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Capsid Protein and Helper Component-Proteinase Function as Potyvirus Cell-to-Cell Movement Proteins

Maria R. Rojas,* F. Murilo Zerbini,† Richard F. Allison,‡ Robert L. Gilbertson,† and William J. Lucas§,[1]

*Department of Agronomy and Range Science, †Department of Plant Pathology, §Section of Plant Biology, Division of Biological Sciences, University of California, Davis, California 95616; and ‡Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824-1312

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The role of bean common mosaic necrosis potyvirus (BCMNV) and lettuce mosaic potyvirus (LMV) proteins was investigated in terms of their capacity to function as viral movement proteins (MPs). Using *Escherichia coli*-expressed proteins and microinjection techniques, direct evidence was obtained that both the potyviral capsid protein (CP) and helper component-proteinase (HC-Pro) function in this capacity, in that both proteins (a) trafficked from cell to cell, (b) induced an increase in plasmodesmal size exclusion limit, and (c) facilitated cell-to-cell movement of viral RNA. CP and HC-Pro mutants were also produced and used in microinjection experiments. Mutations in the core region of the CP either impaired (single and double amino acid substitution mutants) or abolished (triple amino acid substitution mutant) cell-to-cell movement, as did C-terminal deletion mutants in HC-Pro. The BCMNV P1, CI, NIa, and NIb proteins did not exhibit viral MP properties, but NIa and NIb proteins were found to accumulate within the nuclei of injected cells. These results further establish the multifunctional nature of the potyvirus CP and HC-Pro.

INTRODUCTION

Systemic infection of plants by viruses involves at least three steps: (a) viral replication in initially infected cells; (b) cell-to-cell or short-distance movement, where the virus moves out into adjacent cells; and (c) long-distance transport, where spread occurs through the vascular system and the virus infects cells and organs distant from the initial point of entry into the plant. In cell-to-cell and long-distance movement, the infectious form of the virus, which may be virions, viral nucleic acids, viral nucleoprotein complexes, or a combination thereof, must be able to traffic between cells and different cell types. It is generally accepted that plant viruses accomplish this by exploiting plasmodesmata (Deom *et al.,* 1987; Maule, 1991; Lucas and Gilbertson, 1994; Carrington *et al.,* 1996). However, virus movement through plasmodesmata is unlikely to be a passive process (i.e., mediated by diffusion), because dye-coupling studies have established that plasmodesmata have a size exclusion limit (SEL) of 800 to 1000 Da (Tucker, 1982; Goodwin, 1983), which is far too small to allow the free passage of virions and/or free viral nucleic acids (Citovsky and Zambryski, 1991; Deom *et al.,* 1992; Lucas *et al.,* 1993).

It is now well established that many plant viruses encode dedicated movement proteins (MPs) that have the capacity to increase plasmodesmal SEL and mediate the passage of macromolecules between cells (Lucas and Gilbertson, 1994; Wolf *et al.,* 1989; Fujiwara *et al.,* 1993; Noueiry *et al.,* 1994; Waigmann *et al.,* 1994; Ding *et al.,* 1995; Waigmann and Zambryski, 1995). It has also been established that these MPs are able to move cell to cell through plasmodesmata. Furthermore, many viral MPs possess nucleic acid binding properties, consistent with their role in the trafficking of viral nucleic acids. These viral MPs have been characterized for a number of RNA and DNA viruses, including tobamoviruses, dianthoviruses, cucumoviruses, and geminiviruses (Wolf *et al.,* 1989; Citovsky *et al.,* 1990; Fujiwara *et al.,* 1993; Noueiry *et al.,* 1994; Ding *et al.,* 1995). Many of these viruses do not require the capsid protein for cell-to-cell movement, which supports the hypothesis that the infectious form of the virus that moves cell to cell through plasmodesmata is a viral nucleic acid–MP complex. Delivery of this complex to plasmodesmata may involve an interaction with the plant cytoskeleton (Heinlein *et al.,* 1995; Waigmann and Zambryski, 1995; Oparka *et al.,* 1996).

Potyviruses comprise a large and economically important group of plant viruses (Shukla and Ward, 1988). The typical potyvirus genome is a single-stranded positive-sense RNA molecule of about 10,000 nucleotides, with a 5′ end-linked protein (VPg) and a 3′ poly(A) tail (Riechmann *et al.,* 1992). Potyvirus genome expression is accomplished through the translation of the viral RNA into a polyprotein that is cleaved, by three virus-encoded proteinases, into at least eight viral proteins (Dougherty and Carrington, 1988) (Fig. 1). These proteins include the capsid protein (CP); protein 1 (P1), an

[1] To whom correspondence and reprint requests should be addressed. Fax: (916) 752-5410. E-mail: wjlucas@ucdavis.edu.

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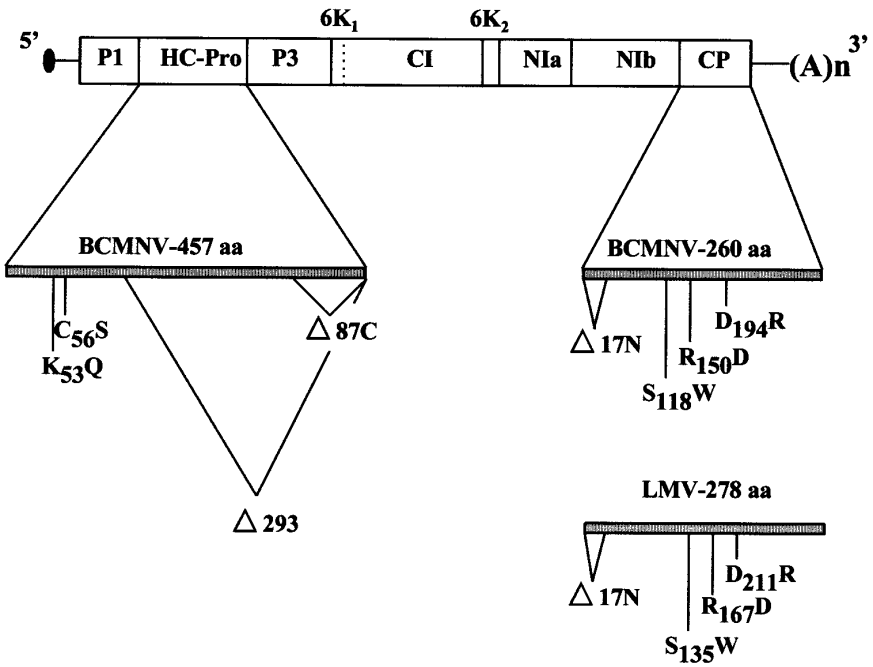

FIG. 1. Genomic organization of a typical potyvirus. Mutations introduced into the helper component-proteinase (HC-Pro) and the capsid protein (CP) genes are indicated.

accessory factor for genome amplification (Verchot and Carrington, 1995); a multifunctional helper component-proteinase (HC-Pro), essential for vector transmission (Atreya and Pirone, 1993; Maia *et al.,* 1996); a cytoplasmic cylindrical inclusion body protein (CI), that has a helicase-like domain (Lain *et al.,* 1991); a 6-kDa membrane-associated protein involved in viral replication (6K) (Restrepo-Hartwig and Carrington, 1994); and two nuclear inclusion body proteins (NIa and NIb). The NIa protein has the VPg at its N-terminus and a proteinase domain at its C-terminus (Carrington *et al.,* 1993), whereas the NIb protein possesses characteristics of an RNA-dependent RNA polymerase (Dougherty and Carrington, 1988). An additional protein, P3, has been detected in infected cells, but its function remains unknown (Rodriguez-Cerezo and Shaw, 1991).

Genetic analyses conducted with a full-length infectious clone of tobacco etch potyvirus (TEV), tagged with the marker gene encoding β-glucuronidase (*GUS*), TEV-GUS, have demonstrated that the CP plays a role in cell-to-cell and long-distance movement (Dolja *et al.,* 1994, 1995) and that the HC-Pro plays a role in long-distance movement (Cronin *et al.,* 1995; Kasschau *et al.,* 1997). However, a dedicated potyvirus MP has yet to be identified by direct experimental analysis, nor has the mechanism(s) by which these proteins facilitate potyvirus movement been established.

