Molecular Studies on Bromovirus Capsid Protein

III. Analysis of Cell-to-Cell Movement Competence of Coat Protein Defective Variants of Cowpea Chlorotic Mottle Virus

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To determine whether the role of coat protein (CP) in cell-to-cell movement of dicot-adapted cowpea chlorotic mottle bromovirus (CCMV) is distinct from that of monocot-adapted brome mosaic bromovirus (BMV), two reporter genes, β-glucuronidase (GUS) and enhanced green fluorescent protein (EGFP), were substituted for the CP in a biologically active clone of CCMV RNA3 (C3). Primary leaves of *Nicotiana benthamiana, Chenopodium quinoa,* and cowpea were co-inoculated with wild-type (wt) CCMV RNA 1 and -2 and either C3/$\triangle$CP-GUS or C3/$\triangle$CP-EGFP and analyzed for GUS activity or the presence of green fluorescence. The visual appearance of infections caused by GUS or EGFP variants indicated that, in CCMV, epidermal cell-to-cell movement can occur without a functional CP. By contrast, inoculation of MP defective variants of C3/$\triangle$CP-GUS or C3/$\triangle$CP-EGFP resulted in subliminal infections. Additional experiments examining the infectivity of wt BMV RNA 1 and -2 and a BMV RNA3 variant bearing the EGFP in the place of CP (B3/$\triangle$CP-EGFP) confirmed previous observations that, unlike CCMV, epidermal cell-to-cell movement of BMV is dependent on the expression of a functional CP. Taken together, the results demonstrate that BMV and CCMV use different mechanisms for initial epidermal cell-to-cell spread, and the individual role played by the respective CP genes in this active process is discussed.   © 1997 Academic Press

INTRODUCTION

In a susceptible host plant, when an invading virus is transported sequentially from initially infected epidermal cells through mesophyll, bundle sheath, and phloem parenchyma, it is said to have moved cell to cell (Carrington *et al.,* 1996). In the absence of such cell-to-cell movement, the infection remains subliminal (Schmitz and Rao, 1996). Genetic analyses of molecularly cloned genomes of several positive-strand RNA viruses of plants have revealed that the active process of cell-to-cell movement is regulated by a cooperative interaction between the host machinery (such as plasmodesmata and various cell and tissue types) and one or more virus-encoded gene products (Carrington *et al.,* 1996; Heinlein *et al.,* 1995; Lucas and Gilbertson, 1994; McLean *et al.,* 1995). For example, in tobacco mosaic tobamovirus (TMV), cell-to-cell movement is mediated by an interaction between the virus-encoded movement protein (MP; Deom *et al.,* 1992) and plasmodesmata (Wolf *et al.,* 1988) while coat protein (CP) is not required for this process (Dawson *et al.,* 1988). In some viruses cell-to-cell movement requires CP, in addition to MP (as in como- and nepoviruses; Ritzenthaler *et al.,* 1995; Van Lent *et al.,* 1991) or MP-like virus-encoded gene products (as in poty- and potexvi-

ruses; Baulcombe *et al.,* 1995; Carrington *et al.,* 1996; Dolja *et al.,* 1992; Oparka *et al.,* 1995). In contrast to viruses from the previously mentioned groups, deletion of CP has no significant effect on either cell-to-cell or long-distance spread of tomato bushy stunt tombusvirus (TBSV) (Scholthof *et al.,* 1993) or barley stripe mosaic hordeivirus (BSMV) (Petty and Jackson, 1990).

Two members of the family *Bromoviridae,* brome mosaic (BMV) and cowpea chlorotic mottle viruses (CCMV), are icosahedral, multipartite, positive-strand RNA viruses that infect plants (Ahlquist, 1994). Essential functions required for replication and subsequent establishment of infection by BMV and CCMV are partitioned among three RNA components (Ahlquist, 1994). Viral RNA replication is dependent on efficient interaction between two nonstructural proteins, 1a and 2a, encoded by the monocistronic RNA 1 and -2, respectively (Ahlquist, 1994; Allison *et al.,* 1988). Genomic RNA3 encodes a nonstructural protein 3a of 32 kDa, designated MP (Mise *et al.,* 1993), and a 20-kDa CP that is translated from subgenomic RNA4 derived from minus-strand RNA3 (Ahlquist, 1994; Allison *et al.,* 1988). These two gene products are dispensable for viral replication, but are required for infection in plants (Allison *et al.,* 1990; DeJong and Ahlquist, 1991). Monocot-adapted BMV and dicot-adapted CCMV have been used as model systems for elucidating the role of viral gene products in replication (Ahlquist, 1994) and virus spread (Mise and Ahlquist, 1995; Mise *et al.,*

