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The Multifunctional Roles of *Apple chlorotic leaf spot virus* 50KP Movement Protein

Masamichi Isogai • Hajime Yaegashi • Nobuyuki Yoshikawa*

Laboratory of Plant Pathology, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka 020-8550, Japan

Corresponding author: * **yoshikawa@iwate-u.ac.jp**

ABSTRACT

Diverse plant virus families encode movement proteins (MPs) in their genomes. MPs are required for cell-to-cell movement of the virus in host plants and modify plasmodesmata in the cell wall, allowing cell-to-cell movement of virus particles or infectious transcripts. In recent years, much progress has been made in determining cell-to-cell movement functions of MPs from herbaceous plant viruses. On the other hand, only a few studies have examined MPs of viruses infecting woody hosts. *Apple chlorotic leaf spot virus* (ACLSV), the type species of the genus *Trichovirus,* is graft-transmissible and causes topworking disease, which induces lethal decline in apple trees grown on Maruba kaido (*Malus prunifolia* var. *ringo*) rootstocks. ACLSV encodes a MP with a mol. wt of 50 kDa (50KP). In this review, we summarize the multifunctional roles of ACLSV MP which has the following characteristics: 1) it localizes to plasmodesmata in infected and transgenic cells, 2) it can spread from cells that initially produce it into neighboring cells, 3) it enables cell-to-cell trafficking of green fluorescent protein (GFP) when 50KP and GFP are co-expressed in the leaf epidermis, 4) it induces the production of tubular structures protruding from the surface of protoplasts, 5) it has two independently active, single-stranded nucleic acid binding domains, 6) it interferes specifically with the functions of the MP encoded by *Grapevine berry inner necrosis virus*, and 7) it acts as a suppressor of systemic silencing without interfering with local silencing, probably by inhibiting the movement of silencing signals.

Keywords: ACLSV, cell-to-cell movement, *Flexiviridae,* MP, MP-derived resistance, RNA-binding domain, RNA silencing suppressor, *Trichovirus*

CONTENTS

INTRODUCTION

Plant viruses replicate their genomes within cells surrounded by impregnable barriers – plant cell walls. To expand viral infections, plant viruses must move from an infected cell to a neighboring cell beyond cell walls (cell-to-cell movement) until they enter the vascular system, which allows rapid movement to distant parts of the plants (long-distance movement). It is generally accepted that cell-to-cell movement proteins (MP) encoded by plant virus genomes localize on plasmodesmata (Pd) and modulate the size exclusion limit (SEL) of the Pd, allowing cell-to-cell movement of viruses (Carrington *et al*. 1996; Lazarowitz and Beachy 1999). Presently, two mechanisms of cell-to-cell movement have been proposed. In the first type (type 1), the MP interacts with RNA to form a MP-RNA complex that is able to transport virus genome to adjacent cells, e.g., *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV). Recent studies have proposed that TMV moves across plasmodesmata as virus replication complexes that contain MPs, viral replicase and genomic RNAs (Kawakami *et al*. 2004), and that its replicase protein affects cell-to-cell movement (Knapp *et al*. 2005). Additionally, coat protein (CP) is essential for the cell-to-cell movement of CMV, although virion assembly is not required (Kaplan *et al*. 1998). In the second type (type 2), mature virions are transported through viral MP-containing tubules that are assembled inside the plasmodesmal pore, e.g., *Cowpea mosaic virus* (CPMV). This type of cell-to-cell movement is called tubule-guided virion movement (van Lent *et al*. 1990; Kasteel *et al*. 1993; Perbal *et al*. 1993; Storms *et al*. 1995; Wieczorek and Sanfaçon 1993). The morphology of the virion-containing tubule suggests that tubule assembly from MP molecules and entrapment of the virion takes place

