

Cell-to-Cell Trafficking of Macromolecules through Plasmodesmata Potentiated by the Red Clover Necrotic Mosaic Virus Movement Protein

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Direct evidence is presented for cell-to-cell trafficking of macromolecules via plasmodesmata in higher plants. The fluorescently labeled 35-kD movement protein of red clover necrotic mosaic virus (RCNMV) trafficked rapidly from cell to cell when microinjected into cowpea leaf mesophyll cells. Furthermore, this protein potentiated rapid cell-to-cell trafficking of RCNMV RNA, but not DNA. Electron microscopic studies demonstrated that the 35-kD movement protein does not unfold the RCNMV RNA molecules. Thus, if unfolding of RNA is necessary for cell-to-cell trafficking, it may well involve participation of endogenous cellular factors. These findings support the hypothesis that trafficking of macromolecules is a normal plasmodesmal function, which has been usurped by plant viruses for their cell-to-cell spread.

INTRODUCTION

Plasmodesmata are plasma membrane-lined cylindrical pores that traverse the cell wall. Each plasmodesma has appressed endoplasmic reticulum in the center that forms an endomembrane continuum between neighboring cells. Proteinaceous particles are embedded in both the plasma membrane and the appressed endoplasmic reticulum membrane of this intercellular organelle, and spaces between these proteinaceous particles form a series of microchannels of ~2.5 nm in diameter within the plasmodesma (Ding et al., 1992b). Although it has long been held that plasmodesmata serve as potential routes for cell-to-cell communication and solute transport during plant growth and development (Gunning and Robards, 1976; Robards and Lucas, 1990; Lucas et al., 1993), how plasmodesmata function in these processes is not well understood. As established by dye-coupling studies, plasmodesmata normally have a size exclusion limit (SEL) of 800 to 1000 D (Tucker, 1982; Goodwin, 1983). Thus, it is generally accepted that transport through plasmodesmata is restricted to only small molecules, which would include metabolites, ions, and hormones.

Plant viral cell-to-cell movement offers a powerful experimental system for studying the function of plasmodesmata. It has been shown that some viruses appear to require capsid protein for cell-to-cell movement, whereas others do not (Culver and Dawson, 1989; Petty and Jackson, 1990; Ziegler-Graff et al., 1991; Dalmay et al., 1992; Xiong et al., 1993). This suggests that in the latter, viral nucleic acids, not virions, move

through plasmodesmata during the infection process. In addition, many known plant viruses encode a specific protein, the movement protein (MP), that mediates in the cell-to-cell spread of viruses during infection (Atabekov and Taliansky, 1990). Several viral MPs have been localized to plasmodesmata in infected plants as well as in transgenic plants that express MP genes (Tomenius et al., 1987; Linstead et al., 1988; Shanks et al., 1989; Atkins et al., 1991; Ding et al., 1992a; Moore et al., 1992), and the 30-kD MP of the tobacco mosaic virus (TMV) has been demonstrated to up-regulate the SEL of plasmodesmata in TMV MP transgenic tobacco plants (Wolf et al., 1989). Furthermore, a number of MPs have been found to bind RNA and single-stranded DNA in a cooperative but nonspecific manner *in vitro* (Citovsky et al., 1990, 1991; Osman et al., 1992; Schoumacher et al., 1992; Giesman-Cookmeyer and Lommel, 1993). It has been hypothesized that such binding leads to unfolding, or relaxation of secondary structures, of viral nucleic acids to form an extended, linear, protein-nucleic acid complex that is then targeted to plasmodesmata for cell-to-cell transport (Citovsky et al., 1990, 1991, 1992; Citovsky and Zambryski, 1991; Citovsky, 1993). These studies suggest that macromolecules can traffic (a presumably active and selective process, in contrast with passive diffusion) cell to cell through plasmodesmata (see also Fisher et al., 1992; Sakuth et al., 1993).

In this study, direct evidence is presented showing that a viral MP and nucleic acids traffic from cell to cell via plasmodesmata in higher plants. The fluorescently labeled 35-kD MP of red clover necrotic mosaic virus (RCNMV), when microinjected into mesophyll cells of a cowpea leaf, moved rapidly from cell

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to cell. The protein also increased the SEL of plasmodesmata to 10-fold higher than the normal value. Furthermore, this protein potentiated plasmodesmal trafficking of the RCNMV RNA, but not single- or double-stranded DNA. Interestingly, electron microscopic studies established that although the RCNMV MP bound to and potentiated trafficking of the RCNMV RNA, it did not unfold and extend these molecules *in vitro*. Thus, if unfolding of RCNMV RNA is a prerequisite for trafficking through plasmodesmata, it may well involve the participation of endogenous cellular factors.

Based on these findings, we propose that trafficking of macromolecules is a normal function of plasmodesmata, which was presumably usurped during coevolution by plant viruses to facilitate cell-to-cell spread of the viral genetic material. For plant growth and development, plasmodesmal trafficking of informational macromolecules potentially provides a means for supracellular (i.e., above the level of individual cells) regulation of gene expression and coordination of physiological function(s) (Lucas et al., 1993).

RESULTS

RCNMV MP Produced in *Escherichia coli* Increases Plasmodesmal SEL

Previously, it was demonstrated that the 30-kD TMV MP increased the SEL of mesophyll plasmodesmata from the normal 800 D to greater than 10 kD in transgenic tobacco expressing this protein (Wolf et al., 1989). To determine whether the 35-kD RCNMV MP also modifies the plasmodesmal SEL, we took an alternative approach and directly coinjected the RCNMV MP produced in *E. coli* and a 9.4-kD fluorescein-conjugated dextran (F-dextran) into mesophyll cells of mature cowpea leaves. As illustrated in Figure 1A, when the 9.4-kD F-dextran alone was microinjected into a mesophyll cell, it remained in the injected cell even after 20 min. However, when coinjected with the RCNMV MP, the 9.4-kD F-dextran spread into the neighboring cells within 30 sec and then further into more distant cells (up to three or more cells away) in less than 5 min (Figures 1B and 1C). This experiment established that the RCNMV MP produced in *E. coli* was biologically active and that it functioned to interact with the cowpea mesophyll plasmodesmata to increase their permeability in a manner analogous to that of TMV MP expressed in transgenic tobacco plants.

This finding was further supported by studies of 12 RCNMV MP mutants. These mutants were generated by alanine scanning mutagenesis, in which clusters of two or three charged residues dispersed throughout the protein were all changed to alanines by site-directed mutagenesis (Giesman-Cookmeyer and Lommel, 1993). These MP mutants were named according to the position of the first amino acid in the mutagenized cluster (except for mutant 278, which was named based on the position of the second amino acid in this cluster to avoid

confusion with TMV MP transgenic tobacco line 277; Giesman-Cookmeyer and Lommel, 1993). As shown in Table 1, when MP mutants 27 to 31, 204, 242, 280, 301, and 305 were coinjected with the 9.4-kD F-dextran, the F-dextran moved from cell to cell as rapidly as when the wild-type MP was coinjected. By contrast, when MP mutants 122, 128, 144, 161, 278, and 291 were coinjected, the 9.4-kD F-dextran remained in the injected cell, even after 20 min (see Figure 1D).