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1993; Rao and Grantham, 1996; Sacher and Ahlquist, 1989; Schmitz and Rao, 1996). Molecular and genetic analysis of the bromovirus MP revealed that this gene is critical for cell-to-cell movement (Mise and Ahlquist, 1995; Mise *et al.*, 1993), dictates host specificity (De Jong and Ahlquist, 1991; Mise and Ahlquist, 1995), and modulates symptom expression in susceptible hosts (Fujita *et al.*, 1996; Rao and Grantham, 1995a). However, recent fluorescence *in situ* localization studies indicated that, in BMV, both MP and CP are essential for cell-to-cell movement (Schmitz and Rao, 1996). Since CCMV is distinct from BMV in host range, it was surmised that the role played by CCMV CP in the cell-to-cell movement process could be different from that of BMV. Therefore, this study reports on cell-to-cell movement characteristics of two CCMV RNA3 variants, each harboring either the β-glucuronidase gene (GUS) or the enhanced green fluorescent protein (EGFP) in the place of its CP.

MATERIALS AND METHODS

Plasmid constructions

Construction of CCMV RNA3 variants. Full-length cDNA clones corresponding to the three genomic RNAs of CCMV, pCC1TP1, pCC2TP2, and pCC3TP4, from which wild-type (wt) infectious RNAs 1 (C1), 2 (C2), and 3 (C3), respectively, can be transcribed *in vitro,* have been described previously (Allison *et al.,* 1988). A variant clone of wt C3 harboring the GUS gene in the place of CP (pCC3/ΔCP-GUS; Fig. 1), was constructed by amplifying the entire GUS sequence from FCP2GUS (Mori *et al.,* 1993; a derivative of BMV RNA3 cDNA provided by T. Okuno) in a PCR with a 5′ oligonucleotide primer (5′ AATGTCGACTTC<u>GTTAAC</u>TGGTAAGAT-GTTA 3′; the underlined bases contain a *Hpa*I restriction site) and a 3′ oligonucleotide primer (5′ GAAGTCATC-GAAA<u>ACTAGT</u>CCTTACGTGCTC 3′; the underlined bases contain a *Spe*I restriction site). The resulting PCR product was digested with *Hpa*I and *Spe*I and ligated into similarly treated pCC3TP4, to yield pCC3/ΔCP-GUS (Fig. 1). A MP defective variant of pCC3/ΔCP-GUS, referred to as pCC3ΔMP/ΔCP-GUS, was constructed by digesting pCC3/ΔCP-GUS with *Bgl*II (present at positions 430 and 1150; Fig. 1) and then treated with mungbean nuclease (Sambrook *et al.,* 1992) prior to re-ligation. To replace the CCMV CP with EGFP (Cormack *et al.,*1996), a PCR product encoding the gene sequence was amplified from plasmid pEGFP (Clontech Laboratories, Palo Alto, CA) using a 5′ oligonucleotide primer (5′ GATCCACCGGT<u>GTTAAC</u>CAT-GGTGAGC 3′: the underlined bases contain a *Hpa*I restriction site) and a 3′ oligonucleotide primer (5′ TGGCTGATT-ATGA<u>ACTAGT</u>GTCGCGGCCGCT 3′; the underlined bases contain a *Spe*I restriction site), digested with *Hpa*I and *Spe*I, and ligated into similarly treated pCC3TP4. The resulting plasmid was designated pCC3/ΔCP-EGFP (Fig. 1). A MP defective variant of pCC3/ΔCP-EGFP, referred to as

pCC3ΔMP/ΔCP-EGFP (Fig. 1), was constructed essentially as described for pCC3ΔMP/ΔCP-GUS.

Construction of the BMV CP variant harboring EGFP. Full-length cDNA clones corresponding to the three genomic RNAs of BMV, pT7B1, pT7B2, and pT7B3, from which wt infectious RNAs 1 (B1), 2 (B2), and 3 (B3), respectively, can be transcribed *in vitro,* have been described previously (Dreher *et al.,*1989). To replace the CP gene of BMV with that of EGFP, a 740-basepair fragment encoding the sequence for the first 10 amino acids of the CCMV CP together with the entire sequence of the EGFP was amplified in a PCR from pCC3/ΔCP-EGFP (Fig. 1) using a 5′ oligonucleotide primer (5′ TTTATC-AT<u>GTCGAC</u>AGTCGGA 3′; the underlined bases contain a *Sal*I restriction site) and a 3′ oligonucleotide primer (5′ TAGTGTCGCG<u>CCCGGG</u>TTACTTGTACAG 3′; the underlined bases contain a *Sma*I restriction site). The resulting product was digested with *Sal*I and *Sma*I and subcloned into *Sal*I- and *Stu*I-treated pT7B3 to yield pT7B3/ΔCP-EGFP (Fig. 1).

