

Endoplasmic reticulum targeting of the *Red clover necrotic mosaic virus* movement protein is associated with the replication of viral RNA1 but not that of RNA2

Masanori Kaido\*, Yasuko Tsuno, Kazuyuki Mise, Tetsuro Okuno

Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

\* Corresponding author. Mailing Address: Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kitashirakawa, Kyoto, 606-8502, Japan. Phone: TEL: +81-75-753-6148, FAX: +81-75-753-6131. E-mail: kaido@kais.kyoto-u.ac.jp

## Abstract

*Red clover necrotic mosaic virus* (RCNMV) is a positive-strand RNA virus with a bipartite genome. The movement protein (MP) encoded by RNA2 is essential for viral movement. To obtain further insights into the viral movement mechanism, subcellular localizations of RCNMV MP fused with green fluorescent protein (MP:GFP) were examined in *Nicotiana benthamiana* epidermal cells and protoplasts. The MP:GFP expressed from the recombinant virus first appeared in the cell wall and subsequently was observed on the cortical endoplasmic reticulum (ER) as punctate spots. In contrast, the MP:GFP expressed transiently in the absence of other viral components was localized exclusively in the cell wall. Transient expression of the MP:GFP with a variety of RCNMV components revealed that the ER localization of the MP:GFP was associated with RNA1 replication, or its negative-strand RNA synthesis, but not those of RNA2 or replicase proteins *per se*. A model of RCNMV cell-to-cell movement is discussed.

Key words: cell-to-cell movement, positive-strand RNA virus, *Dianthovirus*, endoplasmic reticulum, RNA replication, divided genome

## Introduction

Plant viruses encode one or more movement proteins (MPs) to potentiate cell-to-cell and systemic movements. Research on the subcellular localizations of MPs together with other viral proteins and the genome nucleic acid has provided insights into the viral movement mechanism. A variety of MPs localize at plasmodesmata (PD), cytoplasmic channels connecting plant cells, to enlarge the size exclusion limit (SEL) of the PD and enable macromolecules to pass through it (reviewed in Lucas, 2006, and Waigmann et al., 2004). The localization of viral MPs to PD is thought to be essential for viral cell-to-cell movement (Berna et al., 1991; Tremblay et al., 2005). Since the detection of *Tobacco mosaic virus* (TMV) MP on cytoskeletal elements such as actin filaments and microtubules in tobacco protoplasts (McLean et al., 1995), experimental evidence has accumulated and these cytoskeletal elements are believed to be used as the route by which viral MPs and other viral proteins associated with movement are transported to PD (Boyko et al., 2007; Haupt et al., 2005; Kawakami et al., 2004; Liu et al., 2005; Verchot-Lubicz et al., 2007; Waigmann et al., 2004, 2007).

Besides PD and cytoskeleton, several viral MPs have been reported to localize at the endoplasmic reticulum (ER). However, the roles of the ER membranes in viral movement remain uncertain. The recombinant fusion protein of TMV MP with green fluorescent protein (GFP; MP:GFP) was detected on the cortical ER network as punctate regions inducing severe morphological changes to the ER. The TMV replicase component proteins also colocalized with MP:GFP on the cortical ER (Heinlein et al., 1995, 1998; Reichel and Beachy, 1998). These punctates also contained viral coat protein (CP) and genomic RNA and are called 'viral replication complexes' (VRCs) (Asurmendi et al., 2004; Kawakami et al., 2004; Más and Beachy, 1999). The VRCs move along actin filaments and it has been proposed that TMV moves from cell to cell in the form of such complexes (Kawakami et al., 2004; Liu et al., 2005).

However, a TMV MP mutant lacking the C terminal 55 amino acids did not form such complexes but still supported viral cell-to-cell movement (Boyko et al., 2000). This indicates that detectable levels of the TMV MP accumulation in ER are not required for cell-to-cell movement. Furthermore, a pharmacological analysis using brefeldin A (BFA) showed that the ER to Golgi transport pathway is not involved in the movement of TMV (Tagami and Watanabe, 2007; Wright et al., 2007). On the other hand, disruption of the

cortical ER with higher concentrations of BFA inhibited the PD targeting of the MP, suggesting the importance of the ER in the movement of TMV (Wright et al., 2007). Recently it has been proposed that TMV MP associates with ER and targets PD by diffusion within the ER membrane (Guenoune-Gelbart et al., 2008; Sambade et al., 2008).

Mutational analysis of *Potato virus X* (PVX) MPs (TGBp2 and TGBp3) showed that association of these proteins with the ER is important for the cell-to-cell movement of the virus (Krishnamurthy et al., 2003; Mitra et al., 2003). In a recently proposed model for PVX, TGBp2 is hypothesized to recruit TGBp3 to ER-derived granular vesicles; these vesicles align on actin filaments and are believed to play an essential role in viral movement (Ju et al., 2005, 2007; Verchot-Lubicz et al., 2007). TGBp2 and TGBp3 of *Potato mop top virus* (PMTV) also associate with ER-derived vesicles which traffic on actin filaments to PD (Cowan et al., 2002; Haupt et al., 2005; Zamyatnin et al., 2004).

*Red clover necrotic mosaic virus* (RCNMV) is a positive-strand RNA virus in the *Dianthovirus* genus of the *Tombusviridae* family. The genome consists of two RNAs. RNA1 encodes two N terminal overlapping nonstructural proteins (p27 and p88) that are required for viral RNA replication, and CP (Xiong and Lommel, 1989). RNA2 encodes a 35 kDa MP that is required for viral movement (Lommel et al., 1988; Xiong et al., 1993). Translation of RCNMV MP is thought to be linked to the replication of RNA2 (Mizumoto et al., 2006). The MP has the ability to bind single-stranded nucleic acids (Giesman-Cookmyer and Lommel, 1993; Osman et al., 1992, 1993), localizes in the cell wall (Tremblay et al., 2005) and increases the SEL of PD (Fujiwara et al., 1993). Analysis of the MP:GFP fusion protein expressed from recombinant RCNMV showed that targeting of MP to the cell wall is required for viral cell-to-cell movement (Tremblay et al., 2005).

To further investigate the movement mechanism of RCNMV, we have analyzed the subcellular localization of RCNMV MP fused with fluorescent proteins in *Nicotiana benthamiana* epidermal cells and protoplasts. We found that the localization of the fluorescent protein-tagged MP to cortical ER was associated with the replication of RNA1, but not with that of RNA2 or viral replicase component proteins (p27 and p88) *per se*. The mechanism of localization of the RCNMV MP to the ER and its possible significance is discussed.

## Results

### *Subcellular localization of RCNMV MP:GFP expressed from a recombinant virus*

pUCR1-MsG is a plasmid used to transcribe recombinant RCNMV RNA1, in which the CP gene is replaced by the gene encoding the MP:GFP (Fig. 1). MP:GFP is expressed from a subgenomic RNA transcribed with help of co-inoculated RNA2 with a frameshift mutation (Sit et al., 1998, Tremblay et al., 2005). A mixture of *in vitro* transcripts of pUCR1-MsG (R1-MsG) and pRNA2fsMP (R2fsMP, Fig. 1, Tatsuta et al., 2005) was mechanically inoculated to *N. benthamiana* leaves. Fluorescent signals of MP:GFP were first detected in the cell wall at 15 to 20 h post inoculation (hpi; Fig. 2A). No GFP fluorescence was detected in the cytoplasm and the cell wall-fluorescent signals were restricted to single cells at these time points. In addition to localization at the cell wall, small punctates started to appear in the cortical region of the cytoplasm at 18 to 24 hpi (Figs 2B). Most of the fluorescent signals were restricted to single cells at these time points. A lot of cytoplasmic punctates were detected at 20 to 28 hpi (Fig. 2C). At 24 hpi and later, larger cortical fluorescent punctates appeared (Fig. 2D). Those cells with larger punctates contained fewer punctates in the cortical cytoplasm than the cells with smaller punctates (compare Figs 2C and 2D). In those cells, fluorescent signals were often observed in the cell wall of the neighboring cells (data not shown). By 30 hpi, more than 90 % of fluorescent foci were composed of multiple cells (data not shown), showing that the MP:GFP fusion protein was functional for viral cell-to-cell movement. Filamentous fluorescence representing cytoskeletal localization of MP:GFP was not detected (data not shown).