Interestingly, all of the RCNMV MP mutants that elicited an increase in plasmodesmal SEL also potentiated RCNMV cell-to-cell movement in two host plants, cowpea and *Nicotiana benthamiana*, whereas all of the mutants that failed to increase the plasmodesmal SEL did not permit spread of the virus in either host (Table 1). These data demonstrate that increasing the SEL of plasmodesmata is a specific function of the RCNMV MP, which is presumably required to establish effective viral cell-to-cell movement.

RCNMV MP Trafficks from Cell to Cell

Previous studies on TMV did not determine whether the MP itself trafficked from cell to cell, but only that plasmodesmal SEL was increased by the MP to allow diffusion of biologically inactive large dyes such as F-dextran (Wolf et al., 1989). The observation that the 9.4-kD F-dextran, when coinjected with RCNMV MP, moved into cells that were located three or more cells away from the injected site in cowpea mesophyll (cf. Figure 1C) suggested that the MP itself also moved cell to cell to open plasmodesmal microchannels in those distant cells.

To directly test whether the RCNMV MP is indeed able to move from cell to cell, we labeled the 35-kD RCNMV MP purified from *E. coli* with the fluorescent dye fluorescein isothiocyanate (FITC) and microinjected it into cowpea leaf mesophyll cells. As shown in Figure 2, the fluorescence spread from the injected cell into surrounding cells in less than 3 min after injection. By contrast, when MP mutant 278, which was defective in both increasing the plasmodesmal SEL and effecting viral cell-to-cell spread, was similarly labeled and microinjected, the fluorescence remained in the injected cell even after 20 min (Figure 2D). In addition to the fact that free FITC molecules were removed from the protein fractions used for microinjection, these data indicate that the cell-to-cell spread of the fluorescence represents cell-to-cell trafficking of the wild-type RCNMV MP.

Although we cannot provide direct cytological data to show that the cell-to-cell trafficking of the RCNMV MP is indeed via plasmodesmata, evidence from other RNA plant viruses indicates that plasmodesmata are the sole target sites across the cell wall for the viral MPs (Tomenius et al., 1987; Linstead et al., 1988; Shanks et al., 1989; Atkins et al., 1991; Ding et al., 1992a; Moore et al., 1992). Moreover, as shown above, the RCNMV MP increases by 10-fold the SEL of plasmodesmata when microinjected into cowpea leaf mesophyll cells. Based on these data, we conclude that the cell-to-cell trafficking of the FITC-labeled RCNMV MP occurred via plasmodesmata.

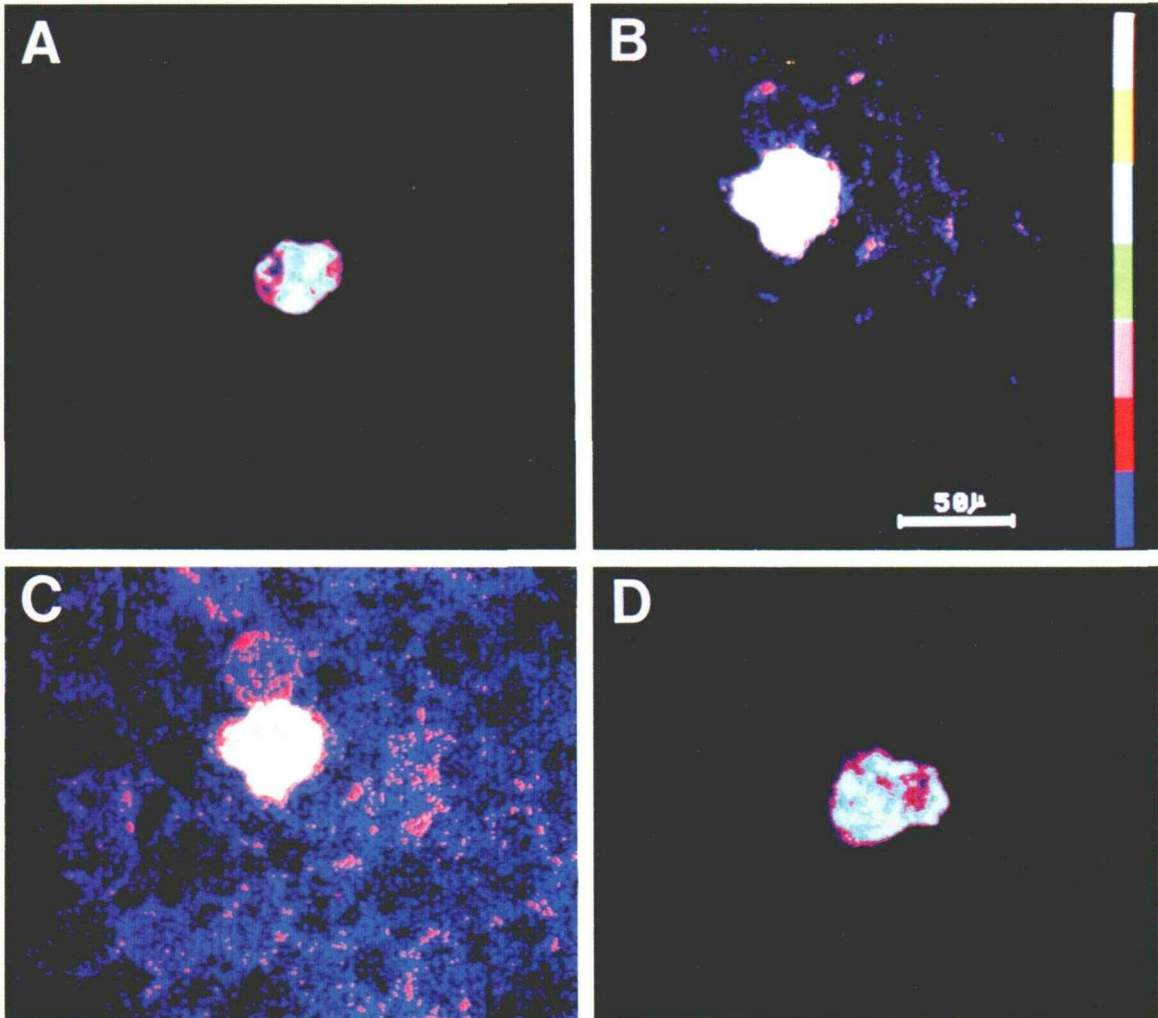


Figure 1. Cell-to-Cell Movement of 9.4-kD F-Dextran Potentiated by RCNMV MP in Cowpea Mesophyll Cells.

The intensities of fluorescence are presented as false-color images. As shown in the color bar in (B), white represents the highest intensity while black is background.

(A) The 9.4-kD F-dextran, injected alone, remains in the injected mesophyll cell (image taken 20 min after initial injection).

(B) and (C) Thirty seconds and 5 min, respectively, after coinjection of the 9.4-kD F-dextran and wild-type RCNMV MP.

