Interference of Long-Distance Movement of *Grapevine berry inner necrosis virus* in Transgenic Plants Expressing a Defective Movement Protein of *Apple chlorotic leaf spot virus*

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ABSTRACT

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Transgenic *Nicotiana occidentalis* plants expressing a movement protein (P50) and partially functional deletion mutants (ΔA and ΔC) of the *Apple chlorotic leaf spot virus* (ACLSV) showed resistance to *Grapevine berry inner necrosis virus* (GINV). The resistance is highly effective and GINV was below the level of detection in both inoculated and uninoculated upper leaves. In contrast, GINV accumulated in inoculated and uninoculated leaves of nontransgenic (NT) plants and transgenic plants expressing a dysfunctional mutant (ΔG). On the other hand, in some plants of a transgenic plant line expressing a deletion mutant ($\Delta A'$, deletion of the C-terminal 42 amino acids), GINV could spread in inocu-

lated leaves, but not move into uninoculated leaves. In a tissue blot hybridization analysis of $\Delta A'$ -plants inoculated with GINV, virus could be detected in leaf blade, midribs, and petiole of inoculated leaves, but neither in stems immediately above inoculated leaves nor in any tissues of uninoculated leaves. Immunohistochemical analysis of GINV-inoculated leaves of $\Delta A'$ -plants showed that GINV could invade into phloem parenchyma cells through bundle sheath of minor veins, suggesting that the long-distance transport of GINV might be inhibited between the phloem cells and sieve element (and/or within sieve element) rather than bundle sheath-phloem interfaces. Immunogold electron microscopy using an anti-P50 antiserum showed that P50 accumulated on the parietal layer of sieve elements and on sieve plates. The results suggested that resistance in P50-transgenic plants to GINV is due to the interference of both long-distance and cell-to-cell movement of the virus.

Apple chlorotic leaf spot virus (ACLSV) has flexuous filamentous particles, approximately 740 to 760 nm in length and is the type species of the genus *Trichovirus* (8,9,19,22,36). The 50-kDa protein (P50) encoded by open reading frame 2 of ACLSV genome is a movement protein (MP), which has the following characteristics: (i) it localizes to plasmodesmata of infected and transgenic plant cells (35), (ii) it can spread from the cells that initially produce it into neighboring cells (24), (iii) it enables cell-to-cell trafficking of green fluorescent protein (GFP) when P50 and GFP are co-expressed in leaf epidermis (24), (iv) it induces the production of tubular structures protruding from the surface of protoplasts (24), and (v) it binds to single-stranded nucleic acids (12).

Transgenic plants expressing viral MPs are reported to be resistant to homologous and heterologous viruses and the phenomenon is called MP-derived resistance (1,2,5,15,18,25). Recently, we reported that transgenic *Nicotiana occidentalis* plants expressing a functional ACLSV P50 show specific resistance to infection with *Grapevine berry inner necrosis virus* (GINV, a member of the genus *Trichovirus*) (34), but not to infection with *Apple stem grooving virus* (genus *Capillovirus*) and *Apple stem pitting virus* (genus *Foveavirus*) (33). The resistance of transgenic plants expressing P50 (P50-plant) to GINV is highly effective, and GINV has not been detected in either inoculated or upper uninoculated leaves by an enzyme-linked immunosorbent assay (ELISA) (33). The analysis of the interactions between P50 and GINV-MP (P39) in P50-plants and nontransgenic (NT) plants showed that a functional P50 specifically interferes with the intracellular and inter-

cellular trafficking and tubule-inducing activity of P39 and vice versa (11). It was suggested that the resistance was not due to gene silencing because the proteins, not mRNAs, were involved in the mutual interference of the functions of P50 and P39 (11). Therefore, it was concluded that the resistance of P50-plants against GINV is mediated through interaction between P50 and P39, i.e., P50 in transgenic plants blocks the functions of P39, and consequently, the cell-to-cell movement of GINV is inhibited in tissues expressing P50 (11).

Transgenic plants expressing the P50-deletion mutants, ΔA and ΔC , which retained movement function (24), were resistant to GINV, whereas transgenic plants expressing a dysfunctional P50 mutant (ΔG) did not show any resistance to the virus (11). In this paper, we report that a transgenic plant line expressing $\Delta A'$ (deletion of the C-terminal 42 amino acids) allowed GINV to spread in inoculated leaves, but not into upper leaves, indicating that $\Delta A'$ expressed in transgenic plants interferes with the long-distance movement of GINV. We also showed that the block in systemic movement of GINV in $\Delta A'$ -plants may occur at the boundary between phloem cells and sieve elements and/or within sieve tubes, not in the bundle sheath-phloem interface.