In vitro transcriptions and biological assays

Full-length and variant cDNA clones of CCMV and BMV were linearized with *Xba*I and *Bam*HI, respectively, and transcribed *in vitro* with T7 RNA polymerase in the presence of 7-methylguanosine (5′)triphospho(5′)guanosine cap (Rao *et al.,* 1994; Rao and Grantham, 1996). Isolation and transfection of protoplasts from barley and *Nicotiana benthamiana,* extraction of total progeny RNA, and analysis of progeny RNA by Northern hybridization using riboprobes of desired specificity were performed as described previously (Rao *et al.,* 1994; Rao and Grantham, 1995a,b). For whole plant inoculations, *N. benthamiana, Chenopodium quinoa,* and cowpea (cv. Black Eye) plants were kept in the dark for at least 18 hr and mechanically inoculated as previously described (Rao *et al.,* 1994). Each experiment was repeated at least three to five times with independently synthesized *in vitro* transcripts. The inoculated plants were kept in the greenhouse at 25° for observation.

GUS assays

In situ GUS assays were done using a colorimetric substrate according to Lapidot *et al.* (1993). *N. benthamiana, C. quinoa,* and cowpea leaves were vacuum infiltrated with a solution containing 50 m*M* sodium phosphate, pH 7.0, 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, 1 m*M* EDTA, pH 8.0, a 1.2 m*M* concentration of the substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, cyclohexylammonium salt (X-gluc, Gold Biotechnology, St. Louis, MO) and 0.5% Triton X-100. Chlorophyll was removed from leaves by soaking in a solution containing 42% ethanol, 10% formaldehyde, and 5% glacial acetic acid and then soaking briefly in 70% ethanol (Lapidot *et al.,* 1993). Infection sites were
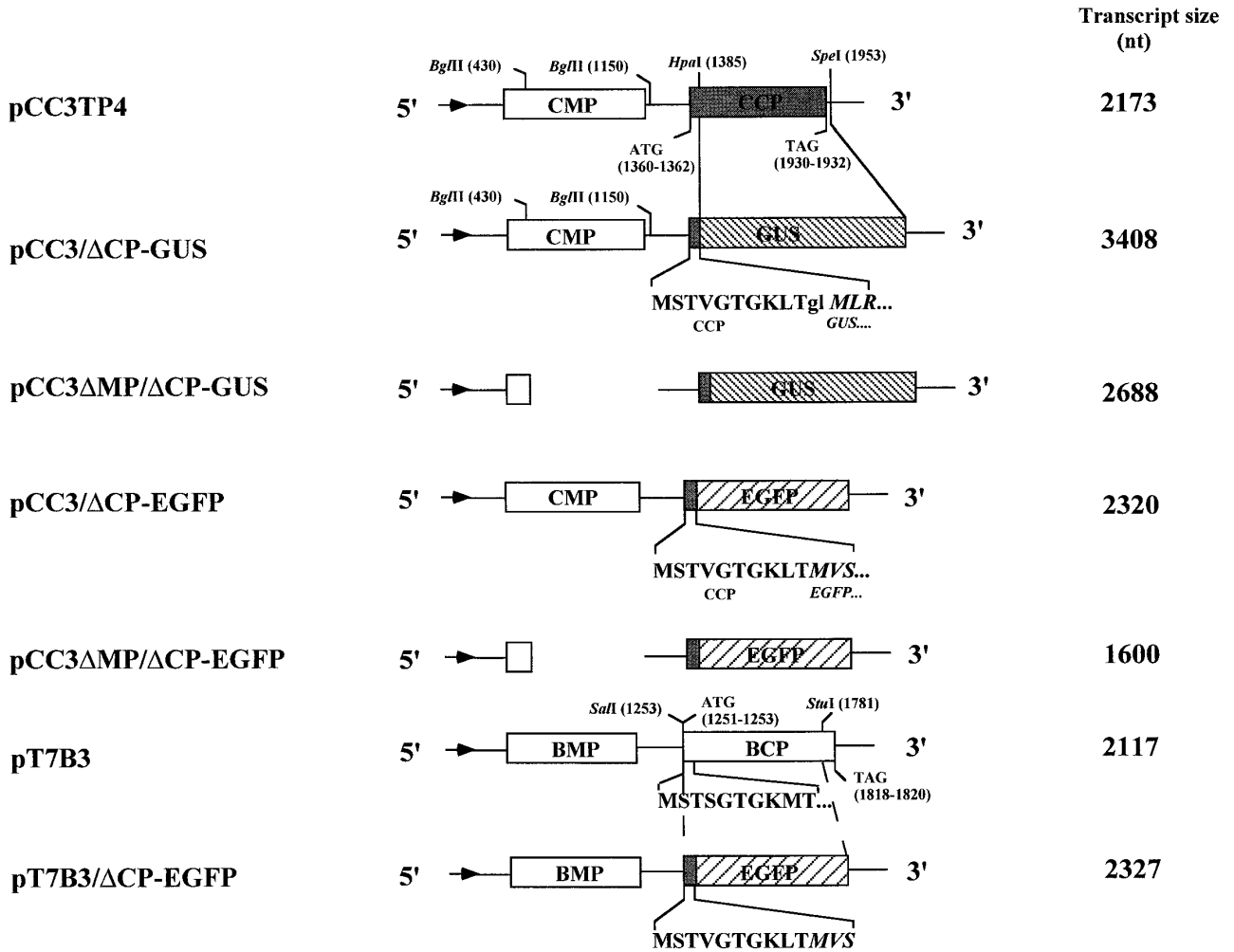
FIG. 1. Schematic representation of alterations introduced into the biologically active clones of wt CCMV RNA3 (pCC3TP4) and BMV RNA3 (pT7B3). The organization of pCC3TP4 is shown, with noncoding sequences represented as single lines and movement protein (CMP) and coat protein (CCP) genes as open and shaded boxes, respectively. The