(D) Fifteen minutes after coinjection of 9.4-kD F-dextran with RCNMV mutant MP 278.

Bar in (B) = 50 μm .

RCNMV MP Potentiates Cell-to-Cell Trafficking of RCNMV RNA

Because the coat protein is not required for cell-to-cell spread of RCNMV (Xiong et al., 1993), it seems that the RCNMV RNA trafficks cell to cell via plasmodesmata. To provide a direct test of this hypothesis, we labeled the *in vitro*-transcribed 3.9- and 1.4-kb genomic RNAs of RCNMV (RNA 1 and RNA 2, respectively) with the nucleotide-specific fluorescent dye TOTO-1 iodide (Glazer and Rye, 1992) and microinjected them into cowpea mesophyll cells. As shown in Figure 3A, when the labeled

RNA 2 was microinjected alone, the fluorescence remained in the injected cell after 5 min. Observations of such injected cells indicated that the fluorescently labeled RCNMV RNA remained within the injected cell for periods greater than 20 to 30 min. However, when unlabeled RCNMV MP was coinjected along with fluorescently labeled RCNMV RNA 2, the fluorescence began to spread from the injected cell into surrounding cell(s) within 30 sec (Figure 3B). After 3 min, the fluorescence associated with RCNMV RNA 2 was detected in cells approximately three cells away from the injection site (Figure 3C). Similar results were obtained when fluorescently labeled

Table 1. Summary of Functions Identified of Wild-Type and Mutant Forms of RCNMV MP

	WT ^a	Mutant											
		27 to 31	122	128	144	161	204	242	278	280	291	301	305
RNA binding ^b (% of WT)	100	20	10	11	32	28	32	56	109	42	59	100	25
Cooperativity ^b	+	-	-	-	+	+	+	+	+	+	+	+	-
Viral infection ^c	+	+	-	-	-	-	+	+	-	(+)	-	+	+
Pd SEL ^d	+	+	-	-	-	-	+	+	-	+	-	+	+
RNA trafficking ^e	+	+	-	-	-	-	+	+	-	-	-	+	+
RNA unfolding ^f	-	-	-	-	-	-	-	-	-	-	-	-	-
DNA trafficking ^g	-	-	-	-	-	-	-	-	-	-	-	-	-

^a WT, wild-type.

^b These data are taken from Giesman-Cookmeyer and Lommel (1993) for the purpose of direct comparison with data obtained during the present study. (+) indicates cooperativity of MP binding to RNA; (-) indicates lack of cooperativity for such binding.

^c These data were obtained from studies on both *N. benthamiana* (Giesman-Cookmeyer and Lommel, 1993) and cowpea. (+) indicates wild-type or mutant MP-potentiated viral infection; (-) indicates inability of a particular MP mutant to effect viral infection. For MP mutant 280, it has to revert to wild type to effect viral infection. See text for details.

^d (+) indicates RCNMV MP-induced increase in plasmodesmal (Pd) SEL, as demonstrated by 9.4-kD F-dextran cell-to-cell movement; (-) indicates no increase in Pd SEL. Four to five plants were used for each RCNMV MP (wild type or mutant) study. Original experimental data (number of injections showing RCNMV MP-potentiated 9.4-kD F-dextran movement per total number of injections) are as follows: F-dextran injected alone (0/9); F-dextran coinjected with the wild-type MP (7/10), 27 to 31 (5/7), 122 (0/5), 128 (0/5), 144 (4/18), 161 (0/6), 204 (4/7), 242 (4/6), 278 (0/4), 280 (6/7), 291 (0/6), 301 (5/7), and 305 (5/7).

^e (+) indicates RCNMV MP-mediated cell-to-cell trafficking of RCNMV RNA 2 through plasmodesmata; (-) indicates no trafficking. Four to five plants were used for each RCNMV MP (wild type or mutant) study. Original experimental data (number of injections showing RCNMV MP-mediated RNA 2 trafficking per total number of injections) are as follows: RNA 2 injected alone (0/10); RNA 2 coinjected with wild-type MP (10/14), 27 to 31 (5/7), 122 (0/7), 128 (0/6), 144 (0/8), 161 (0/6), 204 (5/6), 242 (7/9), 278 (0/12), 280 (0/6), 291 (0/6), 301 (4/6), and 305 (6/7). See text for detailed discussion of RCNMV MP mutant 280.

^f (-) indicates no unfolding of RCNMV RNA 1 and RNA 2 after binding to the RCNMV MP, as determined by electron microscopy. Two to four experiments were conducted in each case.

^g (-) indicates no RCNMV MP-potentiated trafficking of single- or double-stranded DNA. Eight and 14 injection experiments were conducted for single- and double-stranded DNAs, respectively, and in none of these experiments did either of the DNAs show cell-to-cell trafficking in the presence of RCNMV MP.

RCNMV RNA 1 was coinjected with unlabeled RCNMV MP (Figures 3D, 3E and 3F). Because TOTO-1 iodide only fluoresces when bound to nucleotides (Glazer and Rye, 1992), the observed cell-to-cell spread of the fluorescence must be due to trafficking of RCNMV RNA 1 and RNA 2, which is potentiated by the coinjected RCNMV MP, rather than to diffusion of free TOTO-1 iodide. Because plasmodesmata are the sole targeting sites across the cell wall for viral MPs, the RCNMV MP-potentiated cell-to-cell trafficking of RCNMV RNA must be via plasmodesmata.

When the RCNMV MP mutants 27 to 31, 204, 242, 301, and 305 were coinjected with fluorescently labeled RNA 2, the fluorescence also spread rapidly from cell to cell (Table 1). By contrast, when the RCNMV MP mutants 122, 128, 144, 161, 278, 280, and 291 were coinjected with the labeled RNA 2, the fluorescence remained in the injected cell, even after 20 min (Table 1 and Figure 3G). These data, as well as the fact that RNA did not traffic from cell to cell when injected alone, exclude the possibility that breakdown products of RCNMV RNA molecules diffused cell to cell.

Interestingly, the RCNMV MP mutant 280, which did not potentiate RNA trafficking, did appear to potentiate both cell-to-cell movement of RCNMV during infection (Giesman-

Cookmeyer and Lommel, 1993) and an increase in plasmodesmal SEL (Table 1). Thus, an increase in the plasmodesmal SEL is necessary, but not sufficient, for RNA cell-to-cell trafficking. In a previous study (Giesman-Cookmeyer and Lommel, 1993), it was shown that in infected plants, this mutation always reverted to the wild-type sequence, and only wild-type progeny virions were recovered. Hence, MP mutant 280 did not actually effect viral spread, but a reversion to the wild-type MP occurred during replication, and this was the form of the MP that was then able to effect cell-to-cell spread of the virus. These data, therefore, establish that increasing the plasmodesmal SEL and potentiating plasmodesmal transport of RNA are two genetically distinct functions associated with the RCNMV MP that are both required for cell-to-cell movement of RCNMV.