MP:GFP localization was also investigated in *N. benthamiana* protoplasts. Small punctates started to appear near the surface of protoplasts, probably toward the internal surface of the plasmalemma, at 12-18 hpi (Fig. 2E, see also serial sections) and they grew larger by 24 hpi (Figs 2F and 2G). At 30 hpi, about 10% of the infected protoplasts showed string-like structures protruding from the surface (Fig. 2H). Large punctates were often detected around the nucleus at 24 hpi (data not shown).

### *Subcellular localization of RCNMV MP:GFP expressed transiently in the absence of other viral components*

In the previous section we showed that RCNMV MP:GFP expressed from the recombinant virus (R1-MsG + R2fsMP) formed fluorescent punctates both in the cell wall and the cortical region of cytoplasm. To examine the subcellular localization of MP:GFP in the absence of other viral components, we used *Agrobacterium*-mediated expression system. Agroinfiltration of pBICRM<sub>s</sub>G (Fig. 1) that expresses MP:GFP from the *Cauliflower mosaic virus* (CaMV) 35S promoter induced fluorescent punctates exclusively in the cell wall but not in the cytoplasm (Fig. 3A, upper right panel). The signals in the cell wall started to appear at 24 hpi and disappeared by 90 hpi. No cytoplasmic fluorescence was observed at any time points before 24 hpi (data not shown). This result differs from the results reported for TMV MP:GFP fusion protein in transient expression assay, in which TMV MP:GFP produced cytoplasmic fluorescence on the cortical ER as well as in the cell wall (Crawford and Zambryski, 2000; Heinlein et al., 1998).

Agroinfiltration of pBICR1/MsG2fsMP (Fig. 1) that expresses both R1-MsG and R2fsMP induced fluorescent punctates in both cytoplasm and cell wall (Fig. 3A, upper left panel) as observed in leaves mechanically inoculated with a mixture of R1-MsG and R2fsMP (see Fig1C). Fluorescent signals were observed at 28 hpi in a limited number of cells and later spread to the surrounding cells (data not shown). The transition pattern of MP:GFP localization in the epidermal cells was also similar to that observed in inoculation of a mixture of R1-MsG and R2fsMP (data not shown).

Subsequently, we prepared protoplasts from the agroinfiltrated leaves. About 30% of protoplasts prepared from pBICR1/MsG2fsMP-infiltrated leaves showed cortical fluorescence, whereas protoplasts from pBICRM<sub>s</sub>G-infiltrated leaves showed no fluorescence (Fig. 3A, lower panels). Accumulation of MP:GFP in these protoplasts was below the limit of detection by western blot analysis (Fig. 3B), showing that little, if any, MP:GFP accumulated in the cytoplasm of pBICRM<sub>s</sub>G-infiltrated cells.

#### *Formation of cytoplasmic fluorescent punctates in transient expression of RCNMV MP:GFP is associated with the replication of RCNMV RNA1*

It is obvious from Figure 3 that RCNMV MP:GFP by itself does not have the ability to stay and form large complexes in the cytoplasm. Therefore, cytoplasmic localization of

RCNMV MP:GFP expressed from the recombinant virus seemed to require other viral factor(s) or virus replication processes itself.

First, to investigate what viral factor(s) might be responsible for the cytoplasmic localization of MP:GFP, we coinfiltrated *Agrobacterium* containing pBICRM5G together with *Agrobacterium* containing a variety of RCNMV components into *N. benthamiana* leaves. Cytoplasmic fluorescent punctates were easily detected in epidermal cells in the leaves coinfiltrated with pBICR12fsMP (Fig. 1) that expresses both wild type (wt) RNA1 and R2fsMP (Fig. 4A-2). This shows that transiently expressed-MP:GFP also induced cytoplasmic fluorescence in association with RCNMV multiplication. About 5% of the epidermal cells showed cytoplasmic fluorescent punctates at 30 hpi and more than 20% at 48 hpi (data not shown). Similar results were obtained following coinfiltration with pBICRC1 (Figs 1 and 4A-3). Because pBICRC1 expresses only wt RNA1, this result indicates that RNA1 was responsible for the formation of fluorescent punctates of the MP:GFP in the cytoplasm, and CP, which is not expressed without RNA2 (Sit et al., 1998; Tatsuta et al., 2005), was dispensable.

Next to investigate whether the replicase component proteins alone could induce the cytoplasmic fluorescent punctates, we used pBICp27 and pBICp88, which express p27 and p88, respectively (Fig. 1, Takeda et al., 2005). Coinfiltration with pBICp27 or pBICp88 or both, failed to induce fluorescent punctates in cytoplasm (Fig. 4A-5 and data not shown). These results suggest that the replicase component proteins *per se* are insufficient to induce the cytoplasmic fluorescent punctates of MP:GFP.

Next, to investigate whether replication-deficient RNA1 could induce the cytoplasmic localization of MP:GFP, we used pBICRC1GVD that expresses a mutant RNA1 with the GDD motif replaced with GVD in p88 (Fig. 1 and see Takeda et al., 2005). Coinfiltration of pBICRC1GVD did not induce cytoplasmic fluorescent punctates (Fig. 4A-4). Thus, the results described above suggest the association of the viral RNA replication with the cytoplasmic localization of MP:GFP.

The above coinfiltration assay results suggest that the cytoplasmic localization of MP:GFP is associated with the replication of RCNMV RNA1. To test whether the replication of RNA2 also could induce cytoplasmic fluorescent punctates in the cytoplasm, pBICp27, pBICp88 and pBICRC2fsMP (Fig. 1, Takeda et al., 2005) were coinfiltrated with pBICRM5G. Interestingly and surprisingly, no cytoplasmic fluorescent punctates were detected (Fig. 4A-6). Thus, the cytoplasmic localization of MP:GFP is

likely associated with the replication of RCNMV RNA1, but not RNA2.

*Accumulation levels of RCNMV MP:GFP, p27 and negative-strand RNA2 are not related to the induction of cytoplasmic fluorescent punctates*

The above results leave the possibility to be addressed that the accumulation levels of viral proteins or RNAs might be responsible for differential distribution patterns of MP:GFP fluorescence. To test this, we analyzed the accumulation of MP:GFP, p27 and viral RNAs. Western blot results showed that the level of MP:GFP accumulation was similar in all the coinfiltrated leaves (Fig. 4B, lanes 1 to 6). The accumulation levels of p27 were similar in the leaves coinfiltrated with pBICRA12fsMP, pBICRC1, pBICp27+pBICp88 and pBICp27+pBICp88+pBICRC2fsMP (Fig. 4B lanes 2, 3, 5 and 6), but were less than half in the pBICRC1GVD-coinfiltrated leaves (Fig. 4B, lane 4). These results suggest that appearance of MP:GFP in the cytoplasm is not related to the accumulation levels of MP:GFP and p27. Although we failed to detect p88 protein probably because of its low accumulation in plant cells, the accumulation levels of negative-strand RNA2 in the leaves coinfiltrated with pBICp27+pBICp88+pBICRC2fsMP and pBICRA12fsMP (Fig. 4B, lanes 2 and 6) suggest the comparable levels of p88 in these leaves. These results also support that the replication of RNA2 is not related to the induction of cytoplasmic fluorescent punctates of MP:GFP.