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Invited Mini-Review

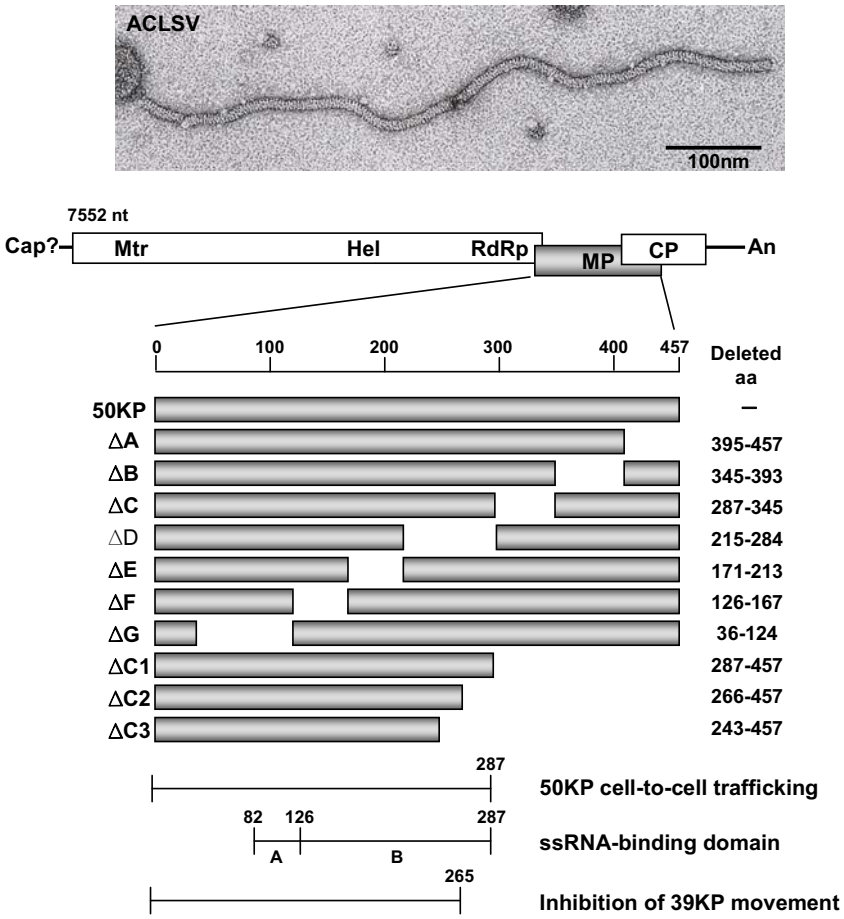

Fig. 1 Particle and genome organization of ACLSV, and a summary of ACLSV MP (50KP) deletion mutants. The regions of the wild-type 50KP that are represented in each 50KP deletion derivative are indicated by gray bars. Amino acid residues 1-287 and 1-267 are essential for 50KP cell-to-cell trafficking and inhibition of 39KP movement, respectively. ssRNA-binding domains A (amino acid residues 82-125) and B (amino acid residues 126-287) have potential to interact with ssRNA.

simultaneously at or near the plasma membrane (Carvalho *et al*. 2003). It has been shown that the MP is a component of the tubule structure (van Lent *et al*. 1990, 1991) and is bound specifically to CPMV virions, and the MP engages in self interactions (Carvalho *et al*. 2003). Recently, Sánchez-Navarro and associates (2006) showed that CPMV MP transports *Alfalfa mosaic virus* (AlMV) genomes to adjacent cells using the type1 movement mechanism when CPMV MP is modified for binding to AlMV CP. Thus, in viruses classified into the type 2 movement group, both type 1 and type 2 movement mechanisms may co-exist, and the host or tissue type may determine the mechanisms used by the virus (Nurkiyanova *et al*. 2001; Carvalho *et al*. 2004; Isogai *et al*. 2006; Sánchez-Navarro *et al*. 2006). As mentioned above, in recent years remarkable progress has been made in the elucidation of virus cell-to-cell movement. However, only a few studies have examined MPs of viruses infecting woody hosts, although some orchard infecting viruses cause critical problems for fruit production.

Apple chlorotic leaf spot virus (ACLSV) is distributed worldwide in fruit trees including apple, peach, pear, plum, cherry and apricot (Lister 1970; Németh 1986; Yoshikawa 2001) and is graft-transmissible (Yanase 1974). In Japan, ACLSV is well known as one of the causative agents of apple topworking disease, and induces lethal decline in apple trees grown on Maruba kaido (*Malus prunifolia* var. *ringo*) rootstock. Additionally, top grafting is a method most often used for renewal of a variety in apple trees even today. Thus, the disease had spread to healthy apple trees and had caused severe damage to them until the cause of this disease was elucidated. ACLSV is classified in the type species of the *Trichovirus* genus, *Flexiviridae* family (Fauquet *et al*. 2005). ACLSV has a flexuous filamentous particle (740 to 760 nm in length and 12 nm in width) and contains a single RNA species and a single coat protein (Yoshikawa and Takahashi 1988) (**Fig. 1**). The ACLSV genome contains three open reading frames which encode a replication-associated protein (216KP), MP (50KP), and CP, respectively (**Fig. 1**; German *et al*. 1990; Sato *et al*. 1993).

