cell-to-cell trafficking of GFP that is expressed in the same cells.

Complementation of local cell-to-cell spread of 50KP-deficient ACLSV with 50KP and 50KP–GFP expressed transiently in leaf epidermis

Two 50KP-deficient mutants (pStuStop and pStuStuNhe) of an infectious ACLSV cDNA clone (pCLSF) (Satoh et al., 1999) were found to replicate in protoplasts from C. quinoa leaves (data not shown). These clones were shown previously to give systemic infection of transgenic N. occidentalis plants that constitutively express 50KP (Yoshikawa et al., 2000). To examine whether 50KP expressed transiently in leaf epidermis can complement the spread of 50KP-deficient virus, pStuStuNhe was co-bombarded with p35S50KP or p35S50KP–GFP into leaf epidermis of C. quinoa plants. Northern hybridization analysis of leaves 3 days after bombardment showed that progeny viral RNA was detected in leaves when pStuStuNhe was co-bombarded with p35S50KP or p35S50KP–GFP (Fig. 4A, lanes 6–9). In contrast, no viral RNA was found in samples bombarded with pStuStuNhe only (Fig. 4A, lanes 3–5). Compared with the band of progeny genomic RNA from leaves inoculated with pCLSF (Fig. 4A, lane 2), the signals for viral RNA from co-bombarded leaves were weak, suggesting that 50KP-deficient virus may spread to a limited area around the cells that originally produce 50KP or 50KP–GFP. These results indicate that 50KP and 50KP–GFP expressed transiently in epidermal cells are functional and can complement local spread of 50KP-deficient virus. Similar complementation of 50KP-deficient virus (pStuStuNhe) was found in leaves co-bombarded with AA, AB or AC (Fig. 4B, lanes 1–3), but not AD, AE, AF or AG (Fig. 4B, lanes 4–7).

The pattern of accumulation of 50KP–GFP and its induction of tubules in protoplasts

Leaf mesophyll protoplasts isolated from N. occidentalis and C. quinoa plants were transfected with p35SGFP, p35S50KP–GFP or p35S50KP deletion mutants and the distribution of fluorescence was observed at 4, 12, 24 and 48 h after transfection. In protoplasts transfected with p35SGFP, the fluorescence was found in nuclei and cytoplasm (Fig. 2A),
**Table 2.** Cell-to-cell trafficking of GFP co-expressed with ACLSV 50KP in leaf epidermis of *N. occidentalis*

Leaves are numbered from the bottom of an *N. occidentalis* plant (12 true-leaf stage). GFP fluorescence was observed 24 h after bombardment. Of those cells that showed GFP fluorescence, the percentage within each category is shown.

<table>
<thead>
<tr>
<th>Expressed protein*</th>
<th>Single cells</th>
<th>2–5 cells</th>
<th>6–10 cells</th>
<th>11–15 cells</th>
<th>&gt; 15 cells</th>
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<td>3rd leaf</td>
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</tr>
<tr>
<td>GFP</td>
<td>98</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFP + 50KP</td>
<td>64</td>
<td>25</td>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5th leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>4</td>
<td>20</td>
<td>44</td>
<td>32</td>
</tr>
</tbody>
</table>

* p35SGFP and p35SGFP plus p35S50KP were bombarded into each half-leaf of split *N. occidentalis* leaves.

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**Fig. 4.** Northern blot analysis of leaves bombarded with p∆StuNhe, p∆StuNhe plus p35S50KP–GFP or p∆StuNhe plus p35S50KP deletion mutants. (A) A control sample without bombardment (lane 1) and samples from leaves bombarded with pCLSF (lane 2), p∆StuNhe alone (lanes 3–5), p∆StuNhe plus p35S50KP (lanes 6 and 7) or p∆StuNhe plus p35S50KP–GFP (lanes 8 and 9). (B) Samples from leaves bombarded with p∆StuNhe plus p35S50KP deletion mutants ∆A (lane 1), ∆B (2), ∆C (3), ∆D (4), ∆E (5), ∆F (6) or ∆G (7). The arrow and the arrowhead respectively indicate the positions of ACLSV RNA and RNA derived from p∆StuNhe.

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consistent with its distribution in epidermal cells. The GFP distribution pattern did not change until 48 h after transfection. In contrast, 50KP–GFP was present as small, irregular spots (Fig. 2J) and fibrous structures forming networks (Fig. 2K, L) at the periphery of protoplasts, as observed in leaf epidermal cells, indicating that fibrous structures were formed without requiring the presence of a cell wall. The time-course of the distribution of 50KP–GFP showed that the fluorescence was present as small, irregular spots in 96% of sampled protoplasts (158/165) and as fibrous structures in 4% of sampled protoplasts (7/165) 4 h after transfection. The percentage of protoplasts showing fibrous network structures (Fig. 2K and L) increased to 37 and 39% (54/145 and 42/107) in two independent experiments 12 h after transfection. The ratio did not change after that. These results suggest that 50KP–GFP may accumulate initially as small, irregular spots in the periphery of the protoplast but then the distribution changes to a fibrous network structure.

Tubular structures protruding from the surface of the protoplasts were also observed in a few protoplasts expressing 50KP–GFP (Fig. 2O–Q). The tubules were of variable lengths and appeared to be very fragile and to fragment easily during observation. The percentages of protoplasts expressing 50KP–GFP with tubules were 0% (1/165), 2% (3/145) and 3% (5/178) at 4, 12 and 24 h after transfection. The percentage did not increase at 48 h after transfection. The tubules were formed in protoplasts transfected with mutants ∆A, ∆B or ∆C but not ∆D, ∆E, ∆F or ∆G (Table 1). Interestingly, small, irregular spots and fibrous network structures were never found in protoplasts containing tubular structures.