*Formation of cytoplasmic fluorescent punctates by the transiently expressed RCNMV MP:GFP is associated with the synthesis of negative-strand RNA1*

To investigate which step in RCNMV RNA1 replication is important for the induction of the cytoplasmic fluorescent punctates, two pBICRC1 derivatives containing deletions in either the 32 or 52 non-coding region of RNA1 (pBICRC1-3D and pBICRC1-5D, Fig. 1) were used. R1-3D expressed from pBICRC1-3D cannot serve as a template for negative-strand RNA1 synthesis (Iwakawa et al., 2007). R1-5D expressed from pBICRC1-5D can serve as a template for negative-strand RNA1 synthesis but cannot lead to the production of positive-strand RNA1 (M. Tsukuda and T. Okuno, unpublished result). Coinfiltration with pBICRC1-3D did not induce cytoplasmic fluorescent



punctates (Fig. 5A, panel 4). On the other hand, coinfiltration with pBICRC1-5D induced cytoplasmic fluorescent punctates similar to those observed with pBICRC1 (Fig. 5A, panels 2 and 3). Thus, the synthesis of negative-strand RNA1 was sufficient for the induction of cytoplasmic fluorescent punctates. In these leaves the accumulation levels of MP:GFP were similar (Fig. 5B). The accumulation level of negative-strand RNA1, was below the limit of detection in pBICRC1-3D-coinfiltrated leaves (Fig. 5B, lane 4). The accumulation level of p27 in pBICRC1-3D-coinfiltrated leaves was less than half of that in pBICRC1 or pBICRC1-5D-coinfiltrated leaves (Fig. 5B, lanes 2 to 4). The lower accumulation of p27 in the pBICRC1-3D-coinfiltrated leaves might reflect the absence of stable viral RNA replication complexes in these cells, because the accumulation of p27 from wt RNA1 and R1-3D is similar in a cell-free extract of evacuated tobacco BY-2 protoplasts (Iwakawa et al., 2007). Together, negative-strand RNA synthesis of RNA1 is sufficient to induce cytoplasmic fluorescent punctates of RCNMV MP:GFP.

#### *RCNMV MP:GFP localizes to the cortical ER*

The localization of RCNMV MP:GFP to the cortical region of cytoplasm prompted us to address whether RCNMV MP localizes to the cortical ER. To test this, we used RCNMV MP fused with DsRed-monomer (DRm) (Clontech, Mountain View, CA, USA). Transgenic *N. benthamiana* plants (Line 16c; Voinnet and Baulcombe, 1997) expressing GFP with an ER localizing signal were infiltrated with the *Agrobacterium* containing pBICR1/MD2fsMP (Fig. 1). The MP:DRm signals were detected as foci with multiple fluorescent cells at 40 hpi (Fig. 6A-b), indicating that MP:DRm was functional for viral cell-to-cell movement. In these epidermal cells, the MP:DRm localized as fluorescent punctates on ER (Figs 6A-a to A-c). Colocalization of the MP:DRm and GFP was also observed in protoplasts prepared from these leaves (Figs 6A-d to A-f). Large cortical punctates of GFP shown in Figures 6A-a and 6A-d were never detected in the mock-inoculated 16c epidermal cells or protoplasts (data not shown). These results suggest that the MP:DRm was recruited to and stayed at the cortical ER.

To determine if the cortical fluorescent punctates observed in transiently expressed MP:GFP in the coinfiltration assays (Figs 4 and 5) also localizes to the cortical ER, we used another plasmid (pBICER:DRm) expressing DRm with an ER localization signal. Coinfiltration of pBICRMsG with pBICER:DRm and pBICRC1 induced cytoplasmic

fluorescent punctates, and they localized on the cortical ER (Fig. 6B). Similar colocalization of MP:GFP and DRm were observed after coinfiltrations with pBICR12fsMP or pBICRC1-5D (data not shown).

#### *RCNMV MP:GFP colocalizes with the viral replication protein*

Previously fusion proteins of GFP and RCNMV replicase component proteins (GFP:p27 and GFP:p88) have been shown to form fluorescent punctates on the cortical ER inducing membrane proliferation in *N. benthamiana* epidermal cells and protoplasts (Turner et al., 2004). These results prompted us to investigate whether the MP:GFP on the cortical ER is colocalized with RCNMV replicase proteins. *N. benthamiana* protoplasts were inoculated with R1-MsG and R2fsMP, and were fixed with formaldehyde to detect the replicase proteins immunologically. As the antibody against p27 recognizes also p88, the red fluorescent signals detected (Fig. 7) should contain both p27 and p88. However, as we mentioned above, the accumulation level of p88 is much lower than p27, so that most of the signals probably represented p27. The MP:GFP and p27 colocalized near the surfaces of protoplasts (Fig. 7). Smaller MP:GFP punctates formed at an early stage of infection (12 hpi), and larger aggregates at a later stage (18 hpi) were both accompanied by p27.

## Discussion

In this study, we have investigated the subcellular localization of RCNMV MP:GFP fusion protein in *N. benthamiana*. The MP:GFP expressed from the recombinant virus (R1-MsG and R2fsMP) infection localized in the cell wall and later on the cortical ER, whereas the MP:GFP expressed transiently without other viral components localized exclusively in the cell wall. The localization of the MP:GFP on the cortical ER is linked to the synthesis of the negative strand RNA1, but not RNA2.

### *Colocalization of RCNMV MP:GFP with p27*

Inoculation of the recombinant virus to *N. benthamiana* induced fluorescent punctates containing both the MP:GFP and the replicase component p27 protein on the cortical ER, concomitant with the morphological changes of ER membrane (Figs 6 and 7). These results suggest that these punctates contain the viral replicase complexes, because p27 and p88 proteins bind to RNA1 and recruit RNA1 to the cellular membrane fraction (H. Iwakawa and T. Okuno, unpublished results). However, the presence of the MP in the punctates may not contribute to the replication of viral RNAs, considering that the expression of RCNMV MP has no effect on the accumulation of the viral RNAs in cowpea protoplasts (Tatsuta et al., 2005) or *N. benthamiana* protoplasts (data not shown). Colocalization of viral replicase component proteins with MP has been reported in *Brome mosaic virus* (BMV) (Dohi et al., 2001) and TMV (Asurmendi et al., 2004; Heinlein et al., 1998). Moreover, both replicase component proteins BMV 2a and TMV 126-kDa are associated with viral cell-to-cell movement processes, respectively (Hirashima and Watanabe, 2001; Traynor et al., 1991). BMV 2a has a polymerase-like domain and TMV 126-kDa has methyltransferase and helicase-like domains (Haseloff et al., 1984; Hodgman et al., 1988; Kamer and Argos, 1984; Koonin and Dolja, 1993). RCNMV p27, in which no functional domains have been reported, might also play roles in virus movement. Besides the MPs mentioned above that belong to '30K' superfamily (Melcher, 2000), the PVX MP (TGBp3) which belongs to triple gene block family has also been reported to colocalize with the replicase protein on the ER (Bamunusinghe et al., 2009). The colocalization of replicase component proteins with MP might indicate that the processes of viral RNA replication and virus movement are functionally linked, as

suggested in the studies on TMV (Christensen et al., 2009; Guenoune-Gelbart et al., 2008; Liu et al., 2005). Alternatively, as discussed below, these replicase component proteins might be involved in the recruitment of the MP through the formation of active viral replicase complexes.