MATERIALS AND METHODS

Plants expressing P50 and its truncated derivatives. *N. occidentalis* plants expressing P50 (line d-5) and its truncated derivatives (ΔA [line A-3], ΔC [line C-3], and ΔG [line G-23]) (11,33) were used in this study. Another *N. occidentalis* deletion mutant, $\Delta A'$, which has amino acids 416 through 457 deleted, was constructed as described previously (33). Transgenic plant line A'-5, which expresses the $\Delta A'$ deletion mutant, was used in this study. Northern blot hybridization and immunoblot assays of R1 seed-

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lings of transgenic plants were conducted to compare the expression level of mRNAs and proteins among lines d-5, A-3, C-3, G-23, and A'-5 as described below.

Total RNA was extracted from transgenic plant leaves (0.1 g) according to Verwoerd et al. (30). LiCl-insoluble RNA was electrophoresed in 1% agarose gel containing formaldehyde and transferred to a Hybond-N⁺ membrane (Amersham, Tokyo). A negative-strand RNA probe of full-length 50KP gene was prepared from a cDNA clone using a digoxigenin RNA labeling kit (Roche Diagnostics, Tokyo, Japan). Prehybridization, hybridization, and chemiluminescent detection were conducted according to the protocols supplied by Roche Diagnostics.

Total proteins were extracted from leaves (0.1 g), electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel, and then analyzed by immunoblotting using an anti-P50 antiserum as described previously (23,33).

Mechanical inoculation of GINV. Two grams of GINV-infected *Chenopodium quinoa* leaves was homogenized in 10 ml of 0.1 M Tris-HCl (pH 7.2) and 0.01 M MgCl₂, and the homogenates were used for inoculation.

Kanamycin-resistant R1 seedlings of transgenic *N. occidentalis* plants (50KP, ΔA', ΔA, ΔC, and ΔG) were grown to the six- to seven-leaf stage in pots. Before virus inoculation, the seedlings were analyzed by immunoblot analysis to confirm the expression of the protein, and only plants expressing the protein were used for inoculation tests. Three leaves per plant were mechanically inoculated with GINV, and inoculated plants were grown in a growth chamber and observed for symptoms. Ten plants per transgenic line were assayed in each infection test, and the experiments were repeated three times.

Analyses of inoculated plants. Inoculated and upper uninoculated leaves of *N. occidentalis* plants were collected at 14 and 21 days postinoculation (dpi), respectively, and the virus accumulation was assayed by ELISA using an anti-GINV antiserum as described previously (33).

Virus infection was also analyzed by a direct tissue immunoblotting (26). Fresh whole leaves harvested at 7 dpi (inoculated leaves) and 14 dpi (upper uninoculated leaves) were placed between two layers of No. 2 filter paper. Blots were made by gently striking the leaves with a rubber hammer. The paper was rinsed in 2% Triton X-100 for 30 min with gentle shaking to remove residual green color and then in phosphate-buffered saline with Tween (PBST)-milk (10 mM NaPO₄, pH 7.2, 0.9% NaCl, 0.1% Tween, and 3% skim milk) for 30 min. The paper was incubated with an antiserum against GINV as a primary antibody, washed with PBST-milk, and incubated with a goat anti-rabbit immunoglobulin G (IgG) conjugated with alkaline phosphatase as a secondary antibody. Nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT-BCIP) was used for color developing.

For tissue blot hybridization analysis, a negative-strand RNA probe was prepared from a cDNA clone of GINV (nucleotides 825 to 2469 of the genome) (34) as described previously. Leaf blades, midribs, and petioles from inoculated and upper uninoculated leaves were cut by a razor blade, and the cut faces were directly touched to a Hybond-N⁺ membrane. In this blotting, leaf blades were rolled and then cut by razor before blotting. Midribs (8 mm long) were cut vertically, and cut faces of two pieces were blotted. The petioles and stems, immediately above the inoculated leaves, were cut horizontally, and the cut face was contacted onto a membrane. Prehybridization, hybridization, and chemiluminescent detection were conducted according to the protocols supplied by Roche Diagnostics.