**Discussion**

A TMV MP–GFP fusion has been shown to co-localize with cortical ER and microtubules (Heinlein *et al.*, 1995, 1998; McLean *et al.*, 1995). It has been reported that AlMV MP–GFP expressed in epidermal cells is also associated with the ER and that the association may be important for intracellular and intercellular movement of AlMV MP (Huang & Zhang, 1999). In order to clarify whether 50KP–GFP was co-localized with the ER and/or microtubules, we conducted several double-labelling experiments with a monoclonal anti-
body against tubulin and rhodamine-labelled secondary antibody (Heinlein et al., 1995) or using the hexyl ester of rhodamine B (Huang & Zhang, 1999). However, we could not obtain definitive evidence of co-localization of 50KP–GFP and ER and/or microtubules (data not shown).

Cell-to-cell trafficking of MP–GFP expressed transiently in leaf epidermis has been reported in CMV and AIMV, in which the protein moves into neighbouring cells from an originally transfected cell and, in contrast, free GFP remains in single cells (Itaya et al., 1997; Huang & Zhang, 1999). Recently, Oparka et al. (1999) reported that the capacity of plasmodesmata to traffic macromolecules depends on the physiological conditions of the leaves; in sink leaves, proteins of up to 50 kDa could move freely through plasmodesmata. Our results presented here show that free GFP (27 kDa) could move from cell to cell in young leaves of *N. occidentalis* and *C. quinoa*, but was mostly restricted to single cells in mature leaves (Fig. 3). This result is in good agreement with the reported non-specific trafficking of GFP in developing tobacco leaves (Oparka et al., 1999). In contrast, 50KP–GFP (77 kDa) spread into neighbouring cells from cells that produced it, even in mature leaves, indicating that 50KP has a specific activity for cell-to-cell trafficking.

It was unexpected that cell-to-cell trafficking of 50KP–GFP was restricted to only a few cells in leaf epidermis of *C. quinoa* plants. There may be structural and/or functional differences between the plasmodesmata that interconnect epidermal cells of *N. occidentalis* and *C. quinoa* plants. 50KP–GFP expressed in epidermal cells of *C. quinoa* leaves was able to complement local spread of the movement-defective virus (Fig. 4). Thus, 50KP–GFP may move from the epidermal cells to underlying mesophyll cells in *C. quinoa* leaves. It has been reported that a CMV mutant (M8) 3a–GFP fusion protein was unable to traffic through plasmodesmata that interconnect epidermal cells, as the wild-type 3a–GFP did, in tobacco (*Nicotiana tabacum*) and *Nicotiana benthamiana* (Canto & Palukaitis, 1999). Because M8 CMV infects tobacco systemically, the virus spread via plasmodesmata to and within mesophyll tissue (Canto & Palukaitis, 1999).

In addition to the cell-to-cell trafficking of 50KP itself, we showed that 50KP can facilitate the cell-to-cell trafficking of GFP when both proteins are co-expressed transiently in epidermal cells of *N. occidentalis* leaves (Table 2). However, there was no facilitation of cell-to-cell movement of GFP when the protein was expressed in leaf epidermis of transgenic *N. occidentalis* constitutively expressing a functional 50KP (data not shown).

Transgenic *N. occidentalis* plants expressing 50KP are known to complement 50KP-deficient ACLSV for movement (Yoshikawa et al., 2000). As shown in Fig. 3, 50KP expressed transiently in leaf epidermis also complemented local spread of 50KP-deficient virus. It has been reported that transgenic plants expressing CMV 3a protein could complement 3a-deficient CMV, but plants expressing 3a protein fused to GFP could not, showing that CMV 3a protein fused to GFP is not biologically functional (Canto & Palukaitis, 1999; Kaplan et al., 1995). We also found that there was no complementation of the movement of 50KP-deficient virus in *N. occidentalis* expressing 50KP–GFP (unpublished results). However, 50KP–GFP could complement local movement of a 50KP-deficient virus when pStuStop was co-bombarded with p35S50KP–GFP. These results suggest that complementation could occur when both p35S50KP–GFP and pStuStop were introduced in cells at the same time. On the other hand, 50KP expressed transiently in cells may be functionally different from that in transgenic plant cells.

We do not know the biological significance of the tubules on the surface of protoplasts induced by 50KP–GFP. No tubular structures spanning cell walls were found in ultrathin sections of tissues infected with ACLSV (Yoshikawa et al., 1997, 1999) and the tubules were observed in only a small portion of protoplasts that expressed 50KP–GFP. This is similar to what has been described for TMV, where fluorescent protrusions were formed in a small percentage of protoplasts infected with TMV MP–GFP (Heinlein et al., 1998). In contrast, CMV 3a–GFP generated tubules on the surface of 14–85% of infected protoplasts (Canto & Palukaitis, 1999). So far, tubule formation on protoplasts has been reported for spherical viruses in the *Conoviridae, Bromoviridae* and *Caulimoviridae* and for *Tomato spotted wilt virus* but not for TMV (Kasteel et al., 1996, 1997; Storms et al., 1995; van Lent et al., 1991; Zheng et al., 1997).

As summarized in Table 1, mutational analysis has shown that the C-terminal region (between aa 287 and 457) of 50KP is not essential for localization to plasmodesmata, cell-to-cell trafficking through plasmodesmata, complementation of local movement of 50KP-deficient virus or tubule formation on the surface of protoplasts. In contrast, deletions in the N-terminal region of 50KP resulted in the complete disruption of all these activities, suggesting that there must be a close correlation between these functions. The expressed proteins (AD to AG) all formed large aggregates in the cytoplasm. It is probable that these proteins are not folded properly and that this makes the protein incapable of interacting with a subcellular structure(s) or undergoing intracellular and intercellular trafficking.

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