#### *Differences between RCNMV MP:GFP and TMV MP:GFP subcellular localization*

RCNMV MP and TMV MP can complement each other functionally, and both viruses are supposed to share in part a common mechanism for cell-to-cell movement (Giesman-Cookmyer et al., 1995). However, two aspects of subcellular localization of MP:GFPs differ between these viruses. (i) Transiently expressed RCNMV MP:GFP in the absence of other viral components was detected only in the cell wall (Fig. 3), whereas the TMV MP:GFP or TMV MP:RFP (red fluorescent protein) formed punctates on the cortical ER, as well as in the cell wall (Crawford and Zambryski, 2000; Kotlizky et al., 2001; Reichel and Beachy, 1998; Sambade et al., 2008). (ii) The filamentous fluorescent structures of TMV MP:GFP detected in the late stage of infection in *N. benthamiana* epidermal cells (Heinlein et al., 1998) were not observed following infection with the recombinant RCNMV (Fig. 2).

These differences might be attributed to the characters of two MPs. Biochemical fractionation, circular dichroism spectroscopy and tryptic digestion analyses have suggested that TMV MP is an integral membrane protein with two  $\alpha$ -helical trans-membrane domains and a C-terminal region exposed to the cytosol, which is needed for dimer formation (Brill et al., 2000, 2004; Fujiki et al., 2006; Reichel and Beachy, 1998). Also RCNMV MP could function as multimers, because two kinds of MP null mutants that lose the ability to target the cell wall and to move cell to cell, complemented each other and restored the targeting and movement function (Tremblay et al., 2005). Although RCNMV MP is reported to form multimers, we failed to detect cytoplasmic fluorescences in cells infiltrated with pBICRMsG alone (Fig. 3). RCNMV MP does not have trans-membrane helices according to the  $\Delta$ G Prediction Server v1.0 (<http://www.cbr.su.se/DGpred/>) (data not shown) and thus might have less affinity to ER membranes than TMV MP. Such characteristics would make RCNMV MP less stable in the cytoplasm and make it difficult to detect cytoplasmic fluorescence of the MP:GFP. Additionally and alternatively, differences in virus infection mechanism might cause

these differences as discussed below.

#### *Possible significance of ER-targeting of RCNMV MP*

What is the significance of the targeting of RCNMV MP to the ER associated with negative-strand RNA synthesis of RNA1 but not RNA2? Cell-to-cell movement of RCNMV does not require CP or virion formation (Xiong et al., 1993), suggesting that the virus moves from cell to cell in the form of a nucleoprotein that contains at least the genomic RNA and the MP. Accordingly, RCNMV MP must capture both genomic RNA1 and RNA2 and transport them to neighboring cells to establish an infection. RCNMV MP does not have sequence specificity for RNA binding (Giesman-Cookmyer and Lommel, 1993; Osman et al., 1992). Therefore, the virus may need to keep the MP and genomic RNAs close each other to facilitate efficient movement. Because the MP is encoded by the RNA2, the newly translated MP is likely to be present close to the RNA2 molecules. The ER-targeting mechanism of RCNMV MP:GFP, depending on the replication of RNA1 that does not encode MP, seems to reflect the strategy to keep the MP close to RNA1 and thereby achieve its intracellular movement followed by intercellular movement of RNA1. Such a strategy would be valid, especially for those viruses with a divided genome. Interestingly, *Tomato mosaic virus* (ToMV), a close relative of TMV with a monopartite genome, shows a strong contrast to RCNMV in cytoplasmic localization of MP:GFP (Tamai and Meshi, 2001). The ToMV MP:GFP fusion protein formed large cytoplasmic aggregates when expressed transiently by particle bombardment in *N. benthamiana* epidermal cells. Co-expression of ToMV MP:GFP and the mutant ToMV genomic RNA lacking the MP gene promoted ToMV MP:GFP targeting to the cell wall and reduced the cytoplasmic aggregates. These results suggest that the ToMV MP:GFP that had accumulated in the cytoplasm was trafficked to the PD in a virus infection-coupled manner. Reverse effects of viral RNA replication on the localization of MP:GFPs between RCNMV and ToMV suggest different multiplication strategies between these two viruses.

#### *What factor(s) recruits RCNMV MP:GFP to the cortical ER?*

A series of agroinfiltration experiments revealed that negative-strand RNA synthesis of

RNA1 but not RNA2 was associated to the recruitment of RCNMV MP:GFP to the cortical ER (Figs 4 and 5). As the replicase component proteins p27 and p88 are not responsible for such recruitment *per se*, there remain two candidates. One is negative-strand RNA1 and/or the resulting putative products such as double-stranded (ds) RNAs of RNA1. However, these are unlikely: although RCNMV MP has the ability to bind single-stranded RNAs, it does not exhibit sequence specificity in RNA binding (Giesman-Cookmyer and Lommel, 1993; Osman et al., 1992). In addition, RCNMV MP does not bind ds-RNA (Giesman-Cookmyer and Lommel, 1993).

The other candidate is the viral replicase complexes formed on RNA1. The replicase complexes formed on RNA1 might be different from those on RNA2 and localize at different sites on the cortical ER. Previously we have shown that RCNMV RNA1 is replicated preferentially by using p88 translated in *cis*, while RNA2 effectively recruits p88 translated from RNA1 (Okamoto et al., 2008). These phenomena suggest differences in the process of viral replicase complex formation leading to different features of the complexes. The viral replicase complexes formed on RNA1 might employ different host factors from those on RNA2. *Vesicular stomatitis virus* uses two kinds of RNA polymerase complexes that contain the viral polymerase protein L in common, but have different viral and host-encoded proteins; one polymerase is responsible for the transcription of mRNA and the other for the replication of viral RNA genome (Qanungo et al., 2004). Only the replicase complexes formed on RCNMV RNA1 or the host factor protein(s) included in the complexes might be able to recruit the MP:GFP to the cortical ER. Alternatively, the replicase complexes formed on RNA2 can recruit the MP:GFP. The replicase complexes formed on RNA2 associated with the recruited MP:GFP might be transported to PD more efficiently than those on RNA1.

## Materials & Methods

### *Plasmid construction*

pUCR1 (Fig. 1 and see Takeda et al., 2005) is a plasmid used to transcribe the RCNMV RNA1 Australian strain from T7 promoter. It was constructed from pRC1|G (Xiong and Lommel, 1991). The R1-MP:GFP plasmid used to transcribe the recombinant RCNMV RNA1, in which the CP gene has been replaced by that encoding MP:GFP, has been described by Tremblay et al. (2005). The XhoI/SmaI fragment (2.7 kb) of R1-MP:GFP containing the MP:GFP gene and 3' noncoding region was transferred to the XhoI/SmaI sites of pUCR1, creating pUCR1-MsG. The reason we constructed pUCR1-MsG is that several base differences were found in the p27 gene of R1-MP:GFP and pUCR1. Despite these base differences in p27 genes between R1-MP:GFP and pUCR1-MsG, the accumulated levels of viral RNA, p27 protein and MP:GFP were similar in the inoculated *N. benthamiana* protoplasts (data not shown).

pUBRC1 (Mizumoto et al., 2006) and pUBRC2 (Takeda et al., 2005) were the plasmids used to transcribe RCNMV RNA1 and RNA2, respectively, from the CaMV 35S promoter. pUC118RA1(AscI) is a derivative of pUBRC1 in which the SmaI cleavage site downstream of the CaMV 35S terminator has been changed to that for AscI by linker insertion. pUC118RA2(AscI) is a derivative of pUBRC2 in which the HindIII site upstream of the 35S promoter has been changed to that for AscI by linker insertion. The start codon of MP ORF of pUC118RA2(AscI) was base-substituted (ATG to ATT) by recombinant PCR to create pUC118RA2dMP(AscI). An XhoI/MluI fragment (2.2 kb) of R1-MP:GFP was inserted into the XhoI/MluI sites of pUC118RA1(AscI) to create pUBR1MsG. A SacI/AscI fragment (5.3 kb) of pUBR1MsG and an AscI/SmaI fragment (2.2 kb) of pUC118RA2dMP(AscI) were inserted into the SacI/SmaI sites of pBIC18 (Takeda et al., 2005) to create pBICR1/MsG2fsMP.