Immunohistochemical analysis. Leaf pieces (5 mm²) from NT and transgenic *N. occidentalis* plants (50KP and ΔA') inoculated with GINV were excised and fixed in 4% formaldehyde in 0.1 M Na-phosphate buffer (pH 7.5) at 4°C overnight. The samples were dehydrated, treated in Histo-clear (National Diagnostics, UK), and embedded in paraffin (Paraplast Embedding Media, Sigma,

Tokyo, Japan). Preparations of thin sections (10 μm) were carried out as described by Karyeija et al. (13). Thin sections were initially incubated in PBS containing 4% bovine serum albumin (BSA) for 30 min and then treated with an antibody against GINV (dilution 1:100) for 3 h at room temperature. After washing with PBS, the samples were incubated with a goat anti-rabbit IgG conjugated with alkaline phosphatase. After washing with PBS, samples were stained with a fuchsin substrate solution as described by Rajamaki and Valkonen (20,21) and observed by a light microscope.

Confocal laser scanning microscopy. Examinations of transgenic plant leaves expressing P50 fused to GFP (EGFP) were conducted under a confocal laser scanning microscope (Leica CLSM; Leica, Wetzlar, Germany) using the following conditions: excitation filter BP488, beamsplitter TK510, and emission filter BP540 as described previously (35).

Immunogold electron microscopy. Leaf pieces, 1 × 10 mm, from transgenic *N. occidentalis* plants expressing P50, ΔA, ΔA', and ΔG proteins, and ACLSV-infected *C. quinoa* plants were fixed in 2% paraformaldehyde plus 0.5% glutaraldehyde overnight. The samples were dehydrated through ethanol series, infiltrated in a graded series of LR White resin at low temperature (−20°C), and polymerized at 58°C for 16 to 20 h as described pre-

Fig. 1. Analyses of transgenic *Nicotiana occidentalis* plants expressing P50 and P50 deletion mutants (ΔA', ΔA, ΔC, and ΔG) of *Apple chlorotic leaf spot virus* (ACLSV). **A**, Northern blot analysis of RNA from a leaf sample of nontransgenic plant (lane NT) and leaf samples from kanamycin-resistant R1 seedlings of transgenic plants expressing P50 (lane 1), ΔA' (lane 2), ΔA (lane 3), ΔC (lane 4), and ΔG (lane 5). **B**, Immunoblot analyses of leaf samples from kanamycin-resistant R1 seedlings of transgenic plants expressing P50, ΔA', ΔA, ΔC, and ΔG. Total proteins from 2.5 mg of leaf tissues were loaded per lane. Arrowheads indicate the positions of expressed P50 and P50 deletion mutants. **C**, Immunoblot analyses of leaf samples from kanamycin-resistant R1 seedlings of transgenic plant expressing P50, ΔA', and ΔA. Lanes 1 to 10, leaf samples from 10 individual transgenic plants. Lane NT, leaf samples from a nontransgenic plant. Arrows indicate the positions of P50, ΔA', and ΔA. Arrowheads indicate the positions of the phosphorylated forms of P50, ΔA', and ΔA.

viously (35). Thin sections (98 nm) were mounted on formvar-coated nickel grids and incubated in 2% BSA in 0.15 M NaCl, 0.1 M phosphate buffer, pH 7.4 (PBS), for 45 min. Sections were then treated with an antiserum against P50 diluted with 1:500 in PBS containing 0.5% BSA for 90 to 120 min. After washing with PBS containing 0.1% BSA, the grids were incubated in a goat anti-rabbit IgG coupled to 10 nm colloidal gold (Zymed Laboratories, CA) for 45 to 60 min. The sections were stained with 1% uranyl acetate. All sections were viewed with a Hitachi H-800 (Tokyo, Japan) electron microscope.