positions of selected restriction sites in pCC3TP4 used to either introduce desired deletions or incorporate foreign genes are shown. The locations of the start (ATG) and stop (TAG) codons of CCP are indicated. In pCC3/ΔCP-GUS, the GUS gene was fused to sequence coding for the first N-terminal 10 amino acids (shown in uppercase letters) of CCP. In this construct, the creation of a compatible HpaI restriction site at the beginning of the GUS gene resulted in the introduction of two additional amino acids (shown in lowercase letters). The first three amino acids of the GUS gene located at the fusion junction are shown in italicized uppercase letters. For pCC3ΔMP/ΔCP-GUS, the space indicates the extent of the sequence deleted as a BglII fragment (present at positions 430 and 1150) in the MP gene. pCC3/ΔCP-EGFP was constructed similarly to pCC3/ΔCP-GUS by incorporation of the entire sequence coding for the EGFP as HpaI and SpeI fragments. In this clone, no additional amino acids are present at the fusion junction. The first 10 amino acids of CCMV CP and the first 3 amino acids of EGFP present at the fusion junction are shown in uppercase and italicized uppercase letters, respectively. Construction of pCC3ΔMP/ΔCP-EGFP was similar to that of pCC3ΔMP/ΔCP-GUS. In pT7B3, the positions of selected restriction sites used to introduce foreign genes are shown. The first 10 N-proximal amino acids of BMV CP (BCP) that share extensive homology to those of CCP are shown in uppercase letters. pT7B3/ΔCP-EGFP was constructed by subcloning a cassette encompassing the first 10 N-terminal amino acids of CCP and the entire EGFP gene from pCC3/ΔCP-EGFP as a SalI and StuI fragment. Thus the composition of EGFP expressed from the subgenomic RNA of B3/ΔCP-EGFP is identical to that of C3/ΔCP-EGFP. The arrow at the 5' end denotes the position of the T7 RNA polymerase promoter. Expected transcript sizes from wt and each RNA3 variant are shown at right.

identified by the presence of an indigo precipitate. They were observed and photographed under bright-field optics using a Nikon photomicroscope.