The DsRed-monomer sequence was amplified by polymerase chain reaction (PCR) using ClaI/DR5' and MluI/DR3' primers and pDRm-actin (Clontech, Mountain View, CA, USA) as the template. It was cut with ClaI/MluI and inserted into the ClaI/MluI sites of R1-MP:GFP, creating pR1-DRm. The MP open reading frame (ORF) cut from R1-MP:GFP by ClaI was inserted to the ClaI site of pR1-DRm to create pR1-MD. The XhoI/MluI fragment of pUC118RA1(AscI) was replaced by the XhoI/MluI fragment (2.1

kb) of pR1-MD, creating pUBRC1-MD. The SacI/AscI fragment of pBICR1/MsG2fsMP was replaced by a SacI/AscI fragment (5.3 kb) of pUBRC1-MD, creating pBICR1/MD2fsMP.

The MP:GFP fragment was amplified by PCR using BamRAMP5' and EcosGFP3' primers and R1-MP:GFP as the template. It was cut with BamHI/EcoRI and inserted into the BamHI/EcoRI sites of pBICP35 (Mori et al., 1992) to create pBICRMsG. pUC118RA2(AscI) was cut with NheI, treated with T4 DNA polymerase and self-ligated. The AscI/SmaI fragment (2.2 kb) of the resultant plasmid and the SacI/AscI fragment (4.6 kb) of pUC118RA1(AscI) were inserted into the SacI/SmaI sites of pBIC18 to create pBICR12fsMP.

The StuI/XhoI fragment of pBICRC1 was replaced by a StuI/XhoI fragment of pBICp88-GVD (Takeda et al., 2005) to create pBICRC1-GVD.

pBICRC1 was cut with Sall, blunt-ended with T4 DNA polymerase and then cut with EcoRI. The resultant fragment (3.8 kb) was ligated with a 12.5 kb fragment of pBICp27 (Takeda et al., 2005) that had been cut with HindIII, blunt-ended with T4 DNA polymerase and then cut with EcoRI, creating pBICRC1-5D.

The PCR fragment produced using 35S/3'D1 and RtSm+ primers and pBICP35R (Takeda et al., 2005) as the template and another PCR fragment produced using S/R1int5'P and 35S/3'D1 primers and pUCR1 as the template were mixed and amplified with S/R1int5'P and RtSm+ primers. SacII/SmaI fragments of pRCP35A1 (Takeda et al., 2005) were replaced with recombinant PCR fragment cut with SacII/SmaI, creating pUBRC1-3D. The smaller SacII/SmaI fragment of pBIC18 was replaced by the 4.5 kb fragment of pUBRC1-3D that had been cut with SacII/SmaI, creating pBICRC1-3D.

### *Plant growth conditions*

*N. benthamiana* was grown on commercial soil at 25 °C. Plants at 5 to 6 weeks-old were used for viral RNA inoculation and agroinfiltration. Eight-week-old plants grown in pots (14 cm diameter and 12 cm deep) were used for the preparation of protoplasts.

### *Protoplast preparation and viral RNA inoculation using polyethylene glycol (PEG)*

*N. benthamiana* protoplasts were prepared and inoculated with viral RNA transcripts



essentially as described by Navas-Castillo et al. (1997) with modifications. The newest fully expanded leaves were cut with a sharp razor at 1 mm intervals, incubated for 16 h at 25 °C in the dark in 1% cellulase: Onozuka RS (Yakult Honsha, Tokyo, Japan) plus 0.5% Macerozyme R-10 (Yakult Honsha) in MMC (0.5 M mannitol, 10 mM CaCl<sub>2</sub>, 5 mM MES, pH 5.7). Protoplasts were collected through three layers of cheesecloth and washed twice in MMC with centrifugation at 45 g for 2 min. Protoplasts ( $2.5 \times 10^5$  concentrated in 0.2 mL MMC) were mixed with 10 µL of inoculum and with 0.4 mL fresh PEG solution: 1 g of PEG 4000 (#81240, Fluka Chemie GmbH, Deisenhofen, Germany), 0.75 mL of distilled water, 0.625 mL of 0.8 M mannitol, 0.25 mL of 1 M Ca(NO<sub>3</sub>)<sub>2</sub>. Immediately after being mixed well, the suspension was diluted with 4 mL MMC and kept on ice for 15 min before being centrifuged at 45 g for 2 min. Protoplasts were washed with 4 mL of MMC twice and incubated in 1 mL of incubation solution (0.5 M mannitol, 1 x Aoki solution pH 6.5, 4 mM MES, 200 µg/mL chloramphenicol) at 17 °C

#### *Agroinfiltration*

Agroinfiltration assay was performed as described (Kaido et al., 2007). In coinfiltration assays, total *Agrobacterium* cultures were suspended in 10 mM MgCl<sub>2</sub> to an OD<sub>600</sub> of 0.8; that is, *Agrobacterium* cells containing pBICRMsG to a final OD<sub>600</sub> of 0.4 each. *Agrobacterium* cells containing an RCNMV component to a final OD<sub>600</sub> of 0.133 and *Agrobacterium* cells containing pBICP35 were used as the filler.

#### *Immunofluorescent labeling*

Fixation of *N. benthamiana* protoplasts and immunolabeling procedure were as described by Liu et al. (2005).

#### *Confocal microscopy*

GFP and DsRed-monomer fluorescence were observed using an Olympus FluoView FV500 confocal microscope (Olympus Optical Co., Tokyo, Japan) equipped with an argon laser, an He:Ne laser and a 60 x Plan Apo oil immersion objective lens (numerical aperture 1.4). The samples were excited with the argon laser for GFP and with the He:Ne

laser for DsRed-monomer. We used a dichroic mirror, DM488/543, a beam splitter, SDM560, and two emission filters: BA505-525 for GFP and BA560IF for DsRed-monomer. In experiments for detecting dual localization, scanning was performed in sequential mode to minimize signal bleed-through. All images shown are from optical sections taken at 1  $\mu\text{m}$  intervals and were processed using Adobe Photoshop CS3 software.

#### *Western and northern blot analyses*

Protein extraction and western blot analyses were performed as described (Takeda et al., 2005). Total RNA extraction from *N. benthamiana* leaves and protoplasts and northern blot analysis were also performed as described (Mizumoto et al., 2002). The probes used for the detection of negative-strand RCNMV RNA1 and RNA2 were as described by Mizumoto et al. (2006). The signals were detected with a luminescent image analyzer (LAS 1000 plus; Fuji Photo Film Co. Ltd., Tokyo, Japan) and the signal intensities were quantified using the Image Gauge program version 3.1 (Fuji Photo Film).

## **Acknowledgement**

We thank Dr. S. A. Lommel and Dr. T. L. Sit for providing plasmids R1-MP:GFP, pRC1|G and pRC2|G, Dr. D. Baulcombe for providing *N. benthamiana* line 16c. We are also grateful to Dr. C. Masuta for advice on preparation of *N. benthamiana* protoplasts, Dr. A. Takeda and M. Tsukuda for the construction of several plasmids and H. Iwakawa and A. Mine for helpful discussion. This study was supported in part by a Grant-in-Aid (17780035) for Young Scientist B, a Grant-in-Aid (19580046) for Scientific Research C from the Japan Society for the Promotion of Science.

## References

Asurmendi, S., Berg, R. H., Koo, J. C. and Beachy, R. N. 2004. Coat protein regulates formation of replication complexes during *Tobacco mosaic virus* infection. Proc. Natl. Acad. Sci. U.S.A. 101: 1415-1420.