RESULTS

Analysis of the transgenic plants expressing P50 and its truncated derivatives. R1 seedlings from transgenic plants used in this study segregated 3:1 for resistance to kanamycin. Northern

Fig. 2. Virus accumulation in nontransgenic (NT) and transgenic *Nicotiana occidentalis* plants (P50, $\Delta A'$, and ΔA) inoculated with *Grapevine berry inner necrosis virus* (GINV). Inoculated and upper uninoculated leaves from each of 10 plants were collected at 14 days postinoculation (dpi) and 21 dpi, respectively, and GINV accumulation was determined by an enzyme-linked immunosorbent assay. H, a healthy leaf sample.

blot and immunoblot analyses of kanamycin-resistant R1 seedlings showed that mRNAs and proteins of P50 and its truncated derivatives were expressed in all samples from transgenic plant lines d-5, A'-5, A-3, C-3, and G-23. No large differences in the amount of mRNAs and the expressed proteins were noted among these lines (Fig. 1A and B).

In a previous report, we showed that transgenic *N. occidentalis* plants expressing P50 complemented the systemic spread of the P50-deficient ACLSV mutants (pStuStop and p Δ StuNhe) (33). When these P50-deficient mutants were inoculated onto A'-5, ΔA -3, ΔC -3, and ΔG -23 plants by particle bombardments, no complementation of the movement of P50-deficient virus was found in inoculated or uninoculated upper leaves of these transgenic plants expressing P50 deletion mutants (data not shown), though $\Delta A'$, ΔA , and ΔC , but not ΔG , retained the activities for localization to plasmodesmata, cell-to-cell trafficking, and tubule formation on surface of protoplasts (24; unpublished data).

Before GINV was inoculated, 10 individual plants of transgenic lines d-5, A'-5, or A-3 were analyzed by an immunoblot analysis. P50 was detected as diffuse bands (Fig. 1C), suggesting that P50 expressed in transgenic plants is phosphorylated as shown in infected leaves (23). Similarly, ΔA protein in all transgenic plant leaves and $\Delta A'$ protein in some plants were seen as two bands (Fig. 1C). The slower migrating bands were suggested to be phosphorylated because the incubation of the protein samples with alkaline phosphatase resulted in the change of the electrophoretic mobility from the slower migrating band to the faster migrating band (data not shown). There were no differences in the amount of the expressed proteins among individual plants of transgenic lines d-5, A'-5, or A-3 (Fig. 1C).

Response of the transgenic plants expressing P50 and P50 deletion mutants to GINV infection. When transgenic (50KP, $\Delta A'$, and ΔA) and NT *N. occidentalis* plants (10 individual plants per line) were inoculated with GINV, the transgenic plants did not develop detectable symptoms in either the inoculated or upper uninoculated leaves. In contrast, the GINV-inoculated NT-plants showed chlorotic spots and mosaic symptoms in uninoculated leaves. ELISA of extracts from P50- and ΔA -plants inoculated

Fig. 3. Virus spread in nontransgenic (NT) and transgenic *Nicotiana occidentalis* plants expressing P50, ΔA , ΔC , ΔG , and $\Delta A'$ inoculated with *Grapevine berry inner necrosis virus* (GINV). Inoculated leaves (IL) and upper uninoculated leaves (UL) were harvested at 7 days postinoculation (dpi) and 14 dpi, respectively, and assayed by a direct tissue immunoblot analysis using an anti-GINV antiserum. Arrowheads in ΔC and $\Delta A'$ indicate small spots showing virus infection.

with GINV using an anti-GINV antiserum indicated that no detectable virus accumulated in inoculated leaves (14 dpi) or in uninoculated leaves (21 dpi) (Fig. 2). In contrast, all NT-plants supported virus accumulation in both inoculated and uninoculated leaves (Fig. 2). These results were consistent with previous results (11,33). Unexpectedly, in $\Delta A'$ -plants inoculated with GINV, some plants allowed virus accumulation in inoculated leaves (Fig. 2, $\Delta A'$ plants 1, 2, and 8), but others did not. However, virus accumulation was never found in uninoculated leaves, even when the plants supported virus accumulation in inoculated leaves (Fig. 2). The experiment was repeated three times and the results were essentially identical. The analysis by a direct tissue immunoblotting using an anti-GINV antiserum also indicated that GINV was detected as spots on inoculated leaves in some $\Delta A'$ -plants, but never in uninoculated leaves in all plants (Fig. 3). GINV was detected in both inoculated and uninoculated leaves in NT-plants. No virus accumulation was found in either inoculated or uninoculated leaves of P50- and ΔA -plants (Fig. 3). In the case of ΔC -plants, small spots were sometimes detected only in inoculated leaves of some plants (Fig. 3). However, virus accumulation in inoculated leaves of ΔC -plants was below the level of detection by ELISA.