In the present study, the role of potyvirus proteins in cell-to-cell movement was investigated using *Escherichia coli*-expressed proteins that were fluorescently labeled and then introduced into host cells using microinjection techniques. Results from these studies provide direct evidence that the HC-Pro and CP can increase plasmodesmal SEL and mediate their own cell-to-cell movement. Furthermore, both proteins facilitate cell-to-cell movement of viral RNA. These findings establish that potyviruses encode two viral MPs, and the role of these MPs in mediating potyvirus infection is discussed.

MATERIALS AND METHODS

Plant materials and viral isolates

Two potyviruses were used in this study: the NL-3 strain of bean common mosaic necrosis virus (BCMNV; previously named bean common mosaic virus) and lettuce mosaic virus (LMV). The complete nucleotide sequence of the isolate of the BCMNV NL-3 strain used in this study was recently published (Fang *et al.,* 1995). An LMV pathotype II isolate from California (LMV-R) was used in this study, and the capsid protein sequence of this isolate was recently published (Zerbini *et al.,* 1995). *Nicotiana benthamiana* Domin and lettuce (*Lactuca sativa L.* cv. "Cobham Green") plants used in microinjection experiments were grown in a greenhouse at 25° under a 16-hr photoperiod and irradiance levels of approximately 700–1000 μmol m^{-2} s^{-1}.

Construction of BCMNV and LMV recombinant plasmids

For BCMNV, DNA sequences encoding P1, HC-Pro, CI, NIa, NIb, and CP genes were amplified from three cDNA clones comprising the complete BCMNV genome

(Fig. 1; Fang *et al.,* 1995). The polymerase chain reaction (PCR) was used with the following parameters: 94° for 1 min (denaturing), 55° for 2 min (annealing) and 72° for 3 min (extension) for 15 cycles. To amplify each gene, specific primers were designed from the BCMNV sequence (Fang *et al.,* 1995). Restriction sites were introduced into the 5′ ends of the oligonucleotides and a stop codon (TAG) was included in the 3′ primer of all the genes, except for the CP gene, which contains the viral polyprotein stop codon. The primers used for each gene were as follows, with primers for the 5′ and 3′ ends of the genes shown, respectively; P1, 5′-A-A-C-T-*C-T-C-G-A-G*-A-T-G-T-T-T-G-G-A-T-C-C-3′ and 5′-C-G-C-G-*G-A-A-T-T-C*-C-T-A-A-T-A-A-T-G-T-T-C-A-A-T-3′, *Xho*I and *Eco*RI sites shown in italics; HC-Pro, 5′-G-C-C-A-*C-T-G-C-A-G*-A-C-G-C-A-C-A-A-A-C-C-G-3′ and 5′-A-T-C-C-*C-T-T-A-A-G*-C-T-A-T-C-C-A-A-C-C-C-T-A-T-A-G-T-G-C-3′, *Pst*I and *Eco*RI sites shown in italics; CI, 5′-C-A-G-G-C-T-C-G-A-G-A-G-C-T-T-A-G-A-T-G-A-C-3′ and 5′-A-A-G-C-C-C-A-T-G-G-C-T-A-T-T-G-T-A-G-A-C-G-A-A-C-3′, *Xho*I and *Nco*I sites shown in italics; NIa, 5′-T-G-C-A-*C-T-C-G-A-G*-G-G-A-A-G-G-A-A-G-C-G-T-3′ and 5′-C-A-G-T-*C-C-A-T-G-G*-C-T-A-C-T-G-C-A-C-A-C-T-G-A-C-3′, *Xho*I and *Nco*I sites shown in italics; NIb, 5′-T-G-G-A-*C-T-C-G-A-G*-A-G-C-A-A-G-A-A-G-G-A-T-3′ and 5′-C-C-A-G-*C-C-A-T-G-G*-C-T-A-T-T-G-T-G-T-T-G-A-C-A-C-3′, *Xho*I and *Nco*I sites shown in italics; and CP, 5′-G-G-C-C-A-A-*C-T-C-G-A-G*-T-C-C-A-G-C-A-A-G-A-A-A-G-3′ and 5′-A-G-A-C-*C-C-A-T-G-G*-T-T-A-C-T-G-C-C-C-A-G-A-3′, *Xho*I and *Nco*I sites shown in italics. The PCR-amplified fragments were cloned using the TA Cloning System (Invitrogen, San Diego, CA) to generate plasmids pBCP1, pBCHC-Pro, pBCNIa, pBCNIb, and pBCCP, respectively. The coding sequence of each gene was excised from the corresponding recombinant plasmid by digestion with the appropriate restriction enzymes and ligated into the *E. coli* expression vector, pRSET (A, B, or C) (Invitrogen), digested with the same restriction enzymes.

For LMV, the CP gene was amplified by PCR from the recombinant plasmid pLMV, which contains a 1.3-kb fragment comprising the 3′-end of the LMV genome (Zerbini *et al.,* 1995). As described for BCMNV, the following primers were used: 5′-C-T-A-G-G-*C-T-G-C-A-G*-G-A-G-A-C-A-C-A-A-AG-C-T-T-G-A-T-G-C-A-G-G-C-3′ and 5′-G-T-G-T-C-*G-A-A-T-T-C*-C-C-T-T-T-A-G-T-G-C-A-A-C-C-C-T-C-T-C-A-C-G-C-T-3′, *Pst*I and *Eco*RI sites shown in italics. The amplified CP fragment was digested with *Pst*I and *Eco*RI and ligated into *Pst*I/*Eco*RI-digested pRSETB, to generate pLMVCP. All PCR-amplified fragments for both BCMNV and LMV were sequenced to confirm their integrity.

Construction of CP and HC-Pro mutants

To engineer BCMNV and LMV CP ΔN mutants, in which the first 17 amino acid residues of the CP were deleted, PCR was used with oligonucleotide primers corresponding to the 5′ nucleotide sequence encoding amino acid residues 18 to 22 of the CP genes, and the CP 3′-end oligonucleotide primer previously described. Single, double, and triple amino acid substitution mutations were introduced into the coding sequence of the core region of the CP by site-directed mutagenesis according to the manufacturer's instructions (5 prime → 3 prime, Inc., Boulder, CO). The Ser_{118}, Arg_{150}, and Asp_{194} codons of the BCMNV CP were changed to Trp, Asp, and Arg, respectively, to generate the $S_{118}W$, $R_{150}D$, and $D_{194}R$ mutants. The Ser_{135}, Arg_{167}, and Asp_{211} codons of the LMV CP were changed to Trp, Asp, and Arg, respectively, to generate the $S_{135}W$, $R_{167}D$, and $D_{211}R$ mutants (Fig. 1). Double amino acid substitution mutants for BCMNV and LMV CPs were generated by introducing the $S_{118}W$ and $S_{135}W$ mutation, respectively, into a recombinant plasmid containing the BCMNV single $R_{150}D$ mutant or the LMV $R_{167}D$ mutant. An LMV triple amino acid substitution mutant was generated with the $S_{135}W$, $R_{167}D$, and $D_{211}R$ mutations.

A double amino acid substitution mutation was introduced into the coding sequence of the N terminus of the BCMNV HC-Pro gene using site-directed mutagenesis. In this mutant, the Lys_{53} and Cys_{56} codons were changed to Gln and Ser, respectively, to generate the $K_{53}Q$ and $C_{56}S$ double mutant (Fig. 1). A BCMNV HC-Pro ΔC mutant, in which the last 87 amino acid residues of the HC-Pro gene were deleted, was generated by PCR using an oligonucleotide primer corresponding to the HC-Pro 3′ end nucleotide sequence encoding amino acids 365 to 370 and the HC-Pro 5′-end oligonucleotide primer previously described. An additional HC-Pro deletion mutant, in which the C-terminal 293 amino acid residues were deleted, was generated by the introduction of a premature stop codon by changing the nucleotide at position 491 (G → A). The presence of all the introduced mutations was confirmed by DNA sequence analysis.