Bamunusinghe, D., Hemenway, C. L., Nelson, R. S., Sanderfoot, A. A., Ye, C. M., Silva, M. A. T., Payton, M. and Verchot-Lubicz, J. 2009. Analysis of potato virus X replicase and TGBp3 subcellular locations. Virology doi:10.1016/j.virol.2009.08.002

Berna, A., Gafny, R., Wolf, S., Lucas, W. J., Holt, C. A. and Beachy, R. N. 1991. The TMV movement protein: Role of the C-terminal 73 amino acids in subcellular localization and function. Virology 182: 682-689.

Boyko, V., Hu, Q., Seemanpillai, M., Ashby, J. and Heinlein, M. 2007. Validation of microtubule-associated *Tobacco mosaic virus* RNA movement and involvement of microtubule-aligned particle trafficking. Plant J. 51: 589-603.

Boyko, V., van der Laak, J., Ferralli, J., Suslova, E., Kwon, M-O. and Heinlein, M. 2000. Cellular targets of functional and dysfunctional mutants of *Tobacco mosaic virus* movement protein fused to green fluorescent protein. J. Virol. 74: 11339-11346.

Brill, L. M., Dechongkit, S., De La Barre, B., Stroebel, J., Beachy, R. N. and Yeager, M. 2004. Dimerization of recombinant *Tobacco mosaic virus* movement protein. J. Virol. 78: 3372-3377.

Brill, L. M., Nunn, R. S., Kahn, T. W., Yeager, M. and Beachy, R. N. 2000. Recombinant *Tobacco mosaic virus* movement protein is an RNA-binding,  $\alpha$ -helical membrane protein. Proc. Natl. Acad. Sci. U.S.A. 97: 7112-7117.

Christensen, N., Tilsner, J., Bell, K., Hammann, P., Parton, R., Lacomme, C. and Oparka, K. 2009. The 5' cap of *Tobacco mosaic virus* (TMV) is required for virion attachment to the actin/endoplasmic reticulum network during early infection. Traffic 10: 536-551.

Cowan, G. H., Lioliopoulou, F., Ziegler, A. and Torrance, L. 2002. Subcellular localisation, protein interactions, and RNA binding of *Potato mop-top virus* triple gene block proteins. *Virology* 298: 106-115.

Crawford, K. M. and Zambryski, P. C. 2000. Subcellular localization determines the availability of non-targeted proteins to plasmodesmatal transport. *Current Biology* 10: 1032–1040.

Dohi, K., Mori, M., Furusawa, I., Mise, K. and Okuno, T. 2001. Brome mosaic virus replicase proteins localize with the movement protein at infection-specific cytoplasmic inclusions in infected barley leaf cells. *Arch. Virol.* 146: 1607-1615.

Fujiki, M., Kawakami, S., Kim, R. W. and Beachy, R. N. 2006. Domains of *Tobacco mosaic virus* movement protein essential for its membrane association. *J. Gen. Virol.* 87: 2699-2707.

Fujiwara, T., Giesman-Cookmeyer, D., Ding, B., Lommel S. A. and Lucas, W. J. 1993. Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the *Red clover necrotic mosaic virus* movement protein. *Plant Cell* 5: 1783-1794.

Giesman-Cookmeyer, D., Silver, S., Vaewhongs, A. A., Lommel, S. A. and Deom, C. M. 1995. Tobamovirus and Dianthovirus movement proteins are functionally homologous. *Virology* 213: 38-45.

Giesman-Cookmeyer, D. and Lommel, S. A. 1993. Alanine scanning mutagenesis of a plant virus movement protein identifies three functional domains. *Plant Cell* 5: 973-982.

Guenoune-Gelbart, D., Elbaum, M., Sagi, G., Levy, A. and Epel, B. L. 2008. *Tobacco mosaic virus* (TMV) replicase and movement protein function synergistically in facilitating TMV spread by lateral diffusion in the plasmodesmal desmotubule of *Nicotiana benthamiana*. *Mol. Plant-Microbe Interact.* 21: 335-345.

Haseloff, J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R. & Kaesberg, P. 1984. Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. *Proc. Natl. Acad. Sci. U.S.A.* 81: 4358-4362.

Haupt, S., Cowan, G. H., Ziegler, A., Roberts, A. G., Oparka, K. J. and Torrance, L. 2005. Two Plant-Viral Movement Proteins Traffic in the Endocytic Recycling Pathway. *Plant Cell* 17:164-181.

Heinlein, M., Epel, B. L., Padgett, H. S. and Beachy, R. N. 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. *Science* 270: 1983-1985.

Heinlein, M., Padgett, H. S., Gens, J. S., Pickard, B. G., Casper, S. J., Epel, B. L. and Beachy, R. N. 1998. Changing patterns of localization of the *Tobacco mosaic virus* movement protein and replicase to the endoplasmic reticulum and microtubules during infection. *Plant Cell* 10: 1107-1120.

Hirashima, K. and Watanabe, Y. 2001. *Tobamovirus* replicase coding region is involved in cell-to-cell movement. *J. Virol.* 75: 8831-8836.

Hodgman, T. C. 1988. A new superfamily of replicative proteins. *Nature* 333: 22-23.

Iwakawa, H., Kaido, M., Mise, K. and Okuno, T. 2007. *cis*-Acting core RNA elements required for negative-strand RNA synthesis and cap-independent translation are separated in the 3'-untranslated region of *Red clover necrotic mosaic virus* RNA1. *Virology* 369: 168-181.

Ju, H-J., Brown, J. E., Ye, C-M. and Verchot-Lubicz, J. 2007. Mutations in the central domain of *Potato virus X* TGBp2 eliminate granular vesicles and virus cell-to-cell movement trafficking. *J. Virol.* 81: 1899-1911.

Ju, H-J., Samuels, T. D., Wang, Y-S., Blancaflor, E., Payton, Mitra, R., M., Krishnamurthy, K., Nelson, R. S. and Verchot-Lubicz, J. 2005. The *Potato virus X*

TGBp2 movement protein associates with the endoplasmic reticulum-derived vesicles during virus infection. *Plant Physiol.* 138: 1877-1895.

Kaido, M., Inoue, Y., Takeda, Y., Sugiyama, K., Takeda, A., Mori, M., Tamai, A., Meshi, T., Okuno, T. and Mise, K. 2007. Downregulation of the *NbNACa1* gene encoding a movement-protein-interacting protein reduces cell-to-cell movement of *Brome mosaic virus* in *Nicotiana benthamiana*. *Mol. Plant-Microbe Interact.* 20: 671-681.

Kamer, G. and Argos, P. 1984. Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res.* 12: 7269-7282.

Kawakami, S., Watanabe, Y. and Beachy, R. N. 2004. *Tobacco mosaic virus* infection spreads cell to cell as intact replication complexes. *Proc. Natl. Acad. Sci. U.S.A.* 101: 6291-6296.

Koonin, E. V. and Dolja, V. V. 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit. Rev. in Biochem. and Mol. Biol.* 28: 375-430.

Kotlizky, G., Katz, A., van der Laak, J., Boyko, V., Lapidot, M., Beachy, R. N., Heinlein, M. and Epel, B. L. 2001. A dysfunctional movement protein of *Tobacco mosaic virus* interferes with targeting of wild-type movement protein to microtubules. *Mol. Plant-Microbe Interact.* 14: 895-904.

Krishnamurthy, K., Heppler, M., Mitra, R., Blancaflor, E., Payton, M., Nelson, R. S. and Verchot-Lubicz, J. 2003. The *Potato virus X* TGBp3 protein associates with the ER network for virus cell-to-cell movement. *Virology* 309: 135-151.

Liu, J., Blancaflor, E. B. and Nelson, R. S. 2005. The *Tobacco mosaic virus* 126-kilodalton protein, a constituent of the virus replication complex, alone or within the complex aligns with and traffics along microfilaments. *Plant Physiol.* 138: 1853-1865.