In recent studies, it was shown that RCNMV MP binds single-stranded DNA cooperatively in vitro (Osman et al., 1992; Giesman-Cookmeyer and Lommel, 1993), and thus experiments were conducted to test whether the RCNMV MP could also potentiate plasmodesmal trafficking of DNA molecules. We labeled single-stranded DNA (produced from the infectious transcript vector pBluescript KS+ containing the coding sequence for RCNMV RNA 1) and double-stranded DNA (pBluescript KS+) with TOTO-1 iodide and coinjected either

one of them with unlabeled RCNMV MP into cowpea mesophyll cells. As shown in Figures 3H and 3I, the single- and double-stranded DNA did not move from the injected cell into surrounding cells, even after 20 min. These results demonstrate that RCNMV MP-potentiated trafficking of nucleic acids via plasmodesmata is a selective process.

In view of the correlation between the abilities to modify plasmodesmal SEL, to traffic RCNMV RNA (1 or 2), and to potentiate cell-to-cell spread of the virus for a given RCNMV MP mutant, we concluded that potentiating plasmodesmal trafficking of RCNMV RNA, like modifying plasmodesmal SEL, is an essential function of the RCNMV MP.

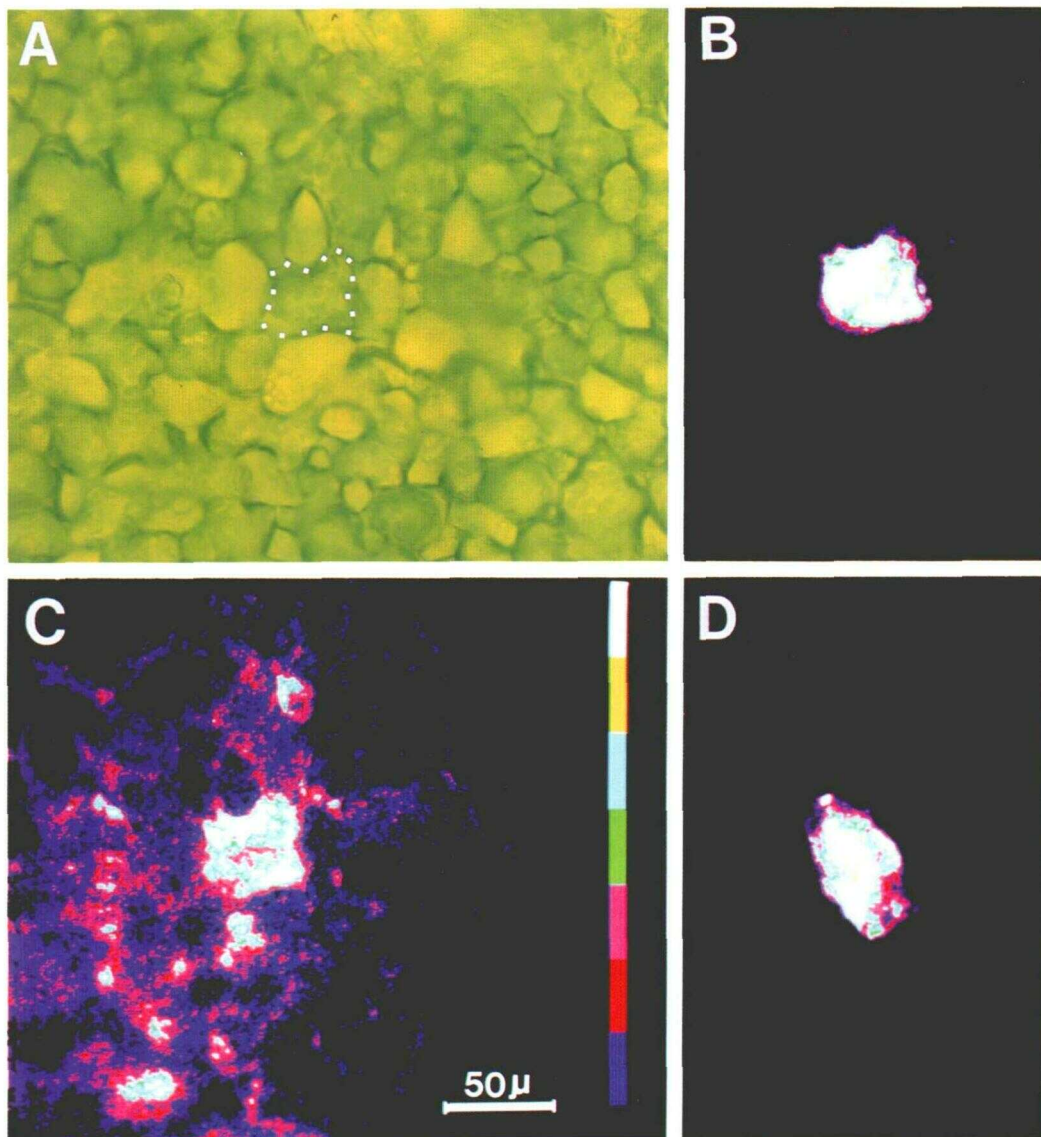


Figure 2. Cell-to-Cell Trafficking of RCNMV MP in Cowpea Mesophyll Cells.

Details relating to fluorescence intensity are as given in Figure 1.

(A) Bright-field image of a portion of the cowpea mesophyll after the epidermis was removed, revealing the cell (outlined by white dots) into which the fluorescently labeled RCNMV MP was injected.

(B) and **(C)** Thirty seconds and 180 sec, respectively, after injection of fluorescently labeled RCNMV MP into the cell marked in **(A)**.

(D) Twenty minutes after the fluorescently labeled RCNMV MP mutant 278 was injected into a mesophyll cell.

Bar in **(C)** = 50 μ m.