Protein expression, purification, and labeling

Recombinant plasmids were transformed into *E. coli* strains BL21::DE3 or JM 109::D3, and protein expression was induced as previously described (Noueiry *et al.,* 1994), except that a 250-ml culture was used. All expressed proteins formed inclusion bodies in *E. coli,* and overexpressed proteins were extracted as described according to Noueiry *et al.* (1994). Proteins were resuspended in 0.1 *M* $NaHCO_3$ containing 0.1% (w/v) SDS. Because the expressed proteins were fusion proteins having an additional 38 to 42 amino acid residues at the N-terminus, including a 6-His tag, they were further purified using Ni-NTA columns according to the manufacturer's instructions (Qiagen, Chatsworth, CA). Column-purified proteins were then labeled with fluorescein isothiocyanate (FITC), according to manufacturer's instructions (Molecular Probes, Eugene, OR), and renatured by stepwise dialysis against native buffer (10 m*M* Tris–HCl,

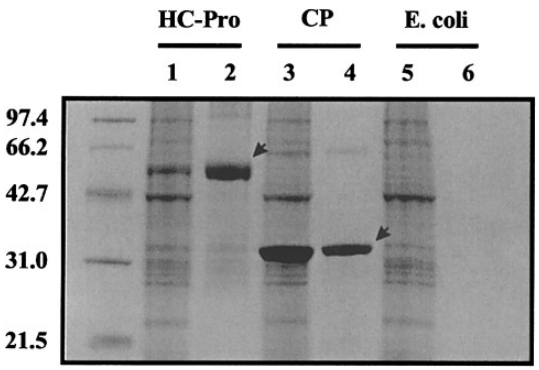

FIG. 2. Expression and purification of BCMNV HC-Pro and CP. SDS–PAGE analysis of total *E. coli* expressed proteins, extracted from *E. coli* transformed with pBCHC-Pro, pBCCP, or pRSET C alone, are shown in lanes 1, 3, and 5, respectively. Purity of expressed HC-Pro and CP, recovered after Ni-NTA column chromatography, is illustrated in lanes 2 and 4, respectively; the purity of these proteins is typical and representative of all of the proteins (mutants and wild-type) that were tested in this study. Lane 6 illustrates the absence of *E. coli* proteins after Ni-NTA column chromatography. Arrows indicate the positions of column-purified HC-Pro and CP.

pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA, 10% [v/v] glycerol, 1 m*M* PMSF, and 1 m*M* DTT) using a microdialyzer (Pierce, Rockford, IL). Protein purity and quantitative analysis were performed after column chromatography (Fig. 2) and again after labeling and renaturing by SDS–polyacrylamide gel electrophoresis (PAGE) and comparison to standards of known quantities. The integrity of the proteins was not altered after labeling and renaturation based on SDS–PAGE analysis (data not shown). The soluble nature of all the *E. coli*-expressed potyviral proteins was established by (a) the absence of a precipitate at room temperature or 4°, (b) the absence of turbidity in the protein preparations, (c) the ability to label the proteins with FITC, and (d) the ability to pressure-inject the proteins through a micropipette (1 μm outside diameter).

Production and labeling of CP BCMNV and LMV RNA transcripts

Plasmids pBCCP and pLMVCP, which contain the coding sequences of the BCMNV and LMV CPs, were linearized at their respective *Nco*I (BCMNV) or *Eco*RI (LMV) sites, respectively. RNA transcripts were synthesized using T7 RNA polymerase and a commercial kit, according to the manufacturer's instructions (Ambion, Austin, TX). Products of the transcription reaction were quantified on agarose gels by comparison to RNA standards of known quantities, and RNA transcripts were stored at −80° until used in microinjection experiments. Nucleic acids (RNA) were labeled with the fluorescent dye TOTO-1-Iodine according to the manufacturer's instructions (Molecular Probes). Nucleic acids also were labeled with chromatide fluorescein-12-UTP (CF-UTP) to produce covalently linked CF-UTP RNA transcripts according to the manufacturer's instructions (Molecular Probes).

Microinjection and confocal laser scanning imaging

Before and after being used in microinjection experiments, aliquots of all FITC-labeled proteins, TOTO-1-labeled RNA, and CF-UTP RNA transcripts were examined by gel electrophoresis to reconfirm the integrity of the injected material. Microinjection was carried out as described (Noueiry *et al.,* 1994).

Movement of fluorescent probes within the mesophyll cells of leaves was observed using either a Leitz Orthoplan epi-illumination microscope, equipped with a Hamamatsu VIM C1966-20 analytical system (Wolf *et al.,* 1989), or a Leica upright confocal laser scanning microscope (CLSM) (model TCS-4D; Leica, Heidelberg, Germany), equipped with a Narishige 4-dimensional hydraulic micromanipulator system (Narishige, Tokyo, Japan). All permanent images were obtained with the Leica CLSM, using long-working distance multimedia objectives (25 and 40X). A low intensity laser (25 mW Krypton/Argon laser) was used to image the spatial distribution of FITC-, TOTO-1-, and CF-UTP-labeled probes. Simultaneous three-channel recordings were made using the following filter sets; FITC and CF-UTP, 488 nm excitation, 525 nm emission; TOTO-1, 514 nm excitation, 533 nm emission; chlorophyll autofluorescence, 488 nm excitation, >590 nm emission.

All experiments were conducted and analyzed with the same laser power and photomultiplier settings. At the conclusion of an experiment, data files were transferred to a Power Mac 8500 workstation. Image analysis, display (adjustments in contrast, brightness, etc.), and preparation for plates were all performed with Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA).

RESULTS

Capsid protein of BCMNV and LMV can move cell to cell

In microinjection experiments, a number of viral MPs have been shown to display three essential functions: the ability to increase plasmodesmal SEL, the capacity to move cell to cell, and the ability to facilitate cell-to-cell movement of viral nucleic acids. To establish the foundation for such experiments it is always necessary to confirm the functional properties of the mesophyll plasmodesmata in test leaves. In their normal state, plasmodesmata allow Lucifer yellow CH (LYCH, 522 Da) to move extensively cell to cell, but do not allow the movement of 10-kDa FITC-labeled dextran (F-dextran). Therefore, in the present study, control injections with LYCH and 10-kDa F-dextran were conducted before and after each series of injection experiments. In these control injections, LYCH was first injected and allowed to move and dissipate throughout the tissue. Subsequently, 10-kDa F-dextran was injected into a cell through which LYCH had passed. In test leaves having functional plasmodesmata,

TABLE 1

The BCMNV and LMV Capsid Proteins Exhibit Cell-to-Cell Movement Protein Properties in *N. benthamiana*