Lommel, S. A., Weston-Fina, M., Xiong, Z., Lomonosoff, G.P., 1988. The nucleotide

sequence and gene organization of *Red clover necrotic mosaic virus* RNA-2. *Nucleic Acids Res.* 16, 8587–8602.

Lucas, W. J. 2006. Plant viral movement proteins: Agents for cell-to-cell trafficking of viral genomes. *Virology* 344: 169-184.

Más, P. and Beachy, R. N. 1999. Replication of *Tobacco mosaic virus* on endoplasmic reticulum and role of the cytoskeleton and virus movement protein in intracellular distribution of viral RNA. *J. Cell Biol.* 147: 945-958.

McLean, B. G., Zupan, J., Zambryski, P. C. 1995. *Tobacco mosaic virus* movement protein associates with the cytoskeleton in tobacco cells. *Plant Cell* 7: 2101-14.

Melcher, U. 2000. The '30K' superfamily of viral movement proteins. *J. Gen. Virol.* 81: 257-266.

Mitra, R., Krishnamurthy, K., Blancaflor, E., Payton, M., Nelson, R. S. and Verchot-Lubicz, J. 2003. The *Potato virus X* TGBp2 protein association with the endoplasmic reticulum plays a role in but is not sufficient for viral cell-to-cell movement. *Virology* 312: 35-48.

Mizumoto, H., Hikichi, Y. and Okuno, T. 2002. The 3'-untranslated region of RNA1 as a primary determinant of temperature sensitivity of *Red clover necrotic mosaic virus* Canadian strain. *Virology* 293: 320-327.

Mizumoto, H., Iwakawa, H., Kaido, M., Mise, K and Okuno, T. 2006. Cap-independent translation mechanism of *Red clover necrotic mosaic virus* RNA2 differs from that of RNA1 and is linked to RNA replication. *J. Virol.* 80: 3781-3791.

Mori, M., Mise, K., Okuno, T. and Furusawa, I. 1992. Expression of brome mosaic virus-encoded replicase genes in transgenic tobacco plants. *J. Gen. Virol.* 73: 169-172.

Navas-Castillo, J., Albiach-Mart, M. R., Gowda, S., Hilf, M. E., Garnsey, S. M. and



Dawson, W. O. 1997. Kinetics of accumulation of *Citrus tristeza virus* RNAs. *Virology* 228: 92-97.

Okamoto, K., Nagano, H., Iwakawa, H., Mizumoto, H., Takeda, A., Kaido, M., Mise, K. and Okuno, T. 2008. *cis*-Preferential requirement of a -1 frameshift product p88 for the replication of *Red clover necrotic mosaic virus* RNA1. *Virology* 375: 205-212.

Osman, T. A. M., Hayes, R. J. and Buck, K. W. 1992. Cooperative binding of the red clover necrotic mosaic virus movement protein to single-stranded nucleic acids. *J. Gen. Virol.* 73: 223-227.

Osman, T. A. M., Thommes, P. and Buck, K. W. 1993. Localization of a single-stranded RNA-binding domain in the movement protein of *Red clover necrotic mosaic dianthovirus*. *J. Gen. Virol.* 74: 2453-2457.

Padgett, H. S., Epel, B. L., Kahn, T. W. Heinlein, M., Watanabe, Y. and Beachy, R. N. 1996. Distribution of tobamovirus movement protein in infected cells and implications for cell-to-cell spread of infection. *Plant J.* 10: 1079-1088.

Qanungo, K. R., Shaji, D., Mathur, M. and Banerjee, A. K. 2004. Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. *Proc. Natl. Acad. Sci. U.S.A.* 101: 5952-5957.

Reichel, C. and Beachy, R. N. 1998. *Tobacco mosaic virus* infection induces severe morphological changes of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 95: 11169-11174.

Sambade, A., Brandner, K., Hofmann, C., Seemanpillai, M., Mutterer, J. and Heinlein, M. 2008. Transport of TMV movement protein particles associated with the targeting of RNA to plasmodesmata. *Traffic* 9: 2073-2088.

Sit, T. L., Vaewhongs, A. A. and Lommel, S. A. 1998. RNA-mediated trans-activation of transcription from a viral RNA. *Science* 281: 829-832.

Tagami, Y. and Watanabe, Y. 2007. Effects of brefeldin A on the localization of *Tobamovirus* movement protein and cell-to-cell movement of the virus. *Virology* 361: 133-140.

Takeda, A., Tsukuda, M., Mizumoto, H., Okamoto, K., Kaido, M., Mise, K. and Okuno, T. 2005. A plant RNA virus suppresses RNA silencing through viral RNA replication. *EMBO J.* 24: 3147-3157.

Tamai, A. and Meshi, T. 2001. Tobamoviral movement protein transiently expressed in a single epidermal cell functions beyond multiple plasmodesmata and spreads multicellularly in an infection-coupled manner. *Mol. Plant-Microbe Interact.* 14: 126-134.

Tatsuta, M., Mizumoto, H. Kaido, M. Mise, K. and Okuno, T. 2005. The *red clover necrotic mosaic virus* RNA2 *trans*-activator is also a *cis*-acting RNA2 replication element. *J. Virol.* 79: 978-986.

Traynor, P., Young, B. M. and Ahlquist, P. 1991. Deletion analysis of Brome mosaic virus 2a protein: Effects on RNA replication and systemic spread. *J. Virol.* 65: 2807-2815.

Tremblay, D., Vaewhongs, A. A., Turner, K. A., Sit, T. L. and Lommel, S. A. 2005. Cell wall localization of *Red clover necrotic mosaic virus* movement protein is required for cell-to-cell movement. *Virology* 333: 10-21.

Turner, K. A., Sit, T. L., Callaway, A. S., Allen, N. S. and Lommel, S. A. 2004. *Red clover necrotic mosaic virus* replication proteins accumulate at the endoplasmic reticulum. *Virology* 320: 276-290.

Verchot-Lubicz, J., Ye, C-M. and Bamunusinghe, D. 2007. Molecular biology of potexviruses: recent advances. *J. Gen. Virol.* 88: 1643-1655.

Voinnet, O. and Baulcombe, D. C. 1997. Systemic signaling in gene silencing. *Nature*

389: 553.

Waigmann, E., Curin, M. and Heinlein, M. 2007. *Tobacco mosaic virus*-a model for macromolecular cell-to-cell spread. In: Waigmann, E. and Heinlein, M. (eds) *Viral transport in plants*. Springer Berlin, Heidelberg, Germany, 29-62.

Waigmann, E., Ueki, S., Trutnyeva, K. and Citovsky, V. 2004. The ins and outs of nondestructive cell-to-cell and systemic movement of plant viruses. *Crit. Rev. Plant Sci.* 23: 195-250.

Wright, K. M., Wood, N. T., Roberts, A. G., Chapman, S., Boevink, P., MacKenzie, K. M. and Oparka, K. J. 2007. Targeting of TMV movement protein to plasmodesmata requires the actin/ER network: evidence from FRAP. *Traffic* 8: 21-31.

Xiong, Z., Kim, K.H., Giesman-Cookmeyer, D. and Lommel, S.A. 1993. The roles of the *Red clover necrotic mosaic virus* capsid and cell-to-cell movement proteins in systemic infection. *Virology* 192, 27–32.

Xiong, Z. and Lommel, S.A. 1989. The complete nucleotide sequence and genome organization of *Red clover necrotic mosaic virus* RNA-1. *Virology* 171, 543–554.

Xiong, Z. and Lommel, S.A. 1991. *Red clover necrotic mosaic virus* infectious transcripts synthesized *in vitro*. *Virology* 182, 388–392.