Inhibition of long-distance movement of GINV in transgenic plants expressing $\Delta A'$. To examine virus distribution within NT and transgenic plants inoculated with GINV, leaf blades, midribs, and petioles of inoculated leaves at 14 dpi and uninoculated leaves at 21 dpi were analyzed by a tissue blot hybridization analysis. Stems at 21 dpi cut from the plants were also subjected to hybridization analysis. In NT-plants, strong hybridization signals were found in leaf blades, midribs, and petioles of both inoculated and uninoculated leaves, and in stems (Fig. 4). No signals were detected from any leaf tissues and stems of P50- and ΔA -plants inoculated with GINV. The results coincided with those obtained from ELISA and the direct tissue immunoblot analysis described above.

When tissue samples from $\Delta A'$ -plants inoculated with GINV were analyzed, positive signals were found in inoculated leaves of 7 of 10 inoculated plants (Fig. 4, plants 1 and 5 to 10). In one particular plant (plant 7), strong signals with almost the same intensity as those of NT-plants were found in the leaf blade and midrib of an inoculated leaf. In this experiment, GINV was detected in midribs from only two plants (plants 5 and 7), and in petioles from three plants (plants 5, 7, and 9). The signals from petioles of inoculated leaves were weak compared with those from NT-plants. Our attempts to detect GINV in stems or in tissues of uninoculated leaves of $\Delta A'$ -plants failed including plant 7, which showed strong hybridization signals from its inoculated leaves (Fig. 4). These results indicated that in some $\Delta A'$ -plants, GINV could spread by cell-to-cell movement within inoculated leaves, but long-distance movement from inoculated leaves to uninoculated leaves was completely inhibited.

GINV distribution in inoculated leaves of transgenic plants expressing $\Delta A'$. We next investigated the distribution of GINV in leaf tissues of NT-, P50-, and $\Delta A'$ -plants by an immunohistochemical analysis using an anti-GINV antiserum. Samples for thin sections were prepared from leaf blades, midribs, and petioles of inoculated leaves at 7 and 14 dpi and of uninoculated leaves at 21 dpi. In cross sections of leaf blades from NT-plant leaves at 7 dpi, GINV was already distributed in the epidermal cells and palisade and spongy parenchyma cells (Fig. 5A) and in bundle sheath cells and phloem cells in minor veins (class IV) of *N. occidentalis* leaves (Fig. 5F). In cross sections from midribs of NT-plant leaves at 14 dpi, a strong immunostaining signal was often detected in vascular bundle cells near abaxial phloem (Fig. 5B). In uninoculated leaves at 21 dpi, virus was detected from leaf mesophyll cells, bundle sheath cells and phloem cells in minor veins, and vascular bundle cells of midribs and petioles (data not shown). In contrast, no signals were found in any types

of tissues from inoculated and uninoculated leaves of P50-plants (data not shown).

In the analysis of inoculated leaves of $\Delta A'$ -plants, GINV accumulation was first verified by direct tissue immunoblotting of half-leaves, and then the opposite half-leaves were fixed for immunohistochemical analysis. In the inoculated leaves of $\Delta A'$ -

Fig. 4. Tissue blot hybridization analyses of nontransgenic (NT) and transgenic *Nicotiana occidentalis* plants expressing $\Delta A'$, P50, and ΔA inoculated with *Grapevine berry inner necrosis virus* (GINV). Leaf blades (LB), midribs (MR), and petioles (P) of inoculated leaves (IL) at 14 days postinoculation (dpi) and upper uninoculated leaves (UL) at 21 dpi, and stems (S) immediately above IL at 21 dpi were analyzed using a negative-stranded RNA probe of GINV genome. Numbers indicate each individual plant inoculated with GINV.

plants at 7 dpi, virus-specific signals were found in palisade and spongy parenchyma cells and in bundle sheath cells of minor veins (Fig. 5C). Immunostaining signals were also found in bundle sheath cells near abaxial phloem in midribs of inoculated leaves at 14 dpi (Fig. 5D), which was consistent with virus distribution in NT-plant leaves. Extensive observation of leaf minor veins at 14 dpi showed that immunostaining signals were found in

phloem cells as well as in the surrounding bundle sheath cells (Fig. 5G, H, and I). The immunostained cells were determined to be phloem parenchyma cells based on cross sections of minor vein (class IV) of a healthy leaf (Fig. 4E) and previous criteria (6,29). The percentage of phloem parenchyma cells containing signals out of minor veins in which bundle sheath cells were infected with GINV was 54% (7 out of 13 phloem cells examined).