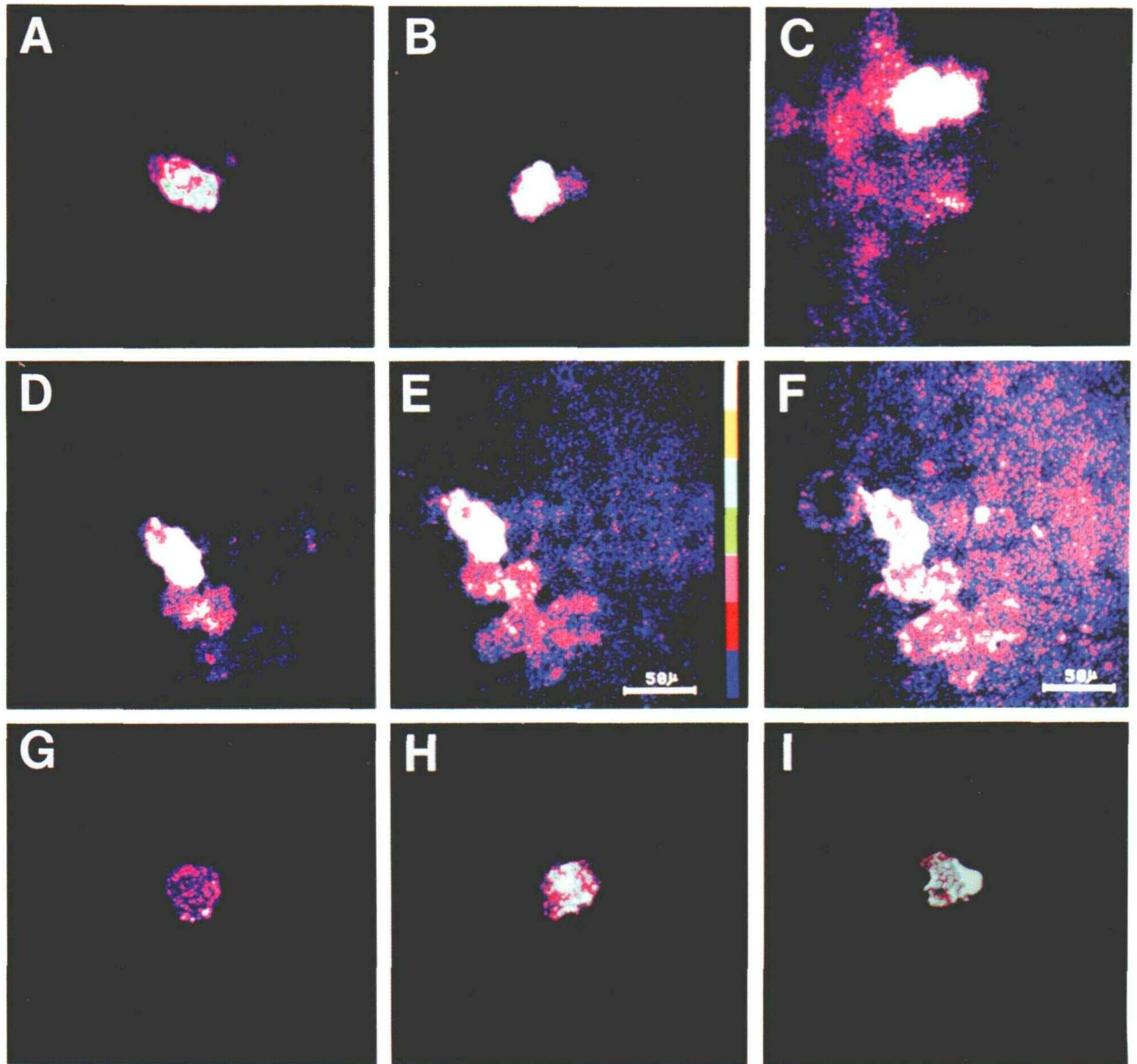


Figure 3. RCNMV MP–Potentiated Cell-to-Cell Trafficking of Fluorescently Labeled RCNMV RNA 1 and RNA 2 between Cowpea Mesophyll Cells.

Fluorescence intensities are as given in Figure 1.

(A) Injection of RNA 2 alone into a mesophyll cell. RNA 2 did not move out of injected cells but was slowly degraded over time (image was taken 5 min after injection). Injection of RNA 1 alone gave a similar result (data not shown).

(B) and **(C)** Thirty and 180 sec, respectively, after coinjection of RNA 2 and wild-type RCNMV MP into a mesophyll cell.

(D), **(E)**, and **(F)** Forty, 80, and 180 seconds, respectively, after coinjection of RCNMV RNA 1 and wild-type RCNMV MP.

(G) Twenty minutes after coinjection of RNA 2 with RCNMV mutant MP 278.

(H) Twenty minutes after coinjection of fluorescently labeled single-stranded DNA (produced from the infectious transcript vector of RCNMV RNA 1) and wild-type RCNMV MP.

(I) Twenty minutes after coinjection of fluorescently labeled double-stranded DNA (pBluescript KS+) and RCNMV MP.

Bars in **(E)** and **(F)** = 50 μ m.

RCNMV MP Does Not Unfold RCNMV RNA

As shown above, increasing the plasmodesmal SEL is required, but not sufficient, to permit cell-to-cell trafficking of RCNMV RNA. Presumably, one of the essential steps of the RNA trafficking process involves interaction(s) between RNA and RCNMV MP and/or certain cellular factors. Based on studies of TMV and cauliflower mosaic virus MPs, it has been proposed that cooperative binding of MP to a viral nucleic acid molecule unfolds the latter to form an extended and linear protein–nucleic acid complex that is then targeted to plasmodesmata for transport (Citovsky et al., 1990, 1991, 1992; Citovsky and Zambryski, 1991; Citovsky, 1993). However, although RCNMV MP is able to bind RNA and single-stranded DNA *in vitro* in a nonspecific and cooperative manner (Osman et al., 1992; Giesman-Cookmeyer and Lommel, 1993), such *in vitro* cooperative binding is not correlated to the ability of RCNMV MP either to effect viral cell-to-cell movement during infection (Giesman-Cookmeyer and Lommel, 1993) or to potentiate cell-to-cell RNA trafficking (Table 1). Thus, *in vitro* protein–RNA binding assays alone appear to be insufficient to explain the mechanisms of RCNMV MP function *in vivo*. It should be noted that previous *in vitro* studies on the TMV and cauliflower mosaic virus MP binding properties were not performed with RNA and DNA species that are known to be trafficked through plasmodesmata or that are of plant viral origin (Citovsky et al., 1990, 1991, 1992). Given these considerations, we undertook an electron microscopic characterization of the structures of complexes formed between RCNMV MP and RCNMV RNA 2 and between RCNMV MP and RCNMV RNA 1 to determine whether RCNMV MP indeed unfolds RCNMV RNA *in vitro* and, if it does, whether a direct correlation exists between the abilities of the RCNMV MP mutants to unfold RNA and to potentiate RNA cell-to-cell trafficking.

The *in vitro*-transcribed RNA 1 and RNA 2 were fluorescently labeled with TOTO-1 iodide. Protein binding experiments were performed as previously described (Giesman-Cookmeyer and Lommel, 1993). The buffer and incubation conditions for the binding experiments were also essentially the same as those used for the RCNMV MP/RNA microinjection experiments. Furthermore, for each binding experiment, an aliquot of the reaction mixture was used for electron microscopic study, and the remaining mixture was loaded onto an agarose gel, as shown in Figure 4. This strategy permitted a direct correlation of electron microscopic observations to the gel shift profiles. It should be pointed out here that incorporation of TOTO-1 iodide into RNA molecules did not appear to affect RCNMV MP binding to RCNMV RNA as compared to binding assay using radiolabeled RNA (Giesman-Cookmeyer and Lommel, 1993).