When scions are infected with graft-transmissible viruses, stocks joined to them are forced to be infected with the viruses, which move into every part of the plants. Thus, top grafting must be handled carefully, especially when scions infected with graft-transmissible viruses show no symptoms because of different cultivar response to viral infection. To understand the mechanisms of virus movement seems to be useful to prevent the viruses from spreading by top grafting. Here we review the multifunctional roles of 50KP as movement protein and RNA silencing suppressor obtained in the last ten years.

AMINO ACID SEQUENCE

The ACLSV (isolate P-205) genome consists of 7552 nucleotides and contains three open reading frames which encode a replication-associated protein, MP, and CP, respectively (**Fig. 1**; Sato *et al*. 1993). In this review, the ACLSV MP is referred to as 50KP. The 50KP-coding region begins at AUG (nucleotides 5727-5729) and terminates at UGA (nucleotides 7098-7100). The protein consists of 457 amino acids, and the predicted molecular mass is 50453 Da. *In vivo* detection of 50KP by immunoblotting, besides a 50 kDa protein, a 52 kDa protein is also detected in the infected *Chenopodium quinoa*. The 52 kDa protein is larger than that predicted by the encoding 50KP ORF (Sato *et al*. 1995). The 52 kDa protein extracted from infected tissues treated with alkaline phosphatase results in a decrease of its molecular mass from 52 kDa to 50 kDa. The results suggest that 50KP is translated *in vivo*, and a part of 50KP is phosphorylated in infected tissues.

Many viral MPs have been assigned to the 30K superfamily independently of the mechanisms of virus cell-to-cell movement (Melcher 2000). The 50KP also has been assigned to the 30K superfamily by comparative sequence analysis (Koonin 1991), and has the principal conserved motif (amino acids residues 85 to 117) in the vast superfamily of the plant MP (Mushegian and Koonin 1993). In the principal conserved motif, one aspartic acid, referred to

as the D motif, is almost absolutely conserved (Koonin *et al*. 1991). An aspartic acid at amino acids residue 112 of 50KP is assigned to the D motif.

SUBCELLULAR LOCALIZATION

In immunoblot analysis, the 50KP is detected in cell wall fraction prepared from infected *C. quinoa* tissues (Sato *et al*. 1995). We tried to examine the intracellular localization of 50KP by immunogold electron microscopy (Yoshikawa *et al*. 1999). Immunoelectron microscopy of ultrathin sections of ACLSV-infected *C. quinoa* leaves using an antibody against 50KP showed that the 50KP was distributed on and near plasmodesmata. These results are convincing in theory and show that plant viruses move from cell-to-cell via plasmodesmata. The ACLSV virions and their associated tubules passing through plasmodesmata are never observed in cell walls (Yoshikawa *et al*. 1999). It is speculated that the ACLSV genome is transported as MP-RNA complex using type 1 transferring mechanisms (see Introduction).

In order to analyze 50KP subcellular localization by a non-destructive test, transgenic *Nicotiana occidentalis* plant leaves expressing 50KP fused to green fluorescent protein (50KP-GFP plant leaves) were observed using fluorescence and confocal laser scanning microscopes (Yoshikawa *et al*. 1999). The 50KP-GFP fluorescence was detected as spots on the cell wall in the epidermal cells (**Fig. 2**). Careful observation by CLSM revealed that the fluorescence spots of 50KP-GFP were visible as strands passing through the cell wall. To test whether the spots were located on plasmodesmata, we detected callose in cell wall using a mouse monoclonal antibody against β-1,3 glucan and an anti-mouse secondary antibody conjugated to rhodamine. The 50KP-GFP fluorescence spots (the GFP fluorescence) and callose fluorescence spots (the rhodamine fluorescence) were co-localized, indicating that the 50KP-GFP fusion targeted to plasmodesmata in the same way as described for 50KP subcellular localization of the ACLSV infected plants. Furthermore, the 50KP-GFP accumulated in sieve elements (SE) and seemed to form an extensive interconnecting network of threadlike structures (**Fig. 2**). The 50KP-GFP was associated with sieve plates as well. More detailed observation of leaf veins showed that the 50KP-GFP fluorescence was associated with the parietal layer of SE and clusters of intense fluorescence interconnected by threadlike structures, which were located at almost regular intervals. The localization of 50KP in SE of transgenic *N. occidentalis* plants expressing 50KP (50KP plants) and ACLSV-infected *C. quinoa* plants was also investigated by immunogold electron microscopy using anti-50KP antiserum (Yoshikawa *et al*. 2006). In 50KP plants and ACLSV-infected *C. quinoa* plants, gold particles were often found on the parietal layer of SE, sieve plates, and the inside of SE as small aggregates. Thus, the accumulation and distribution of 50KP in SE of 50KP plants and ACLSV-infected plants almost coincided with those of 50KP-GFP examined using a confocal laser scanning microscope. Therefore, it is conceivable that the 50KP in the parietal layer of SE plays a role in facilitating the long distance movement of the virus as well as the cell-to-cell movement.