Fig. 5. Immunohistochemical analyses of leaves of nontransgenic (NT) and transgenic ($\Delta A'$) *Nicotiana occidentalis* plant leaves inoculated with *Grapevine berry inner necrosis virus* (GINV) using an anti-GINV antiserum. **A**, A thin section of leaf blade of an NT plant at 7 days postinoculation (dpi) showing the staining signals of virus infection in epidermal cells (EC), palisade parenchyma cells (PC), bundle sheath cells (BS), and spongy parenchyma cells (SC). **B**, A thin section of midrib from an NT plant (14 dpi) showing the staining signals of virus infection in BS. **C and D**, Thin sections of leaf blade (7 dpi) and midrib (14 dpi) of $\Delta A'$ plants, respectively, showing the staining signals of virus infection in BS. **E to I**, Cross section of minor vein (class IV) of a healthy leaf (**E**), an inoculated leaf of NT plant at 7 dpi (**F**), and inoculated leaves of $\Delta A'$ plants at 14 dpi (**G**, **H**, and **I**). Arrows in **G**, **H**, and **I** indicate the staining signals of virus infection in phloem parenchyma cells. C, companion cells; P, phloem parenchyma cells; S, sieve elements; X, xylem.

The results indicated that GINV could enter into phloem parenchyma cells from the surrounding bundle sheath cells in inoculated leaves. No immunostaining signals were detected in leaf blade, midribs, and petioles of upper uninoculated leaves of $\Delta A'$ -plants at 21 dpi (data not shown).

Accumulation and localization of P50 in sieve elements of transgenic and ACLSV-infected plant leaves. We previously re-

ported that P50-EGFP fusion accumulated in sieve elements (SE) and formed an extensive interconnecting network of threadlike structure in transgenic plants (35). P50-EGFP was also heavily associated with sieve plates (Fig. 6A). More detailed observation of leaf veins showed that P50-EGFP fluorescence is associated with the parietal layer of SE and clusters of intense fluorescence interconnected by threadlike structures, which were located at almost regular intervals (Fig. 6B and C). The localization of an MP in SE is reported for *Cucumber mosaic virus 3a* (CMV-3a)-GFP fusion protein (3).

The localization of P50 and its truncated derivatives in SE of transgenic *N. occidentalis* and ACLSV-infected *C. quinoa* plants was also investigated by immunogold electron microscopy using an anti-P50 antiserum. In P50-plant leaves, gold particles were often found on the parietal layer of SE (Fig. 7A and B) and on sieve plates (Fig. 7E). Gold particles were also observed in the inside of SE as small aggregates (Fig. 7C and D), but were not found on P-protein in SE. Similar localization of gold particles were observed in phloem cells of $\Delta A'$ - and ΔA -plants and ACLSV-infected *C. quinoa* plants (Fig. 7F). Thus, the accumulation and distribution of P50 in SE of transgenic and infected leaves almost coincided with those of P50-GFP examined by a confocal laser scanning microscope.

DISCUSSION

Analyses of P50- and ΔA -plants inoculated with GINV by direct tissue immunoblotting and tissue blot hybridization indicated that no virus was detected in either inoculated or uninoculated leaves of inoculated plants. These results reconfirm the previous results obtained by ELISA (11,33). Some of the $\Delta A'$ -plants supported the accumulation of GINV in inoculated leaves, though

Fig. 6. Detection of fluorescence in sieve elements (A to C) of transgenic *Nicotiana occidentalis* leaves expressing P50 fused to green fluorescent protein by a confocal laser scanning microscope. Arrowheads in B and C indicate clusters of intense fluorescence thought to be located in plasmodesmata connecting sieve element and companion cells. SP, sieve plate.