Injected agent[a]	Coinjected agent	Results[b]
Lucifer yellow	—	70 (70)
10 kDa F-dextran	—	0 (68)
FITC-*E. coli* proteins	—	0 (10)
FITC-BCMNV CP	—	30 (37)
FITC-LMV CP	—	25 (34)
BCMNV CP	10 kDa F-dextran	32 (40)
LMV CP	10 kDa F-dextran	27 (38)
BCMNV CP	20 kDa F-dextran	0 (9)
LMV CP	20 kDa F-dextran	0 (10)
BCMNV CP	BCMNV RNA-TOTO	10 (12)
BCMNV CP	BCMNV RNA CF-UTP	6 (8)
BCMNV CP	LMV RNA-TOTO	14 (15)
BCMNV CP	LMV RNA CF-UTP	8 (10)
LMV CP	LMV RNA-TOTO	14 (15)
LMV CP	BCMNV CP RNA-TOTO	9 (10)
BCMNV CP RNA-TOTO	—	0 (12)
BCMNV CP RNA CF-UTP	—	0 (6)
LMV CP RNA-TOTO	—	0 (10)
LMV CP RNA CF-UTP	—	0 (8)
FITC-BCMNV CP Δ17N mutant	—	13 (17)
FITC-LMV CP Δ17N mutant	—	10 (14)
BCMNV CP Δ17N mutant	10 kDa F-dextran	7 (10)
LMV CP Δ17N mutant	10 kDa F-dextran	8 (12)
FITC-BCMNV CP $R_{150}D$ mutant	—	17 (20)
FITC-LMV CP $R_{167}D$ mutant	—	15 (18)
BCMNV CP $R_{150}D$ mutant	10 kDa F-dextran	12 (15)
BCMNV CP $R_{150}D$ mutant	LMV RNA-TOTO	8 (10)
BCMNV CP $R_{150}D$ mutant	LMV RNA CF-UTP	5 (8)
LMV CP $R_{167}D$ mutant	10 kDa F-dextran	12 (15)
FITC-BCMNV CP $S_{118}W,R_{150}D$ mutant	—	12 (15)
FITC-LMV CP $R_{167}D,D_{211}R$ mutant	—	10 (12)
BCMNV CP $S_{118}W,R_{150}D$ mutant	10 kDa F-dextran	10 (12)
BCMNV CP $S_{118}W,R_{150}D$ mutant	LMV RNA-TOTO	10 (12)
LMV CP $R_{167}D,D_{211}R$ mutant	10 kDa F-dextran	8 (10)
FITC-LMV CP $S_{135}W,R_{167}D,D_{211}R$ mutant	—	0 (18)
LMV CP $S_{135}W,R_{167}D,D_{211}R$ mutant	10 kDa F-dextran	15 (17)
LMV CP $S_{135}W,R_{167}D,D_{211}R$ mutant	LMV RNA-TOTO	10 (13)[c]
LMV CP $S_{135}W,R_{167}D,D_{211}R$ mutant	BCMNV RNA CF-UTP	6 (10)[c]

[a] For description of BCMNV and LMV proteins and mutants, see legend to Fig. 1 and under Materials and Methods.
[b] Results are presented as number of injections in which movement to neighboring cells was observed (total number of injections performed).
[c] When movement was detected, the fluorescence associated with RNA-TOTO-1 or CF-UTP RNA was limited to adjacent cells.

fluorescence of the 10-kDa F-dextran was observed to be confined to the injected cell (Table 1 and Fig. 3C). All experimental injections were then performed using fields of cells characterized in this manner. In addition, as the proteins to be tested were produced in *E. coli,* it was essential to establish whether *E. coli* proteins were capable of modifying plasmodesmata. For these control experiments, extracted *E. coli* proteins (as typified in lane 5 of Fig. 2), were FITC-labeled, renatured, and used in microinjection assays. These *E. coli* proteins were incapable of moving cell to cell (Tables 1 and 3).

Microinjection of FITC-labeled CP (FITC-CP) of BCMNV into *N. benthamiana* mesophyll cells resulted in movement of fluorescence into the surrounding cells (Table 1 and Figs. 3A and 3B). Identical results were obtained in parallel experiments in which the LMV FITC-CP was employed (Table 1). Movement of both CPs was observed in approximately 75% of the injections and, normally, it took from 30 to 60 sec for fluorescence to be detected in the neighboring cells, whereas it took up to 2 min for all cells in the microscope field to show fluorescence (12–18 cells at 160× magnification).

When the BCMNV or LMV CPs were coinjected with FITC-labeled 10-kDa F-dextran, movement of fluorescence into neighboring mesophyll cells was observed (Table 1 and Fig. 3D). The cell-to-cell movement of the coinjected 10-kDa F-dextran was similar to that of the labeled CPs, with movement occurring in approximately

75% of the injections and with a lag period of approximately 60 sec before fluorescence was detected in neighboring cells. In similar experiments, 20-kDa F-dextran remained confined to the injected cell when coinjected with BCMNV or LMV CP (Table 1). These results establish that both potyvirus CPs interact with plasmodesmata and increase mesophyll plasmodesmal SEL.

Coinjection of TOTO-1-labeled BCMNV and LMV CP RNAs, transcribed from recombinant plasmids containing cDNA clones of the CP genes from each virus, with unlabeled BCMNV or LMV CPs, resulted in cell-to-cell movement of the viral CP RNA (Table 1 and Fig. 3E). In control experiments, TOTO-1-labeled viral RNA remained confined to the injected cell, indicating that viral CP RNA does not have the capacity to move by itself (Table 1 and Fig. 3F). To validate our findings with TOTO-1-labeled RNA, we next microinjected covalently-linked fluorescence-conjugated CF-UTP LMV CP RNA transcripts. These microinjection experiments demonstrated that the CP mediated cell-to-cell movement of CF-UTP RNA in an identical manner to that observed using TOTO-1-labeled (noncovalently bound) RNA (Table 1 and Fig. 3G). Furthermore, the ability of the LMV and BCMNV CPs to mediate cell-to-cell movement of CP RNA was not virus sequence-specific; i.e., the BCMNV CP facilitated the movement of the BCMNV and LMV CP RNAs, and vice versa (Table 1).

To study the host specificity of the potyvirus CP–plasmodesmal interaction, BCMNV and LMV CPs were injected into lettuce mesophyll cells. BCMNV and LMV FITC-CP moved cell to cell in lettuce in the same way that was observed in *N. benthamiana* (Table 2). Because lettuce is not a known BCMNV host, these results suggest that the cell-to-cell movement function of the potyvirus CP, in mesophyll cells, is not host-specific.

Construction and analysis of coat protein mutants

To further investigate the capacity of the BCMNV and LMV CPs to interact with mesophyll plasmodesmata, a series of mutant CPs were generated. An N-terminal deletion of 17 amino acids (CP Δ17N) was made in both CPs, and single, double, and triple amino acid substitution mutants were generated using site-directed mutagenesis. The amino acids altered in these substitution mutants were three highly conserved residues (serine, arginine, and aspartic acid) located in the core region of the CP (Dolja *et al.,* 1991). Amino acid substitutions were as follows: BCMNV CP $S_{118}W$ and LMV CP $S_{135}W$, conserved serine residue changed to tryptophan; BCMNV CP $R_{150}D$ and LMV CP $R_{167}D$, conserved arginine residue changed to aspartic acid; and BCMNV CP $D_{194}R$ and LMV CP $D_{211}R$, conserved aspartic acid residue changed to arginine (Fig. 1). Experiments were performed with the single amino acid substitution mutants BCMNV CP $R_{150}D$ and LMV CP $R_{167}D$, the double amino acid substitution mutants BCMNV, $S_{118}W$, $R_{150}D$, and LMV $S_{135}W$, $R_{167}D$, and the LMV CP triple amino acid substitution mutant, $S_{135}W$, $R_{167}D$, $D_{211}R$.

In microinjection experiments in *N. benthamiana* mesophyll cells, both the BCMNV and LMV CP Δ17N mutants moved cell to cell and facilitated movement of the 10-kDa F-dextran in a manner similar to that observed for wild-type CPs (Table 1). These results establish that the CP N-terminal 17 amino acids are not essential for eliciting an increase in plasmodesmal SEL, nor for mediating CP cell-to-cell movement. Single and double amino acid substitution mutants in the core region of the CP impaired, but did not completely prevent, cell-to-cell movement. After injection into *N. benthamiana* mesophyll cells, these mutants moved with the same frequency (75%) as wild-type CP (Table 1), but the extent of movement was reduced, with fluorescence being observed mainly within neighboring cells (up to eight cells), whereas wild-type FITC-CP moved extensively beyond the injection site (Fig. 3B). The FITC-labeled LMV CP triple amino acid substitution mutant did not move out of the injected cell (Table 1 and Fig. 3H).