As shown in Figure 5A, when RNA 2 molecules spread in the presence of 10% formamide were visualized in the electron microscope, they appeared as short, linear strands with an average measured length of 165 ± 26 nm (50 RNA molecules were measured). This would suggest that the “base-to-base” spacing for the measured RNA 2 was ~ 1.2 Å. In the

presence of RCNMV MP at a saturating concentration, i.e., the protein concentration at which all of the RNA molecules were bound by the protein as shown in gel shift studies (see Figure 4A), the RNA 2–MP complexes also appeared as short strands similar to the RNA 2 alone (Figure 5B). These strands had an average measured length of 157 ± 29 nm (50 RNA molecules were measured), which was not different from the value obtained for the RNA 2 alone. To prove that the RCNMV MP was indeed bound to the RNA 2 molecules, we treated the reaction mixture with RNase A. We hypothesized that if the RCNMV MP was bound to the single-stranded regions of RNA 2, it should protect these regions from RNase A digestion. Double-stranded regions, such as stem-loops at the 3' end (Lommel et al., 1988), would presumably be protected without MP binding. Both gel shift and electron microscopic examinations demonstrated that after RNase A treatment, the RNA 2 molecules were degraded in the absence of the RCNMV MP, whereas they were protected in the presence of the RCNMV MP (Figures 4B, 5C, and 5D).

Because the measured length of RNA 2 molecules was not altered after RCNMV MP binding, it was possible that the RNA 2 molecules were already unfolded and extended in the absence of RCNMV MP so that binding of this protein did not have any further effect on these molecules. Alternatively, it was possible that RNA 2 molecules were folded, but RCNMV

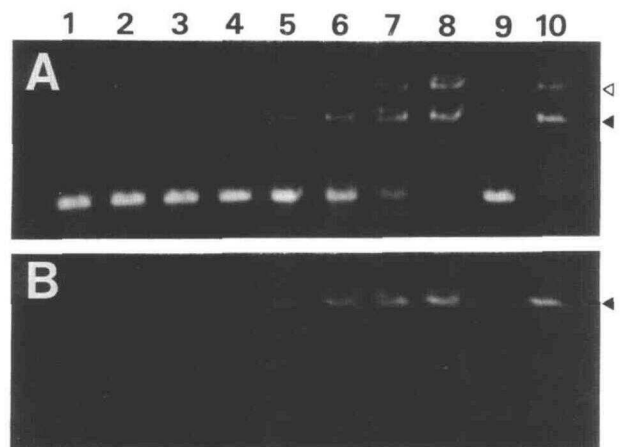


Figure 4. In Vitro RNA Binding Gel Shift Assay.

(A) Binding of wild-type RCNMV MP to fluorescently labeled RCNMV RNA 2. In lanes 1 to 8, 0.0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.5, and 3.0 μg of protein, respectively, were used to bind 50 ng of RNA 2. In lanes 9 and 10, 0.0 and 3.0 μg of protein were used, respectively, and the binding reaction was conducted in 10% formamide. The solid dart indicates the position of RNA 2 bound by the RCNMV MP. The open dart points to the position of loading wells of the agarose gel, and the fluorescence present in these wells is from aggregates of some RNA 2 molecules that are formed in the presence of RCNMV MP, as determined by electron microscopy.

(B) Protection of fluorescently labeled RNA 2 by the wild-type RCNMV MP against RNase A digestion. The solid dart indicates the position of RNA 2 bound by the RCNMV MP.

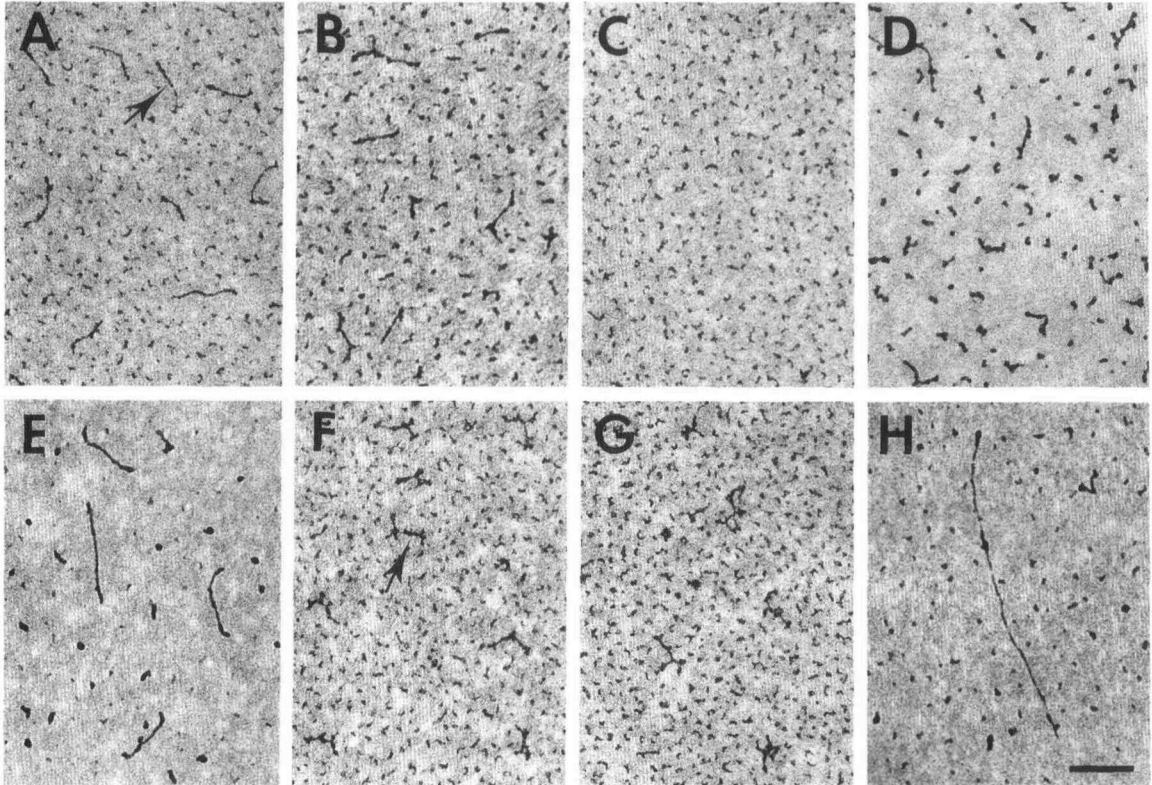


Figure 5. Electron Microscopic Characterization of RCNMV RNA Molecules and Wild-Type RCNMV MP-RNA Complexes.

- (A) RNA 2 alone spread in the presence of 10% formamide. The arrow points to an RNA 2 molecule.
 (B) RNA 2-RCNMV MP complexes spread in the presence of 10% formamide.
 (C) RNA 2 after RNase A digestion spread in 10% formamide.
 (D) RNA 2-RCNMV MP complexes after RNase A treatment spread in 10% formamide.
 (E) RNA 2 alone spread in 80% formamide.
 (F) RNA 1 alone spread in 10% formamide. An RNA 1 molecule is indicated by the arrow.
 (G) RNA 1-RCNMV MP complexes spread in 10% formamide.
 (H) RNA 1 alone, spread in 80% formamide.

Black stipples in the background are cytochrome *c*. Bar in (H) = 0.2 μm .