CELL-TO-CELL MOVEMENT AND INCREASING THE PLASMODESMATAL SIZE EXCLUSION LIMIT

Cell-to-cell trafficking of MP-GFP expressed transiently in leaf epidermis has been reported in CMV and AlMV, in which the protein moves into neighboring cells from originally transfected cells (Itaya *et al*. 1997; Hung and Zhang 1999).

To demonstrate cell-to-cell trafficking of 50KP, the 50KP-GFP were transiently expressed under the control of the 35S promoter in the epidermis of *N. occidentalis* (Satoh *et al*. 2000). Fluorescence microscopy showed that the 50KP-GFP (ca. 77kDa) move from the cells that produce it into the neighboring cells in mature leaves, and in contrast,

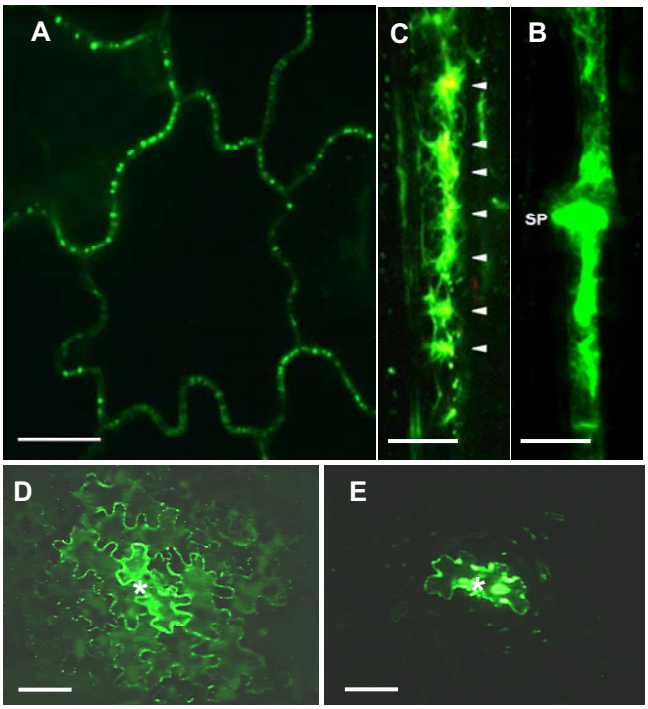

Fig. 2 Analysis of movement proteins fused to GFP using fluorescence and confocal laser scanning microscopes. A-C: Detection of 50KP fused to GFP (50KP-GFP) in leaf epidermal cells (**A**) and sieve elements (**B** and **C**) of transgenic *Nicotiana occidentalis* plants expressing 50KP-GFP by a confocal laser scanning microscope. Arrowheads in **C** indicate clusters of intense fluorescence thought to be located next to plasmodesmata connecting sieve element and companion cells. SP, sieve plate. **D** and **E**: Fluorescence of GINV 39KP fused to GFP expressed transiently in leaf epidermis of nontransgenic (**D**; NT plant) and transgenic *Nicotiana occidentalis* expressing 50KP (**E**; 50KP plant) by a fluorescence microscope. The 39KP-GFP was confined in a single cell and formed aggregates in cytoplasm in 50KP plant (**E**), in contrast to 39KP-GFP in NT plant showing cell-to-cell movement (**D**). Asterisks indicate cells originally producing 39KP-GFP. Scale bars represent 20 μm (**A-C**) and 50 μm (**D**, **E**).

free GFP remained in single cells. At higher magnification, the 50KP-GFP in cells was detected as spots or strands passing through the cell wall, indicating that fluorescence was located at the plasmodesmata, as reported for transgenic *N. occidentalis* plants expressing 50KP-GFP (see Subcellular Localization). Molecules with a mass of <1 kDa can passively diffuse through plasmodesmata under normal conditions (Carrington *et al*. 1996). Thus, these results suggest that 50KP-GFP may increase the SEL of the plasmodesmata up to at least 77 kDa and traffic itself through these plasmodesmata.