The 10-kDa F-dextran was used in coinjection studies to explore the effect of the CP mutations on their ability to induce an increase in plasmodesmal SEL. In these experiments, the single and double amino acid substitution CP mutants facilitated 10-kDa F-dextran movement in approximately 75% of the injections (Table 1). As with experiments conducted with FITC-labeled CP mutants, the extent of the F-dextran movement was limited to within the vicinity of the injected cell (up to eight cells). Interestingly, when the 10-kDa F-dextran was coinjected with the LMV CP triple amino acid substitution mutant, fluorescence was detected in adjacent cells (Table 1 and Fig. 3I). This result suggests that this CP mutant, which was unable to move cell to cell, retained the ability to induce an increase in plasmodesmal SEL.

Experiments in which the CP single or double amino acid substitution mutants were coinjected with TOTO-1- or CF-UTP-labeled RNA revealed that the pattern of fluorescence movement was identical to that observed with 10-kDa F-dextran, indicating that these mutants facilitated cell-to-cell movement of viral RNA (Table 1). In similar experiments performed with the LMV CP triple amino acid substitution mutant, TOTO-1- or CF-UTP-labeled RNA was detected in adjacent cells (Table 1).

Cell-to-cell movement of the BCMNV HC-Pro

The FITC-labeled BCMNV HC-Pro moved rapidly into neighboring cells and, within 15–30 sec, had moved into 20–30 cells (Figs. 4A and 4B, and Table 3). Controls identical to those employed in CP experiments were used to indicate the functional nature of mesophyll plasmodesmata in leaves used for these experiments. HC-Pro moved in every injection (Table 3; cf. 75% for the

TABLE 2

BCMNV and LMV Capsid Proteins Move Cell to Cell in Lettuce

Injected agent[a]	Results[b]
Lucifer yellow	7 (7)
10 kDa F-dextran	0 (7)
FITC-LMV CP	6 (8)
FITC-BCMNV CP	5 (7)

[a] For a description of BCMNV and LMV proteins, see under Materials and Methods.

[b] Results are presented as number of injections in which movement to neighboring cells was observed (total number of injections performed).

CP, Table 1). Moreover, HC-Pro increased plasmodesmal SEL to a greater extent than the CP, in that it allowed movement of 20-kDa (Table 3 and Fig. 4C) and 39-kDa F-dextran (Table 3). Additionally, the BCMNV HC-Pro mediated cell-to-cell movement of BCMNV or LMV CP RNA (Table 3 and Fig. 4D). Finally, the BCMNV HC-Pro also moved cell-to-cell through lettuce mesophyll cells, indicating that its ability to interact with plasmodesmata is not host-specific (Table 3). Collectively, these findings establish that HC-Pro possesses the essential properties of a viral MP.

Construction and analysis of HC-Pro mutants

A series of mutations were engineered in the BCMNV HC-Pro gene with the goal of producing proteins having an altered capacity to interact with mesophyll plasmodesmata. Two deletion mutants were made: HC-Pro Δ87C, in which the C-terminal 87 amino acids were deleted, and HC-Pro Δ293, in which all but the N-terminal 164 amino acids were deleted. A double amino acid substitution mutant in the N-terminus of the BCMNV HC-Pro was generated using site-directed mutagenesis. The amino acids altered in this mutant were the lysine and cysteine residues that are highly conserved in the HC-Pro N-terminus and, when altered in the infectious clone of tobacco vein mottling potyvirus (TVMV), dramatically reduced viral pathogenicity (Atreya and Pirone, 1993). This double amino acid substitution mutant in the BCMNV HC-Pro, $K_{53}Q$ and $C_{56}S$, had the conserved lysine residue at position 53 changed to an aspartate residue, and the conserved cysteine residue at position 56 changed to a serine residue.

In microinjection experiments in *N. benthamiana* mesophyll cells, the FITC-labeled HC-Pro Δ87C mutant exhibited a decrease in the rate and frequency (Table 3) of cell-to-cell movement compared to that observed for wild-type HC-Pro. Movement of this FITC-HC-Pro mutant was only observed in 55% of the injections. Introduction of the FITC-labeled HC-Pro Δ293 mutant into mesophyll cells failed to result in the movement of fluorescence out of the injected cell (Table 3). In contrast, the double amino acid substitution mutant in the N-terminus, HC-Pro $K_{53}Q$ and $C_{56}S$, moved cell to cell with the same frequency (100%) as wild-type HC-Pro, and, as 10-kDa F-dextran moved in coinjection experiments, this mutant was still capable of inducing an increase in plasmodesmal SEL (Table 3). However, it is important to note that, although this mutant possessed viral MP function, its rate of movement was slower than that observed for wild-type HC-Pro. It required 40–50 sec for the double amino acid substitution mutant to move into adjacent cells, whereas movement of the wild-type HC-Pro was observed in less than 5 sec. Together, these results suggest that regions of the HC-Pro C-terminus and, possibly, the N-terminus, may be involved in mediating the interaction between this protein and plasmodesmata.

HC-Pro facilitates CP movement

The potential interaction between HC-Pro and the CP in potyvirus cell-to-cell movement was next examined. Coinjection of unlabeled BCMNV HC-Pro with BCMNV or LMV FITC-CP resulted in enhanced movement, in that, in the presence of HC-Pro, both BCMNV and LMV CPs moved in 100% of the injections (cf. Tables 1 and 3). Movement of FITC-CP was more rapid and extensive when coinjected with HC-Pro than that observed when FITC-CP was injected alone. Moreover, BCMNV HC-Pro facilitated the cell-to-cell movement of the LMV FITC-CP triple amino acid substitution mutant (Table 3 and Fig. 4E). These results suggest that the HC-Pro may interact with the CP, during cell-to-cell movement, in a non-virus-specific manner.

Microinjection studies performed on additional potyvirus proteins

To investigate whether other BCMNV proteins may possess viral MP properties, FITC-labeled P1, CI, NIa, and NIb were individually microinjected into *N. benthamiana* mesophyll cells or coinjected with 10-kDa F-dextrans. P1, CI, NIa, and NIb neither moved out of injected cells nor induced an increase in plasmodesmal SEL (Table 4). However, NIa and NIb were both found to accumulate within the nucleus of injected cells (Figs. 4G and 4I, respectively), whereas CI appeared to form fluorescent aggregates in the cytoplasm of injected cells (Fig. 4F).

DISCUSSION

In this study, the role of various potyvirus proteins was investigated in terms of their capacity to function as viral MPs. Using purified *E. coli*-expressed proteins and microinjection techniques, direct evidence was obtained that both the potyviral CP and HC-Pro function in this capacity. Both proteins (a) trafficked from cell to cell, (b) induced an increase in plasmodesmal SEL, and (c) facilitated cell-to-cell movement of viral RNA. Thus, these