MP binding did not cause unfolding of these molecules. To test these possibilities, RNA 2 molecules were spread in 80% formamide for electron microscopic examination. Such a high concentration of formamide would melt out random intramolecular base-to-base interactions and extend RNA molecules (Ferguson and Davis, 1978). As shown in Figure 5E, RNA 2 molecules became elongated in the presence of 80% formamide, having an average measured length of 270 ± 52 nm (66 RNA molecules were measured) or a base-to-base distance of ~ 1.9 Å. This represents a 36% increase over the value obtained for RNA 2 molecules spread in 10% formamide. Obviously, RCNMV MP binding did not unfold and extend RNA 2 molecules, an observation which is in disagreement with that reported for the TMV MP-hepatitis delta virus (HDV) RNA complex (Citovsky et al., 1992).

These findings were further supported by the characterization of RNA 1 molecules and RNA 1-RCNMV MP complexes.

When RNA 1 molecules alone were spread in 10% formamide and examined in the electron microscope, they appeared highly structured and folded (Figure 5F). After binding to the RCNMV MP (in the presence of 10% formamide), the RNA 1 molecules still retained this highly structured form and were clearly not unfolded and extended (Figure 5G). When spread alone in the presence of 80% formamide, all of the RNA 1 molecules lost their complex structural features and appeared as extended, linear entities (Figure 5H). Length measurement of these RNA 1 molecules yielded a value of 865 ± 116 nm, representing a base-to-base distance of ~ 2.2 Å (30 RNA molecules were measured).

These data demonstrate unequivocally that RCNMV MP did not unfold RCNMV RNA molecules *in vitro*, although it was indeed bound to them. Here it is important to point out that the experimental conditions we used in this study to spread RCNMV RNA molecules as well as RCNMV MP-RNA

complexes for electron microscopic characterizations were similar to those used to spread the HDV molecules and TMV MP-HDV complexes (Citovsky et al., 1992). In particular, 10% formamide, which does not denature RNA molecules, was used in both studies. Our gel shift assays indicated that the presence of 10% formamide did not affect the RNA binding characteristics of the RCNMV MP (Figure 4A).

We tested all of the 12 RCNMV mutant MPs to determine what effect, if any, mutations at certain amino acids might have on the structure of the RCNMV MP-RNA complex. In all cases, the mutant MPs had no apparent effect on the appearance of either RNA 1 or RNA 2 molecules (data not shown). Hence, none of the mutant MPs was capable of RNA unfolding (Table 1). The differences among these RCNMV mutant MPs in their abilities to potentiate plasmodesmal trafficking of RCNMV RNA must, therefore, be due to differences in other functional aspects, e.g., in their ability to induce an increase in plasmodesmal SEL, to recognize RCNMV RNA molecules, or to target viral RNA to plasmodesmata.

Because unfolding RCNMV RNA *in vitro* is not a function of RCNMV MP, the RCNMV MP may interact with RCNMV RNA in a manner that remains to be determined to potentiate cell-to-cell trafficking of these RNA molecules. Alternatively, unfolding of RNA, if necessary for trafficking, may well involve participation of cellular factors.

DISCUSSION

Our results establish that macromolecules, such as viral proteins and nucleic acids, are able to traffic from cell to cell via plasmodesmata in higher plants. Because such trafficking events take place within a few seconds of the test molecule being introduced into the target cell, we propose that endogenous mechanisms exist for macromolecular trafficking through plasmodesmata, and that such trafficking must be going on at all times. In a parallel study on the bean dwarf mosaic virus, it was demonstrated that the fluorescently labeled BL1 movement protein and nucleic acids of this virus also trafficked cell to cell (A. Noueiry, W.J. Lucas, and R. Gilbertson, manuscript submitted). Collectively, these findings bring us one step closer to developing an understanding of the complex functions performed by plasmodesmata, as well as an understanding of viral cell-to-cell movement and systemic infection and disease. We suggest that selective trafficking of macromolecules is a normal function of plasmodesmata and that some plant viruses have usurped this function to enable the spread of their own genetic material from cell to cell. It is possible that plant viruses acquired the genetic information encoding for their MPs from endogenous plant movement protein genes during coevolution (Lucas and Wolf, 1993).

Although the detailed mechanism(s) remains to be elucidated, plasmodesmal trafficking of macromolecules apparently shares certain parallel features with nucleocytoplasmic

transport (Lucas et al., 1993). The fact that proteins of more than 30 kD and RNA molecules of 4 kb traffic rapidly from cell to cell indicates that the normal plasmodesmal SEL of 800 to 1000 D, as established by dye-coupling studies, represents the limit for passive diffusion of small molecules (see also Ding et al., 1993). If the protein molecule is in the globular form and moves through the cytoplasmic annulus, passage of the 35-kD RCNMV MP would require that the diameter of microchannels within the plasmodesma be dilated from the normal 2.5 nm to ~8 to 9 nm. Such an increase in the physical dimension of these plasmodesmal microchannels is conceivably temporary and highly controlled, much like the transport channels within the nuclear pore complex (Akey and Goldfarb, 1989; Ding et al., 1993; Lucas et al., 1993).

Like nucleocytoplasmic transport, plasmodesmal trafficking of macromolecules is a selective process: whereas RCNMV MP potentiates trafficking of RNA, but not any DNA (Figure 3), bean dwarf mosaic virus BL1 protein potentiates trafficking of double-stranded DNA, but not single-stranded DNA or RNA (A. Noueiry, W.J. Lucas, and R. Gilbertson, manuscript submitted). The mechanisms underlying such selectivity remain to be elucidated.

The finding that RCNMV MP does not unfold the RCNMV RNA molecules *in vitro*, although it does bind to them *in vitro* and potentiate their trafficking through plasmodesmata *in vivo*, suggests that RCNMV MP interacts with these RNA molecules in an unidentified manner to permit their targeting to and trafficking through plasmodesmata. Alternatively, if unfolding is required at any step during trafficking, it may well involve the participation of cellular factors (i.e., certain plasmodesmal proteins). At present, we do not know whether the RNA molecule alone, an RNA-MP complex, an RNA-cellular factor complex, or an RNA-MP-cellular factor complex trafficks through plasmodesmata.

Our preliminary results showed that RCNMV MP-potentiated cell-to-cell trafficking of RNA did not appear to be sequence specific. A 3.5-kb RNA was transcribed *in vitro* from the Scal-linearized plasmid pBSIL9, which contains sequences from the isocitrate lyase gene of *Brassica napus* and the *E. coli* plasmid pBS (Comai et al., 1989). Like RCNMV RNA 1 and RNA 2, this nonviral RNA trafficked cell to cell in the presence of RCNMV MP (data not shown). This lack of sequence specificity is consistent with results obtained from *in vitro* RNA binding studies with RCNMV MP (Osman et al., 1992; Giesman-Cookmeyer and Lommel, 1993). It is possible that the three RNAs share a structural feature, rather than a sequence element, that is recognized by the RCNMV MP. This and other possibilities will be tested further.

It is of special interest that although the RCNMV MP does not potentiate plasmodesmal trafficking of single-stranded DNA, it is able to bind such DNA cooperatively *in vitro* (Osman et al., 1992; Giesman-Cookmeyer and Lommel, 1993). This finding, therefore, reveals a level of specificity of RCNMV MP function *in vivo* which is not observed *in vitro*. Hence, the *in vitro* RNA binding assay alone provides limited information on the function of the MP, and the biological relevance of the

nucleic acid binding activity *in vitro* by plant viral MPs remains to be determined.