To further investigate whether 50KP can facilitate the transport of other molecules, free GFP (27 kDa) were co-expressed with 50KP transiently in the epidermis of *N. occidentalis* (Satoh *et al*. 2000). The fluorescence of GFP spread more widely from the cells that initially produced it when co-expressed with 50KP. In contrast, GFP cell-to-cell movement was rare when GFP was singly expressed as described above. Thus, we showed that 50KP could facilitate transport of other molecules as well as its own cell-to-cell movement. It can be concluded from these data that the 50KP plays an important role in viral movement: the 50KP increases SEL and transfers the viral genome complex to remote uninfected cells.

TUBULE-INDUCING ACTIVITY

In the type 2 cell-to-cell movement, mature virions are transported through viral MP-containing tubules that are assembled inside the plasmodesmata pore (see Introduction). In addition, the tubules protruding from infected protoplast were also observed in the type 2 trafficking. Thus, the tub-

ules in infected protoplast seem to be related to the type 2 trafficking mechanism. However, some viral MPs that perform the type 1 trafficking form tubules on the surface of protoplasts, although tubular structure is not observed in plasmodesmata of virus-infected tissues (Kasteel *et al*. 1997; Canto and Palukaitis 1999). The 50KP-GFP also induces the production of fluorescent tubular structures protruding from the surface of protoplasts (Satoh *et al*. 2000). The reason these type1 viral MPs assembles the tubules in protoplasts has not yet been revealed.

We analyzed the correlations among the abilities of 50KP to assemble tubules in protoplasts, to localize plasmodesmata, and to traffic itself from cell to cell (Satoh *et al*. 2000). We also constructed truncated forms of 50KP (**Fig.1**; ΔA to ΔG). When protoplasts were transfected with the mutants fused to GFP, ΔA, ΔB and ΔC formed fluorescence tubular structure, but ΔD, ΔE, ΔF and ΔG did not. In addition, ΔA, ΔB and ΔC retained their abilities to move from cell-to-cell and to localize to plasmodesmata, but ΔD, ΔE, ΔF and ΔG did not retain these abilities. Furthermore, we examined complementation of cell-to-cell movement of 50KP-defective virus when the 50KP mutants were expressed transiently in leaf epidermis. In advance, we revealed that the 50KP-deficient mutant (pΔStuNhe) of infectious ACLSV cDNA clone (pCLSF) was found to replicate in protoplast from *C. quinoa* leaves and was complemented in the viral cell-to-cell movement in transgenic *N. occidentalis* plants expressing 50KP (50KP plants) (Satoh *et al*. 1999; Yoshikawa *et al*. 2000). Similar complementation of the 50KP-deficient virus was found in leaves co-bombarded with ΔA, ΔB and ΔC, but not with ΔD, ΔE, ΔF and ΔG. These mutational analyses indicate that the 50KP domain that is necessary to form tubular structure is in accord with the domain that is necessary for localization to plasmodesmata, cell-to-cell movement of the 50KP, and the ACLSV cell-to-cell movement.

RNA-BINDING ACTIVITY

The 50KP localizes to plasmodesmata within virus-infected cells without formation of tubules; virions are not observed in cell-wall plasmodesmata. Additionally, the 50KP function as follows: 1) the 50KP spread from the cells that produce the protein into neighboring cells, 2) the 50KP facilitate transport of other molecules. Therefore, the ACLSV genome is thought to transport from cell-to-cell as a 50KP-RNA complex that uses the ability of the 50KP to move cell to cell (type 1 mechanism). In type 1, it is reasonable for the viral MP to have the ability to bind RNA because the MP directly transfers virus genomes or viral transcripts to adjacent cells.