Fig. 7. Immunoelectron microscopy of transgenic *Nicotiana occidentalis* leaves expressing P50 (A to E) and *Chenopodium quinoa* leaf infected with *Apple chlorotic leaf spot virus* (ACLSV) (F) using an anti-P50 antiserum. Gold particles were observed on the parietal layer of sieve elements (SE) (A and B) and in the inside of SE (arrowheads in C and D). Gold particles were also located on sieve plates of P50 plant and ACLSV-infected plant (E and F). CW, cell wall; SP, sieve plate.

virus spread was reduced in comparison with that in NT-plants. However, long-distance movement from inoculated leaves to uninoculated leaves was completely inhibited in these $\Delta A'$ -plants. At present, we are not able to explain why only $\Delta A'$ -plants permit virus spread in inoculated leaves, because the number of deleted amino acids of $\Delta A'$ ($\Delta 416-457$) is fewer than that of ΔA ($\Delta 395-457$). Immunoblot analysis did not show large differences in the amount of expressed proteins among P50-, $\Delta A'$ -, and ΔA -plant leaves (Fig. 1). It is also unknown why only some plants from the inoculated $\Delta A'$ -plants were able to allow virus to spread in inoculated leaves and others did not. Immunoblot analysis showed that $\Delta A'$ protein was detected from both plants that allow GINV to accumulate in inoculated leaves and plants that do not allow GINV accumulation in inoculated leaves (Fig. 1C). Polymerase chain reaction analysis also showed that $\Delta A'$ -transgene with the same size was amplified from both transgenic plants (data not shown).

Plant viruses which are replicated in initially infected cells spread from cell to cell in the epidermal and mesophyll cells, and then are capable of passage through bundle sheath, vascular parenchyma, and/or companion cells into SE for systemic movement (4,16,17). The interfaces between nonvascular and vascular tissue appear to be a major barrier for systemic virus movement. In fact, the bundle sheath-phloem interface is known to be a barrier to entry into the phloem long-distance transport system for several virus-host combinations (6,7,10,14,27,28,31,32). In $\Delta A'$ -plants, GINV could not be detected from stems immediately above inoculated leaves in which virus accumulation was found (Fig. 4), suggesting that blocking of GINV systemic movement might occur either at the bundle sheath-phloem interface or between phloem cells and SE (and/or within SE) in inoculated leaves. Immunohistochemical analysis of GINV-inoculated leaves of the plants expressing $\Delta A'$ showed that GINV could invade phloem parenchyma cells through bundle sheath of minor veins (Fig. 5), suggesting that the long-distance transport of GINV might be inhibited between phloem cells and SE and/or within SE rather than between the bundle sheath-phloem interfaces.

CMV-3a protein accumulated in the parietal layer of SE (3). The P50-EGFP fusion is also accumulated in SE and is associated with sieve plates in transgenic plant leaves (35). As shown in this study, GFP fluorescence was found on the parietal layer of SE and clusters of intense fluorescence were located at regular intervals (Fig. 6). The results were confirmed by immunogold electron microscopy of transgenic and infected plant leaves in which gold particles were often found on the parietal layer of SE and sieve plates (Fig. 7). As P50 itself has a specific activity for cell-to-cell trafficking (24), the proteins produced in the outside cells surrounding phloem cells and/or in the phloem parenchyma and companion cells may move into SE and are associated with the parietal layer of SE. Movement proteins in the parietal layer of SE may play a role to facilitate the long-distance movement of the virus in SE.

P50 may bind to GINV-P39 directly or indirectly and interferes with the intracellular and intercellular trafficking of P39 in leaf epidermal cells (11). It therefore seems likely that the movement and function of P39 will be blocked by P50 on the parietal layer of SE, even if GINV could invade into phloem parenchyma cells, companion cells, and SE through bundle sheath in inoculated leaves of $\Delta A'$ -plants. Consequently, virus trafficking within SE may be hindered in $\Delta A'$ -plant leaves.

In this paper, we show that $\Delta A'$ -plants inhibit the long-distance movement of GINV from inoculated leaves to uninoculated leaves. P50 expressed in transgenic plant leaves might not only interfere with the cell-to-cell movement of GINV, but might also block its systemic trafficking through SE. Even if GINV overcomes the inhibition of cell-to-cell movement in inoculated leaves as shown in $\Delta A'$ - and ΔC -plants (Fig. 3), the P50 accumulated on the parietal layer of SE can invalidate the functions of P39 and inhibit long-distance movement of the virus. This is why the

resistance is highly effective and systemic movement of GINV was never observed in transgenic plants expressing P50. The mechanism described here will lead to the design of new virus resistance strategies in plants.

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