Although the RCNMV MP, with a molecular mass of 35 kD, readily trafficked from cell to cell, it rarely facilitated cell-to-cell movement of F-dextrans larger than 9.4 kD. Presumably, the F-dextran movement through plasmodesmata is via passive diffusion that is limited solely by the effective pore size of the microchannels, whereas macromolecular trafficking via plasmodesmata, like nucleocytoplasmic transport of macromolecules, is achieved by an actively controlled process (Silver, 1991; Lucas et al., 1993).

Our analysis of the RCNMV MP mutants suggests that RCNMV MP possesses a number of distinct functional domains. For example, increasing plasmodesmal SEL and potentiating RNA trafficking through plasmodesmata are two genetically distinct functions. Precise mapping of these functional domains must await the development of a three-dimensional structural model and more detailed mutational analyses of this MP.

The biological significance of plasmodesmal trafficking of macromolecules can be far-reaching. As previously discussed (Lucas et al., 1993), whereas nucleocytoplasmic transport of macromolecules provides a direct means of regulating gene expression in individual eukaryotic cells, plasmodesmal trafficking of informational macromolecules could potentially exert an elevated, supracellular coordination of these events in higher plants. The selectivity in terms of trafficking of specific target molecules would establish a considerable advantage over the diffusion of small molecules. Future studies will concentrate on identifying these putative endogenous plant proteins that are capable of moving through plasmodesmata to control plant development and physiological processes.

METHODS

Plant Material

Cowpea (*Vigna unguiculata*) plants were grown in a growth chamber under a temperature regime of 25°C/18°C (day/night) with a 16-hr photoperiod at a PAR level of 230 to 280 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. The first or second fully expanded trifoliate leaf was used for microinjection experiments.

Microinjection

Attached to the plant, the leaf was fixed on the microscope stage adaxial side up, and a small portion of the lower epidermis was peeled off. Distilled water was applied to the exposed region immediately. Details regarding the instrumentation and procedure for microinjection were as previously described (Ding et al., 1992a).

The wild-type and mutants of red clover necrotic mosaic virus (RCNMV) movement protein (MP) were expressed in *Escherichia coli* and stored in B1 buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol, and 200 mM NaCl) as previously described (Giesman-Cookmeyer and Lommel, 1993). Protein concentrations (wild-type and mutants) were between 1.0 and 3.0 mg/mL.

To assess the effect of RCNMV MP on the plasmodesmal size exclusion limit (SEL), a 5 mM solution of 9.4-kD fluorescein-conjugated dextran (F-dextran) (Molecular Probes, Eugene, OR) was prepared in 5 mM KHCO_3 , filtered through a 0.5 μm -pore nylon syringe filter, and stored at 4°C. For experiments in which 9.4-kD F-dextran and RCNMV MP were to be coinjected, the injection medium was prepared by mixing equal volumes of F-dextran and MP.

To determine whether RCNMV MP trafficks cell to cell, the RCNMV MPs (wild-type and mutants) purified from *E. coli* were fluorescently labeled with fluorescein isothiocyanate (FITC) following the method of A. Noueiry, W.J. Lucas, and R. Gilbertson (manuscript submitted).

RNA 1, RNA 2, and single-stranded DNA (7.2 kb) were produced as previously described (Xiong and Lommel, 1991; Giesman-Cookmeyer and Lommel, 1993). The plasmid pBluescript KS+ (3.0 kb) was purified from *E. coli* (Sambrook et al., 1989). The nucleic acids were labeled with the nucleotide-specific fluorescent dye TOTO-1 iodide (Molecular Probes Inc.) as follows. Briefly, a 5- μL nucleic acid (0.5 to 2 $\mu\text{g}/\mu\text{L}$) solution was mixed with 0.5 μL of TOTO-1 iodide and 5 μL of B1 buffer. The mixture was incubated for 1 hr at room temperature and was then diluted in 40 μL of B1 and stored at -20°C prior to use. The nucleic acid solution was injected alone or was mixed with an equal volume of RCNMV MP (5 mg/mL) prior to being used in injection experiments.

In Vitro Assay of RCNMV MP Binding to RCNMV RNA

In vitro-transcribed RCNMV RNA 2 was fluorescently labeled by incubating 1.25 μg of RNA with 1 μL of 1 mM TOTO-1 iodide in 15 μL of Tris-EDTA (TE) for 1 hr at room temperature. The mixture was diluted to 25 ng/ μL by adding 35 μL of B1 buffer. RNA (50 ng) was then incubated with wild-type RCNMV MP in 8 μL of B1 buffer for 10 min on ice. After incubation, a 2- μL aliquot was removed from the reaction mixture which contained 3.0 μg of protein (lane 8, Figure 4) for spreading for electron microscopic characterization, and 2 μL of 50% glycerol/0.25 M EDTA was then added to each of the remaining reaction mixtures. The samples were loaded onto a 1% agarose gel and subjected to a current of 40 mamps for 30 min in TBE buffer (Tris-borate-EDTA) at room temperature. The gel was photographed on a FOTODYNE UV (FOTODYNE Inc.) light source using Polaroid Type 665 film. For RNaseA treatment, the agarose gel described in Figure 4A was incubated for 1 hr in 10 $\mu\text{g}/\text{mL}$ RNaseA at room temperature before being photographed as described above.

Electron Microscopy

A 2- μL aliquot of the reaction mixture obtained from the binding experiment, which involved 50 ng of RNA and 3.0 μg of RCNMV MP (lane 8, Figure 4A), or from control reaction without RCNMV MP, as described in Figure 4, was added to 8 μL of B1 buffer. For spreading in 10% formamide (as per Citovsky et al., 1992), this diluted mixture was added to a solution containing 28 μL of glass-distilled H_2O , 5 μL of TE (2 M Tris, pH 8.3, 0.2 M EDTA), 5 μL of formamide (highest purity grade; Electron Microscopy Sciences, Philadelphia), and 3 μL of cytochrome *c* (Sigma) (4 mg/mL in H_2O). This preparation was then spread onto glass-distilled H_2O to form a monolayer film of macromolecules. For spreading in 80% formamide, 5 μL of an RNA sample was added to a solution containing 40 μL formamide, 3 μL TE, and 2 μL cytochrome *c*. This preparation was spread onto 50% formamide buffered by TE (0.25 M Tris, pH 8.3, 0.025 M EDTA). Formvar-coated copper grids

were used to pick up the monolayer film. Grids were immediately soaked in a solution of uranyl acetate (0.1 µg/mL in 90% ethanol) for 10 sec and then transferred to 90% ethanol for an additional 10 sec before being air dried. Specimens were examined on a transmission electron microscope (model 410LS; Philips, Eindhoven, The Netherlands). Micrographs were taken at a magnification of 10,400. Length measurement was conducted on straight RNA molecules or RNA-MP complexes.

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