To analyze RNA-binding properties of 50KP, the protein was expressed in *Escherichia coli* and was used in UV cross-linking analysis using a digoxigenin-UTP-labelled RNA probe and gel retardation analysis (Isogai and Yoshikawa 2005). The analyses demonstrated that 50KP bound cooperatively to ssRNA. Most of the 50KP could bind to ssRNA in binding buffer with 1 M NaCl concentration when analyzed for dependence of the RNA-binding activity of the MP on NaCl. Furthermore, competition binding experiments showed that 50KP preferentially bound to ssRNA and ssDNA without sequence specificity. The 50KP deletion mutants were used to identify the RNA-binding domain by UV cross-linking analysis. The regions between amino acid residues 82 to 126 and 127 to 287 potentially contain two independently active single-stranded nucleic acid binding domains (**Fig. 1**). Thus, 50KP could transfer viral RNA to adjacent cells if the 50KP-binding viral RNA sustained the ability to move from cell-to-cell. The 50KP and TMV MP are only two examples containing two adjacent RNA-binding domains, even though *Apple latent spherical virus* MP contains at least three independent single-stranded nucleic acid-binding domains (Citovsky *et al*. 1992; Isogai *et al*. 2006).

MP-DERIVED RESISTANCE

The 50KP plants complement the systemic spread of the 50KP-defective mutants of pCLSF (infectious cDNA clone of ACLSV), indicating that 50KP in the 50KP plants is functional. Severity of symptoms is greatly enhanced, and accumulation of virus in upper leaves is increased in 50KP plants inoculated with pCLSF or ACLSV compared with that in nontransgenic control plants (NT plants). However, most 50KP plants inoculated with *Grapevine berry inner necrosis virus* (GINV), another species of the genus *Trichovirus*, neither develop obvious symptoms nor support virus accumulation in inoculated or upper leaves (Yoshikawa *et al*. 2000). When viruses classified into other genera (*Apple stem grooving virus*: genus *Capillovirus*, and *Apple stem pitting virus*: genus *Foveavirus*) are inoculated, there is no difference in symptom development and virus accumulation between 50KP and NT plants. Our results indicate that transgenic plants expressing a functional 50KP are more susceptible to a homologous virus, and on the contrary, show strong resistance specific to GINV, another species of the genus *Trichovirus*. Transgenic plants expressing viral MP are reported to be resistant to homologous and heterologous viruses (Isogai *et al*. 2003). The phenomenon is called MP-derived resistance. However, there is no report of the MP-derived resistance specific to another species in the same genus, except that 50KP plants show resistance specific to GINV.

Inhibition of GINV 39KP cell-to-cell movement

To understand why 50KP plants show specific resistance to GINV, we first investigated the behavior of the GINV MP fused to GFP (39KP-GFP) transiently expressed in NT- and 50KP plant cells (Isogai *et al*. 2003). In NT plants, 39KP-GFP spread from initial proteins producing cells into neighboring cells in leaf epidermis (**Fig. 2**). However, when expressed in cells of 50KP plants, 39KP-GFP formed aggregates in the cytoplasm, and its normal intercellular trafficking was strongly blocked (**Fig. 2**). In contrast, cell-to-cell trafficking of 50KP-GFP was never disturbed in 50KP plant cells. Thus, it is reasonable to conclude that 50KP expressed in transgenic plant cells specifically blocked 39KP cell-to-cell movement.

In order to monitor the cell-to-cell trafficking of both 39KP and 50KP simultaneously, we used two different fluorescent proteins [yellow and cyan fluorescent proteins (YFP and CFP)]. When 39KP-YFP was co-expressed with 50KP-CFP in leaf epidermis of NT plants, the fluorescence of both 39KP-YFP and 50KP-CFP was restricted to originally bombarded single cells in about 90% of the fluorescent cells. Both YFP and CFP fluorescence cells were found in the same cells, and they formed aggregates similar to those found in epidermal cells from the 50KP plant that transiently expresses 39KP-GFP. The spatial distribution of 39KP-YFP aggregates are consistent with those of 50KP-CFP, indicating that 39KP and 50KP colocalize in the same sites of the cytoplasm. The cell-to-cell trafficking of 39KP-YFP was never inhibited when the protein was coexpressed with ACLSV CP fused to CFP, and the 50KP-YFP could spread normally when coexpressed with GINV CP. These results indicate that 39KP-YFP and 50KP-CFP specifically interfere with cell-to-cell trafficking of each other in leaf epidermis.