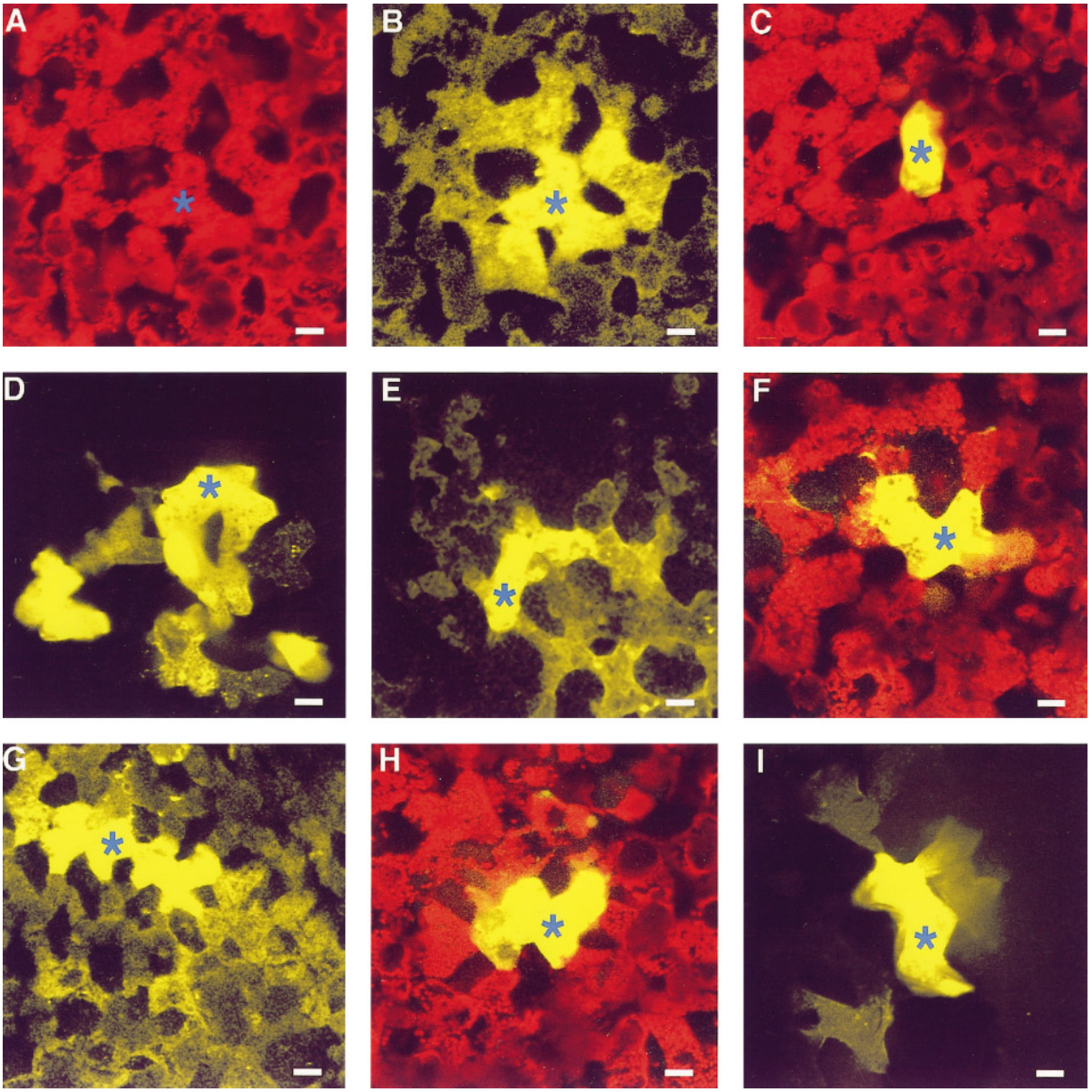

FIG. 3. Cell-to-cell trafficking of potyvirus CP and its effect on the plasmodesmal SEL in *N. benthamiana* mesophyll cells. BCMNV and LMV wild-type and mutant CPs were expressed in *E. coli* and purified proteins were FITC-labeled and renatured prior to being used in microinjection studies. All images for microinjection experiments were collected 5 min after the fluorescent agent was introduced by pressure injection (asterisks indicate location of injected cells) (scale bars represent 50 μm). (A) Cellular arrangement of mesophyll illustrated using chlorophyll autofluorescence. Image composed of eight optical-stacked sections (chlorophyll fluorescence channel). (B) FITC-BCMNV CP moved cell to cell in mesophyll cells. Image composed of eight optical-stacked sections. (C) 10-kDa F-dextran, injected alone, remained in the injected mesophyll cell. To illustrate the spatial relationship between this cell and the surrounding mesophyll, images were simultaneously collected in the FITC and chlorophyll channels and two optically stacked images were combined to generate the image presented. (D) Unlabeled BCMNV CP coinjected with 10-kDa F-dextran resulted in cell-to-cell movement of the F-dextran. Image composed of a single optical section collected in the FITC channel. (E) Unlabeled LMV CP coinjected with TOTO-1-labeled BCMNV CP RNA resulted in cell-to-cell movement of RNA. Image composed of eight optical-stacked sections collected in the FITC channel. (F) TOTO-1-labeled LMV CP RNA, when injected alone, remained in the injected mesophyll cell. As in C, image produced by combining four consecutive optical-stacked sections collected in the FITC and chlorophyll fluorescence channels. (G) Unlabeled LMV CP coinjected with BCMNV CP CF-UTP RNA (covalent label) resulted in cell-to-cell movement of RNA. Image composed of four optical-stacked sections collected in the FITC channel. (H) FITC-LMV CP triple amino acid substitution mutant, $S_{135}W$, $R_{167}D$, $D_{211}R$, remained in the injected mesophyll cell. As in C, image produced by combining four optical-stacked sections collected in the FITC and the chlorophyll channels. (I) Unlabeled LMV CP triple amino acid substitution mutant, $S_{135}W$, $R_{167}D$, $D_{211}R$, coinjected with 10-kDa F-dextran allowed limited movement of the F-dextran into neighboring cells. Image composed of eight optical-stacked sections collected in the FITC channel.