Zamyatnin, Jr., A. A., Solovyev, A. G., Savenkov, E. I., Germundsson, A., Sandgren, M., Valkonen, J. P. T. and Morozov, S. Y. 2004. Transient coexpression of individual genes encoded by the triple gene block of *Potato mop-top virus* reveals requirements for TGBp1 trafficking. *Mol. Plant-Microbe Interact.* 17: 921-930.

## Figure legends

Fig. 1. Genome maps of *Red clover necrotic mosaic virus* (RCNMV) and various derivative constructs. Plasmids containing the prefix 'pUC' and pRNA2fsMP were cut with *Sma*I and used as templates for *in vitro* transcription. Plasmids containing the prefix 'pBIC' were used for agroinfiltration. Shaded boxes labeled GFP show the open reading frame (ORF) of green fluorescent protein. Shaded boxes labeled DRm show the ORF of DsRed-monomer protein, dashed boxes show the untranslated MP ORF; and fs is the four-nucleotide insertion for a frameshifting mutation. Bold lines show the RCNMV untranslated sequences. Key: T7, T7 promoter; Pro, *Cauliflower mosaic virus* (CaMV) 35S promoter; Ter, CaMV terminator; Rz, ribozyme sequence; *Sma*I, *Sma*I recognition sequence.

Fig. 2. Confocal images of RCNMV movement protein/green fluorescent protein (MP:GFP) fusion marker localization in *Nicotiana benthamiana* epidermal cells (A)-(D) or protoplasts (E)-(H) inoculated with transcripts from pUCR1-MsG and pRNA2fsMP. (A)-(D), (G) and (H) show the GFP channel images. (E) and (F) and inset in (A) show merged images of differential interference contrast (DIC) and GFP channels. All images are confocal projections composed of 20 optical sections taken at 1  $\mu$ m intervals, covering from the surface to the middle of epidermal cells or protoplasts.

In the mechanically inoculated leaves, MP:GFP fluorescence was detected exclusively in the cell wall at 15-20 hours post inoculation (hpi) (A). Inset shows a higher magnification image. At 18-24 hpi, small number of fluorescent punctates started to appear in the cortical region of the cells (B). At 20-28 hpi, a lot of fluorescent punctates were detected in the cortical region of the cells (C). At 24 hpi and later, larger fluorescent punctates started to appear in the cortical region of the cells (D). In the inoculated protoplasts, small fluorescent punctates started to appear near the surface of the protoplasts at 12-18 hpi (E and serial sections). At 14-20 hpi, the numbers increased and the fluorescent punctates grew larger (F). At 18 hpi and later, larger fluorescent punctates were detected (G). At 30 hpi, string-like fluorescent structures protruding from the surface were detected in about 10% of protoplasts (H). Bars = 10  $\mu$ m.

Fig. 3. Different localization patterns of MP:GFP. (A) Confocal images of RCNMV

MP:GFP localization in *N. benthamiana* epidermal cells or protoplasts prepared from the leaves infiltrated with *Agrobacterium* containing pBICR1/MsG2fsMP (left) or pBICRMsG (right). Epidermal cells were observed at 30 hpi or 48 hpi and protoplasts were then prepared from the leaves. DIC and GFP channel images were merged. All images are confocal projections composed of 20 optical sections taken at 1  $\mu\text{m}$  intervals, covering from the surface to the middle of epidermal cells or protoplasts. Bars = 10  $\mu\text{m}$ . (B) Western blot analysis of MP:GFP in the infiltrated leaves (total) and the protoplasts prepared from those leaves. Samples loaded in 'total' lanes contained 2 mg of fresh weight leaf and those in 'protoplast' lanes contained  $3 \times 10^5$  protoplasts. MP:GFP was detected using a rabbit anti-GFP antibody. The lower panel (CBB) is a Coomassie brilliant blue-stained gel image showing Rubisco large subunit proteins.

Fig. 4. Localization of RCNMV MP:GFP as cortical punctates depended on the replication of RNA1. *N. benthamiana* leaves were coinfiltrated with *Agrobacterium* containing pBICRMsG in combination with various RCNMV components. About 30 h later, the infiltrated leaves were detached and subjected to confocal microscopy. (A) Cell wall localization of the MP:GFP was detected under all conditions (panels 1 to 6). Cortical fluorescent punctates were detected concomitant with the replication of RNA1 (panels 2 and 3). The DIC and GFP channel images were merged. All images are confocal projections composed of 20 optical sections taken at 1  $\mu\text{m}$  intervals, covering from the surface to the middle of epidermal cells. Bars = 5  $\mu\text{m}$ . (B) From the same leaves observed in (A), protein and RNA were extracted and subjected to western and northern blotting, respectively. Leaf proteins (2 mg fresh weight of leaves) or 2  $\mu\text{g}$  of total RNA were loaded to each lane. MP:GFP and p27 were detected using rabbit polyclonal antibodies against GFP and p27, respectively. CBB is a Coomassie brilliant blue-stained gel image showing Rubisco large subunit proteins. Negative-strand RNA1 and RNA2 were detected using specific riboprobes; rRNA is an ethidium bromide-stained agarose gel image as the loading control.

Fig. 5. Localization of RCNMV MP:GFP as cortical punctates depended on the synthesis of negative-strand RNA1. *N. benthamiana* leaves were coinfiltrated with *Agrobacterium* containing pBICRMsG and various derivatives of RCNMV RNA. (A) At 30 hpi, cortical fluorescent punctates were detected in leaves coinfiltrated with pBICRC1 or

pBICRC1-5D. DIC and GFP channel images were merged. All images are confocal projections composed of 20 optical sections taken at 1  $\mu\text{m}$  intervals, covering from the surface to the middle of epidermal cells. Bars = 10  $\mu\text{m}$ . (B) Western and northern blotting results are in the lower panels. Leaf proteins (2 mg fresh weight of leaves) or 2  $\mu\text{g}$  of total RNA were loaded to each lane. MP:GFP and p27 were detected using appropriate rabbit polyclonal antibodies. CBB is a Coomassie brilliant blue-stained gel image showing Rubisco large subunit proteins. Negative-strand RNA1 was detected using a specific riboprobe; rRNA is an ethidium bromide-stained agarose gel image as the loading control.

Fig. 6. Localization of RCNMV MP to the endoplasmic reticulum (ER) membrane. (A) Confocal images of RCNMV MP:DRm localization in epidermal cells (a-c) or protoplasts (d-f) of 16c transgenic *N. benthamiana*. The 16c plants were infiltrated with *Agrobacterium* containing pBICR1/MD2fsMP and subjected to confocal microscopy at 40 hpi and then used for preparation of protoplasts. Bars = 10  $\mu\text{m}$ . (B) Confocal images of RCNMV MP:GFP localization in *N. benthamiana* epidermal cells. *N. benthamiana* leaf was infiltrated with *Agrobacterium* cultures containing pBICRMsG and pBICRC1 and pBICER:DRm. At 45 h after infiltration, the leaf was subjected to confocal microscopy. Bars = 10  $\mu\text{m}$ . a~c images are confocal projections composed of 12 optical sections taken at 0.25  $\mu\text{m}$  intervals, covering the surface of epidermal cells. The other images are composed of 20 optical sections taken at 1  $\mu\text{m}$  intervals.

Fig. 7. Colocalization of RCNMV MP:GFP and p27 protein in *N. benthamiana* protoplasts. *N. benthamiana* protoplasts were inoculated with transcripts from pUCR1-MsG and pUCR2fsMP and were incubated at 22°C. At 12 and 18 hpi, the protoplasts were fixed with formaldehyde. A rabbit anti-p27 was used as the primary antibody and an ALEXA594-conjugated goat antibody against rabbit IgG was used as the secondary antibody. All images are confocal projections composed of 10 optical sections taken at 1  $\mu\text{m}$  intervals, covering from the surface to the middle of the protoplasts. Bars = 10  $\mu\text{m}$ .