Mapping 50KP domain involved in the inhibition of 39KP cell-to-cell movement

To analyze the 50KP domain involved in the interference of 39KP movement, a series of 50KP deletion mutants fused to CFP (Fig. 1; ΔA-CFP to ΔG-CFP) were used (Isogai *et al*. 2003). Both YFP and CFP fluorescence was confined within single cells in about 80% of the cases in which both CFP and YFP were present as aggregates in the same sites in the cytoplasm in leaf epidermis of NT plants when 39KP-YFP

was coexpressed with ΔA-CFP, ΔB-CFP, or ΔC-CFP. In contrast, the mutants (ΔD-CFP, ΔE-CFP, ΔF-CFP, and ΔG-CFP) did not affect the movement of 39KP-YFP. These results suggest that the amino acid residues 1-286 (ΔC1) are involved in the interference of 39KP movement (**Fig. 1**). To further identity the 50KP domain of inhibition of 39KP cell-to-cell trafficking, we constructed other deletion mutants expressing ΔC1-CFP, ΔC2-CFP, and ΔC3-CFP (**Fig. 1**). ΔC1-CFP spread from the cells that produced it into neighboring cells and was targeted to plasmodesmata, whereas ΔC2-CFP and ΔC3-CFP were restricted to single cells and formed aggregates. In coexpression experiments, the cell-to-cell trafficking of 39KP-YFP was blocked in the presence of ΔC1-CFP and ΔC2-CFP, but not in the presence of ΔC3-CFP. Thus, we revealed that amino acid residues 1-265 of 50KP (ΔC2) inhibit the 39KP cell-to-cell movement (**Fig. 1**).

The relationship between inhibition of 39KP cell-to-cell trafficking and resistance against GINV

To clarify the relationship between the 50KP's role in hindering cell-to-cell trafficking of 39KP and its role in the resistance against GINV seen in plants expressing 50KP, we constructed transgenic plants expressing 50KP deleted proteins (ΔA, ΔC, and ΔG), and then analyzed their responses against inoculations of ACLSV and GINV (Isogai *et al*. 2003). The transgenic plants expressing 50KP, ΔA, ΔC, or 50KP-GFP did not develop symptoms after inoculation with GINV. In contrast, plants expressing ΔG or GFP showed typical symptoms of GINV. Enzyme-linked immunosorbent assays (ELISA) indicate that GINV could not accumulate in either the inoculated or upper leaves of 50KP, ΔA, ΔC, and 50KP-GFP expressing plants. However, GINV accumulated in both inoculated and upper leaves of plants expressing ΔG or GFP. All plants expressing 50KP, the deletion mutants, 50KP-GFP, and GFP were susceptible to ACLSV infection, and there were no differences among these plants in virus accumulation in inoculated and upper leaves. The results indicate that transgenic plants expressing the 50KP-deletion mutants that can block cell-to-cell trafficking of 39KP show resistance against GINV.

Inhibition of GINV long-distance movement

We constructed another transgenic *N. occidentalis* plant expressing the 50KP deletion mutant (ΔA'; deletion of the C-terminal 42 amino acids). To examine virus distribution within 10 transgenic plants expressing ΔA' (ΔA' plants) inoculated with GINV, inoculated and upper uninoculated leaves were analyzed by direct tissue immunoblot analysis, and tissue blot hybridization analysis (Yoshikawa *et al*. 2006). Unexpectedly, positive signals were detected in inoculated leaves of 7 of 10 inoculated plants, but not in the others. However, virus accumulation was never found in systemic leaves, even when the plants supported virus accumulation in inoculated leaves. These results indicate that in some ΔA' plants, GINV can spread by cell-to-cell movement within inoculated leaves, but long-distance movement from inoculated leaves to uninoculated leaves was completely inhibited, although ΔA' completely contains the ΔC2 domain that inhibits 39KP cell-to-cell movement (**Fig. 1**). Furthermore, immunohistochemical analysis of ΔA' plants leaves inoculated with GINV showed that GINV entered into phloem parenchyma cells from the surrounding bundle sheath cells in inoculated leaves, 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. Additionally, we revealed that ΔA' is accumulated in the parietal layer of SE and sieve plates by immunogold electron microscopy, similar to those of the 50KP (**Fig. 2**) (see Subcellular Localization). It therefore seems likely that the movement and function of 39KP will be blocked by the ΔA' on the parietal layer of SE, even though GINV could invade into phloem parenchyma cells, companion cells, and SE through bundle sheath in inoculated leaves of ΔA' plants. Consequently, virus trafficking within SE may be hindered in ΔA' plant leaves. However, at this time, we are not able to explain why only ΔA' plants permit virus spread and why only some plants from the inoculated ΔA' plants were able to allow the virus to spread in inoculated leaves and others were not.