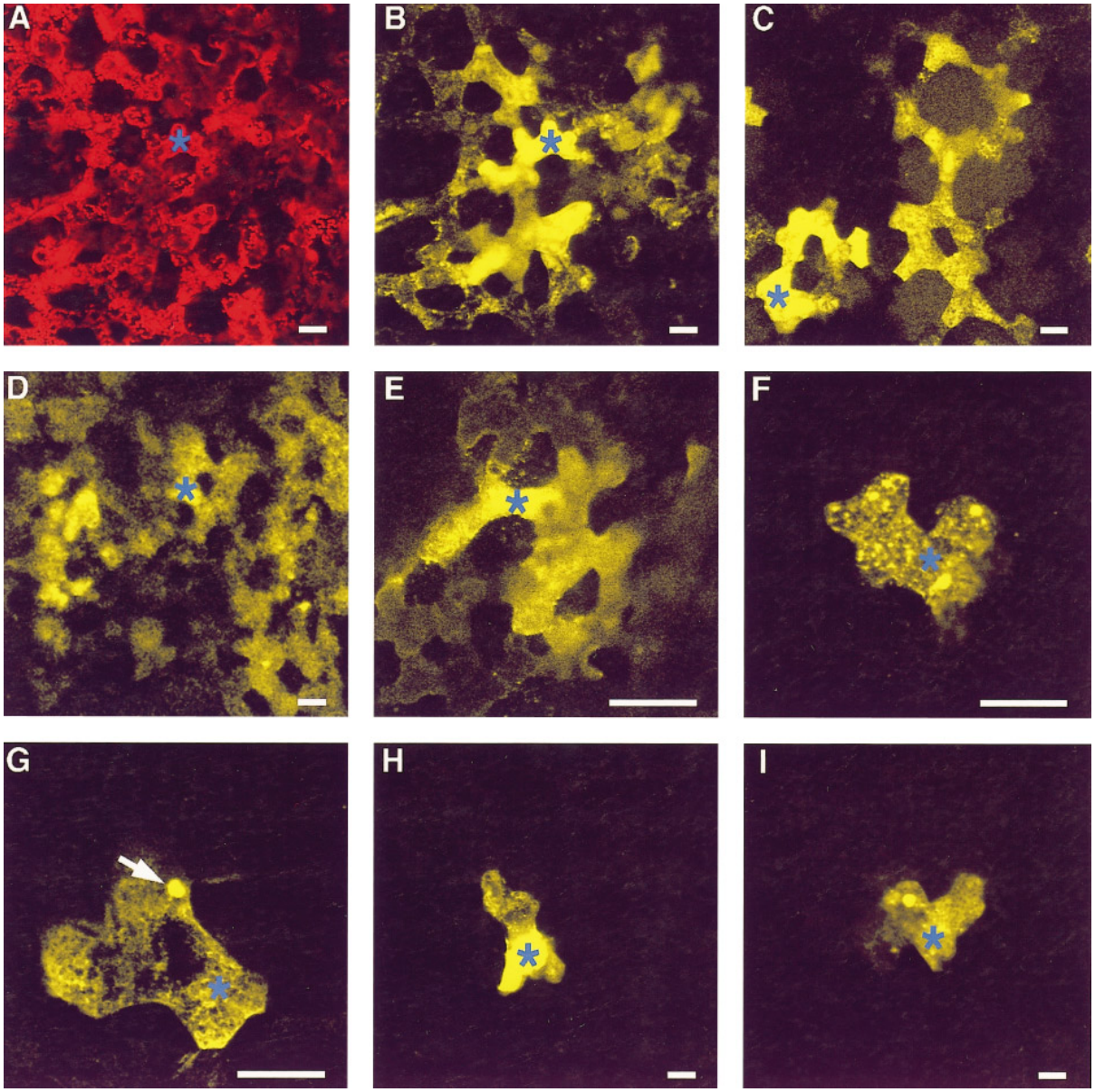

FIG. 4. Cell-to-cell trafficking of BCMNV HC-Pro and its effect on the plasmodesmal SEL in *N. benthamiana* mesophyll cells. BCMNV HC-Pro, CI, NIa, and NIb proteins were expressed in *E. coli* and purified proteins were FITC-labeled and renatured prior to being used in microinjection studies. (A–F) Images were collected 5 min after pressure injection; (G–I) images were collected 30 min after pressure injection (asterisks indicate location of injected cells) (scale bars represent 50 μm in A–D, H, and I, and 70 μm in E–G). (A) Cellular arrangement of mesophyll illustrated using chlorophyll autofluorescence. Image composed of two optical-stacked sections (chlorophyll fluorescence channel). (B) FITC-BCMNV HC-Pro moved cell to cell in mesophyll cells. Image was generated by combining two optical sections collected in the FITC channel. (C) Unlabeled BCMNV HC-Pro coinjected with 20 kDa F-dextran resulted in cell-to-cell movement. Image of the F-dextran is composed of four optical-stacked sections collected in the FITC channel. (D) Unlabeled BCMNV HC-Pro coinjected with TOTO-1-labeled LMV RNA resulted in cell-to-cell movement of RNA. Image composed of eight optical-stacked sections collected in the FITC channel. (E) Unlabeled BCMNV HC-Pro coinjected with FITC-LMV CP triple amino acid substitution mutant, $S_{135}W$, $R_{167}D$, $D_{211}R$, resulted in cell-to-cell movement of the LMV CP mutant. Image composed of eight optical-stacked sections (cf. with Fig. 3H). (F) FITC-BCMNV CI protein remained in the injected cell and fluorescence appeared to aggregate within the cytoplasm of the injected cell. Image composed of four optical-stacked sections collected in the FITC channel. (G) FITC-BCMNV NIa protein remained in the injected cell and fluorescence appeared to accumulate in the nucleus of the injected cell (arrow points to the nucleus as identified using DAPI stain). Image composed of four optical-stacked sections collected in the FITC channel. (H) 10-kDa F-dextran coinjected with unlabeled BCMNV NIa protein remained in the injected cell. Image composed of four optical-stacked sections. (I) FITC-BCMNV NIb protein remained in the injected cell and fluorescence appeared to accumulate in the nucleus of the injected cell. Image composed of four optical-stacked sections.

TABLE 3

BCMNV Helper Component-Proteinase Exhibits Cell-to-Cell Movement Properties in *N. benthamiana* and Lettuce

Injected agent[a]	Coinjected agent	Results[b]
Lucifer yellow	—	46 (46)
10 kDa F-dextran	—	0 (55)
FITC-*E. coli* proteins	—	0 (12)
FITC-BCMNV HC-Pro	—	50 (50)
FITC-BCMNV HC-Pro[c]	—	6 (6)
BCMNV HC-Pro	10 kDa F-dextran	38 (38)
BCMNV HC-Pro	20 kDa F-dextran	17 (19)
BCMNV HC-Pro	39 kDa F-dextran	9 (9)
BCMNV HC-Pro	BCMNV CP RNA-TOTO	15 (15)
BCMNV HC-Pro	LMV CP RNA-TOTO	12 (12)
BCMNV HC-Pro	FITC-BCMNV CP	22 (22)
BCMNV HC-Pro	FITC-LMV CP	14 (15)
BCMNV HC-Pro	FITC-LMV CP $S_{135}W,R_{167}D,D_{211}R$	24 (24)
BCMNV HC-Pro $K_{53}Q$, $C_{56}S$	—	10 (10)
BCMNV HC-Pro $K_{53}Q$, $C_{56}S$	10 kDa F-dextran	10 (10)
BCMNV HC-Pro Δ87C	—	7 (13)
BCMNV HC-Pro Δ293	—	0 (6)

[a] For a description of BCMNV and LMV proteins and mutants, see legend to Fig. 1 and under Materials and Methods.
[b] Results are presented as number of injections in which movement to neighboring cells was observed (total number of injections performed).
[c] Results obtained from experiments performed on lettuce.

results suggest that the CP and HC-Pro are involved in the cell-to-cell movement of the potyviruses. The P1 protein has been shown to have a limited degree of amino acid sequence similarity with the tobacco mosaic tobamovirus 30-kDa MP (Domier *et al.,* 1987) and to possess RNA binding properties (Brantley and Hunt, 1993; Soumounou and Laliberte, 1994), but it did not possess the properties of a *bone fide* viral MP based on our microinjection experiments. The CI protein, which forms the characteristic potyvirus pinwheel inclusion bodies in infected cells, is found in close association with plasmodesmata (Lawson and Hearon, 1971; Lawson *et al.,* 1971; Andrews and Shalla, 1974) and has been suggested to play a role in potyvirus cell-to-cell movement (Langenberg, 1986). Our microinjection experiments performed with CI established that this 70-kDa protein is not a viral MP, but the aggregation of fluorescently labeled CI in the cytoplasm of injected cells is consistent with the formation of pinwheel inclusion bodies in potyvirus-infected cells (Lawson *et al.,* 1971; Restrepo-Hartwig *et al.,* 1990). It is possible that the close proximity of pinwheel inclusion bodies to plasmodesmata may reflect a role of CI as part of a membrane-associated replication complex (Restrepo *et al.,* 1990) that allows for the targeting of viral nucleoprotein complexes to plasmodesmata.

TABLE 4

The BCMNV P1, CI, NIa, and NIb Proteins Do Not Move Cell to Cell in *N. benthamiana*

Injected agent[a]	Coinjected agent	Results[b]
Lucifer yellow	—	14 (14)
10 kDa FITC-dextran	—	0 (12)
FITC-BCMNV P1	—	0 (21)
BCMNV P1	10 kDa F-dextran	0 (20)
FITC-BCMNV CI	—	1 (12)
BCMNV CI	10 kDa F-dextran	0 (10)
FITC-BCMNV NIa	—	0 (15)[c]
BCMNV NIa	10 kDa F-dextran	0 (19)
FITC-BCMNV NIb	—	0 (23)[c]
BCMNV NIb	10 kDa F-dextran	0 (17)

[a] For a description of BCMNV and LMV proteins see under Materials and Methods.
[b] Results are presented as number of injections in which movement to neighboring cells was observed (total number of injections performed).
[c] Fluorescence accumulated in the nuclei of the injected cells.

Our microinjection experiments also established that NIa and NIb are not viral MPs. However, fluorescently labeled NIa and NIb proteins accumulated in the nucleus of injected cells, which is consistent with the targeting of these proteins to the nucleus. These results are in agreement with previous immunolocalization and transgenic plant studies showing that the TEV NIa and NIb proteins have the capacity to enter and accumulate in the nucleus (Restrepo-Hartwig *et al.,* 1990; Carrington *et al.,* 1991). The fact that nuclear targeting of the potyviral NIa and NIb proteins was also observed in microinjection studies, provides further support for the validity of this technique as a method to probe the *in vivo* function of viral proteins.