RNA SILENCING SUPPRESSOR

In plants, RNA silencing functions as an immune system against viruses and transposons (Vance and Vaucheret 2001; Baulcombe 2004; Ding *et al*. 2004; Voinnet 2005; Wang and Metzlaff 2005; Wang *et al*. 2006). The pathway is initially triggered by double-stranded RNAs, which are processed into small interfering RNAs (siRNAs) of 21 to 25 nucleotides (nt) by an RNase III-like enzyme called Dicer (Hamilton and Baulcombe 1999). These siRNAs are incorporated into a protein complex called RNA-induced silencing complex (RISC), and guide the RISC to degrade target RNAs that have identical sequences to the siRNAs (Hammond *et al*. 2000). When RNA silencing is induced at one site, silencing signals spread systemically (Palauqui *et al*. 1997; Voinnet and Baulcombe 1997; Voinnet *et al*. 1998; Guo and Ding 2002; Himber *et al*. 2003), and trigger systemic silencing of target RNA in distant tissues of plants. If the silencing signals induced by virus replication spread in advance of virus movement, sequence-specific virus resistance may be established in whole plants, and the virus cannot infect systemically. To counteract RNA silencing and establish systemic infection, many viruses have evolved RNA silencing suppressors (Roth *et al*. 2004).

To investigate whether 216KP, 50KP, and/or CP encoded by ACLSV-RNA can function as a suppressor of RNA silencing, we conducted *Agrobacterium* infiltration assay in the GFP-expressing *N. benthamiana* line 16c (Yaegashi *et al*. 2007). When GFP plus 216KP, 50KP, or CP was expressed in leaves of 16c plants by agroinfiltration, none of these proteins suppressed local silencing in the infiltrated region. Subsequently, we tested whether 216KP, 50KP, or CP can interfere with the induction of systemic silencing of GFP in upper leaves of 16c plants. The results showed that systemic silencing in upper leaves induced by both single- and double-stranded RNA could be suppressed by 50KP, but not by a frame-shift mutant of 50KP, 216KP, or CP. It has been reported that short (21-22 nt) and long (25 nt) siRNAs may be involved in cell-to-cell and long-distance movement of silencing signals, respectively (Hamilton *et al*. 2002; Himber *et al*. 2003). The analysis of the accumulation level of these two classes of siRNA in leaves infiltrated with GFP plus 50KP indicated that 50KP does not inhibit the production of both short and long siRNA. In the following experiments, we investigated whether 50KP suppress systemic silencing by inhibiting the movement of silencing signals by an assay described by Guo and Ding (2002), in which the CMV-2b was locally expressed along the presumed path of movement of silencing signals. When 16c plants were simultaneously infiltrated with GFP on the tip and 50KP on the basal portion of a leaf, uninfiltrated upper leaves of 50% of infiltrated plants did not show systemic silencing of GFP at 14 dpi. All 16c plants infiltrated with 50KP on the tip and GFP on the basal portion of a leaf exhibited systemic silencing of GFP in upper leaves. These data strongly suggest that the suppression of systemic silencing by 50KP is due to inhibition of the movement of silencing signals into upper leaves, not due to suppression of the production of signals.

The suppressor activity of 50KP is relatively unusual among suppressors encoded by plant viruses reported so far, in that 50KP interferes with the spread of systemic silencing, but not with local silencing. Similar behavior for suppressors encoded by plant viruses have been reported for P1 protein of *Rice yellow mottle virus* (RYMV) and a coat protein (CP) of *Citrus tristeza virus* (CTV) (Hamilton *et al*. 2002; Himber *et al*. 2003; Lu *et al*. 2004). Interestingly, both ACLSV and CTV are fruit tree viruses, and a suppres-

sor activity that only suppresses systemic silencing might be favorable for persistent infection in fruit trees.

Although the nature of the systemic silencing signals remains to be understood (Mlotshwa *et al*. 2002; Voinnet 2005), the signals move from cell-to-cell through plasmodesmata and systemically through phloem in a manner similar to that of virus movement (Palauqui *et al*. 1997; Voinnet *et al*. 1998). Localization of 50KP in plasmodesmata and its accumulation on the parietal layer of SE and on sieve plates (Yoshikawa *et al*. 1999, 2006) may correlate with the ability of 50KP to prevent systemic silencing. Additionally, two recent papers strongly suggested that small RNA binding is a common feature of RNA silencing suppressor (Lakatos *et al*. 2006; Merai *et al*. 2006). Further study will elucidate whether the RNA binding ability of 50KP (Isogai and Yoshikawa 2005; see RNA-binding activity) contributes to the suppression of systemic silencing.

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