The CP has been implicated in potyviral cell-to-cell movement in genetic studies conducted with TEV-GUS. A distinct role for the CP in cell-to-cell movement, separable from virion assembly, was demonstrated based on

the inability of CP core mutants to move out of inoculated cells or to form virions when their movement was complemented in TEV CP transgenic plants (Dolja *et al.,* 1994, 1995). As previously pointed out by Dolja *et al.* (1994, 1995), the potyvirus CP must interact with additional viral-encoded proteins and/or host factors to facilitate viral movement. Our results suggest that the CP interacts with plasmodesmal proteins to facilitate viral movement. In addition, cell-to-cell movement mediated by the potyvirus CP does not appear to be responsible for host specificity in that the BCMNV CP moved cell to cell and increased plasmodesmal SEL in a nonhost (lettuce) and facilitated the movement of LMV CP RNA in *N. benthamiana.* The latter finding is consistent with those of Dolja *et al.* (1994), in which movement defective TEV-GUS CP mutants were rescued in transgenic plants expressing the CP of the heterologous potyvirus, potato virus Y (PVY).

Although it is difficult to directly compare results from microinjection experiments with those derived from genetic studies, such as those in which plants are infected with a "tagged" virus such as TEV-GUS, it is possible to compare results obtained in each system with similar mutants. Overall, results from both systems support the conclusion that the CP plays a role in potyvirus cell-to-cell movement, that the N-terminus of the CP is not needed for cell-to-cell movement, and that the core region of the CP may play a role in cell-to-cell movement. However, similar mutants did not behave identically in the two experimental systems. For example, the ΔN mutant in the TEV-GUS system showed impaired cell-to-cell movement (Dolja *et al.,* 1994). In microinjection studies, ΔN mutants displayed viral MP properties that were indistinguishable from those of the wild-type CP. These results indicate that the deleted N-terminal region is not essential for the potyvirus CP to interact with plasmodesmata. However, this region of the CP could be involved in interaction(s) with additional potyviral proteins and/or host factors that are necessary for efficient cell-to-cell movement of the viral genome. Inefficient cell-to-cell movement may allow for activation of a host defense response that could limit cell-to-cell movement and block systemic infection (Mise and Ahlquist, 1995). Such complex interactions would not be detected in microinjection experiments, in which the function of individual proteins is examined.

TEV-GUS mutants with single ($R_{154}D$), or double amino acid substitutions ($R_{154}D$ and $D_{192}R$) in the core region of the CP were confined to the inoculated cells of tobacco plants (Dolja *et al.,* 1994, 1995). Similar mutations caused an impairment in CP cell-to-cell movement in microinjection experiments, and only the LMV CP triple amino acid substitution mutant was confined to injected cells. Again, the differences in the behavior of these CP mutants in the two experimental systems may be due to the requirement for the interaction of the CP with other viral-encoded factors, or the viral genome, for TEV-GUS cell-to-cell movement.

The role of the viral CP in long-distance movement of many viruses is well documented (Gilbertson and Lucas, 1996). More recently, a role for the CP in the cell-to-cell movement of a number of plant virus groups, in addition to the potyviruses, is emerging; e.g., brome mosaic bromovirus (Rao and Grantham, 1996) and potato virus X potexvirus (Oparka *et al.,* 1996). Interestingly, in the case of PVX, the CP is targeted to plasmodesmata but does not appear to mediate an increase in the plasmodesmal SEL. Clearly, the manner in which the viral CP interacts with viral and/or host factors to mediate cell-to-cell movement is likely to differ among different virus groups. In this regard, microinjection studies provide a powerful approach for investigating the role of viral CPs in cell-to-cell movement.

Our results also provide direct evidence that the BCMNV HC-Pro (56 kDa) possesses characteristics of a viral cell-to-cell MP and suggest that potyviruses encode two MPs (CP and HC-Pro). In fact, our results indicate that the HC-Pro is a remarkably efficient viral MP, as compared with the CP. HC-Pro moved cell to cell more frequently, much faster, and to a greater extent than the CP, and HC-Pro increased the plasmodesmal SEL to a greater value (39-kDa F-dextran) than the CP (10-kDa F-dextran). HC-Pro also facilitated the cell-to-cell movement of viral RNA, which is consistent with a recent report establishing that the PVY HC-Pro possesses nucleic acid binding properties (Maia and Bernardi, 1996). Furthermore, the BCMNV HC-Pro also facilitated the cell-to-cell movement of the movement-defective LMV CP triple amino acid substitution mutant. This finding suggests that efficient potyvirus cell-to-cell movement may involve a direct interaction between HC-Pro and the CP. Alternatively, this interaction could be indirect; i.e., simply mediated by an increase in plasmodesmal SEL by HC-Pro. This latter interpretation finds support in the observation that HC-Pro had the capacity to potentiate movement of 39-kDa F-dextran (the LMV CP fusion protein is 35 kDa). In addition, HC-Pro was also shown to mediate the cell-to-cell movement of a 37-kDa movement-defective geminivirus MP (BC1 Asp78; Noueiry *et al.,* 1994; data not shown). Again, as with the CP, the HC-Pro MP properties do not appear to be virus-specific, in that the BCMNV HC-Pro rescued movement of the LMV CP triple amino acid substitution mutant and facilitated the cell-to-cell movement of LMV CP RNA. This finding is consistent with the infectivity of a TVMV chimera, which contained the heterologous zucchini yellow mosaic potyvirus HC-Pro gene (Atreya and Pirone, 1993).

Recent genetic analyses of HC-Pro function, using TEV-GUS, suggested that HC-Pro plays a role in long-distance transport based upon the identification of mutants that replicated and moved cell to cell in inoculated leaves, but were defective in long-distance transport

(Cronin *et al.,* 1995; Kasschau *et al.,* 1997). However, a number of alanine scanning mutants in the TEV-GUS HC-Pro central domain appeared to move, cell to cell, less efficiently than TEV-GUS, suggesting that HC-Pro may enhance cell-to-cell movement (Kasschau *et al.,* 1997). These results are not inconsistent with our finding that HC-Pro possesses viral MP properties, but raise questions about the precise role of HC-Pro in potyviral cell-to-cell movement. It is possible that the mechanism by which HC-Pro functions in long-distance transport is through an interaction with the specialized SE/CC plasmodesmata.

Elucidation of the precise role of HC-Pro in potyviral cell-to-cell movement has been complicated by the role of HC-Pro in replication (Kasschau *et al.,* 1997). However, genetic studies with an infectious TVMV clone have suggested a role for HC-Pro in cell-to-cell movement. For example, an HC-Pro mutant having a 4-amino acid insertion in the N-terminus was able to replicate in protoplasts but failed to spread in inoculated leaves (Klein *et al.,* 1994). Moreover, other single amino acid substitution or deletion mutants in the 5′ end of the TVMV HC-Pro gene were not infectious (Atreya and Pirone, 1993). In the present study, BCMNV HC-Pro mutants, either having two amino acid substitutions identical to those in noninfectious TVMV mutants or a C-terminal deletion (Δ87C), showed impaired cell-to-cell movement, supporting a role for HC-Pro in cell-to-cell movement. Additional studies, possibly using the noninvasive reporter gene, the green fluorescent protein, are needed to elucidate the precise role of HC-Pro in potyviral cell-to-cell movement.

What is the form and mechanism by which potyviruses move cell to cell? Our results establish that both the CP and HC-Pro may be involved in potyvirus cell-to-cell movement. Furthermore, these proteins probably interact, directly or indirectly, with viral RNA to form a nucleoprotein complex that moves through plasmodesmata. The assembly of this complex and its subsequent delivery to plasmodesmata may be facilitated by CI (Lawson and Hearon, 1971; Lawson *et al.,* 1971; Andrews and Shalla, 1974; Langenberg, 1986). The association of free CP with cell walls (Baunoch *et al.,* 1988) and with cylindrical inclusion bodies (Langenberg, 1986) is consistent with the involvement of the CP in cell-to-cell movement. It will be of interest to determine if HC-Pro shows a similar pattern of localization. It also remains to be determined whether the CP and HC-Pro act cooperatively to mediate cell-to-cell movement of the infectious potyvirus form(s). An alternate possibility is that the CP and HC-Pro function, independently, to mediate in the trafficking of the nucleoprotein complex across specific cellular boundaries within the plant.

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