Detection of EGFP in whole leaves

Leaves of *N. benthamiana, C. quinoa,* and cowpea inoculated with EGFP constructs were illuminated with a hand-held long-wavelength UV light (366 nm) to detect

fluorescent zones of infection. Whole leaves were also viewed under a Nikon Labophot microscope equipped with a super-high-pressure mercury lamp as a source for blue light (Nikon HB 10101AF), an HFX-IIA epifluorescence attachment, and an FITC filter set XF23 (Omega Optical) containing a 488DF22 excitation filter, a 505DRLP02 dichroic filter, and a 535DF35 barrier filter. Photographs were taken with Kodak Ektachrome 400 ASA slide film. Images were scanned from slides with a

Nikon scanner (LS 1000) and graphically arranged using Adobe Photoshop 4.0 software (Adobe Systems Inc., Mountainview, CA).

RESULTS

Characteristics of C3/ΔCP-GUS and C3ΔMP/ΔCP-GUS variants

Schmitz and Rao (1996) found that, in addition to MP, a functional CP is also required to mediate the cell-to-cell movement of BMV. To investigate whether cell-to-cell spread of dicot-adapted CCMV was likewise dependent on the expression of functional CP, a series of C3 variants was constructed (Fig. 1). All C3 plasmids have the prefix pC and RNA transcripts produced by *in vitro* transcription are referred to by the plasmid name without the prefix. For example, RNA transcripts derived from plasmid pCC3/ΔCP-GUS are designated C3/ΔCP-GUS. In bromoviruses the first 24 nucleotides (nt) of the CP gene that code for the N-terminal 8 amino acids are required for the efficient translation of inserted foreign genes from the CP subgenomic RNA (Mori *et al.,* 1993). Therefore, the entire GUS gene sequence was fused to the sequence coding for the first N-proximal 10 amino acids of the CCMV CP using a unique *Hpa*I site located after 25 nt with respect to the start of CCMV CP open reading frame (Fig. 1). Thus, C3/ΔCP-GUS RNA transcripts are 1230 nt longer than those of wt C3 (Fig. 1). Plasmid pCC3ΔMP/ΔCP-GUS is characterized by a deletion of 720 bases from the MP gene sequence (Fig. 1). C3ΔMP/ΔCP-GUS RNA transcripts are 515 nt longer than those of wt C3 and 720 nt shorter than those of its parent C3/ΔCP-GUS (Fig. 1). The two C3 variants, C3/ΔCP-GUS and C3ΔMP/ΔCP-GUS, were independently co-inoculated with C1 + C2 to *N. benthamiana* protoplasts. After 24 hr of incubation, addition of the GUS substrate to these samples resulted in an indigo precipitate within 1 to 2 hr (data not shown), suggesting that both C3 variants replicated in protoplasts and produced subgenomic RNA capable of efficient GUS expression.

C3/ΔCP-GUS is competent for cell-to-cell movement

To assess the competence of C3/ΔCP-GUS in cell-to-cell movement, three hosts susceptible to CCMV infection, *N. benthamiana, C. quinoa,* and cowpea were used. In *N. benthamiana,* CCMV causes symptomless systemic infections (Mise *et al.,* 1993; Table 1). In *C. quinoa,* wt CCMV induces characteristic necrotic local lesions at 5 days postinoculation (dpi) and the upper noninoculated leaves remain uninfected (Table 1). Cowpea is a natural systemic host for CCMV, exhibiting characteristic chlorotic mottling symptoms (Mise *et al.,* 1993; Table 1). None of the plants inoculated with C1 + C2 + C3/ΔCP-GUS developed symptoms on either inoculated or noninoculated upper leaves (Table 1). Since in C3/ΔCP-GUS the CP gene was replaced

with GUS, histochemical analysis of the inoculated leaves for GUS activity would permit visualization of cell-to-cell movement (Dolja *et al.,* 1992; Schmitz and Rao, 1996). Primary leaves of three hosts were inoculated with C1 + C2 + C3/ΔCP-GUS and excised at desired time points, vacuum infiltrated with GUS substrate, and periodically examined for an indigo precipitate under the microscope.

Time course analysis of GUS expression in primary leaves of *N. benthamiana* inoculated with C1 + C2 + C3/ΔCP-GUS revealed that indigo precipitate could be detected in single epidermal cells as early as 6 hr postinoculation (hpi) with a microscope (data not shown). Leaves processed 1 dpi displayed infection foci as macroscopic blue spots (Fig. 2, IA). Microscopic examination of these samples revealed that nearly 50% of the infection foci contained 5–10 blue cells (Fig. 2, I D) while the remaining 50% contained 11–20 blue cells (Table 2). At subsequent time points, the size of the infection foci expanded radially and were readily visible to the naked eye as distinct blue spots (Fig. 2, I B and C). At 3 dpi, the majority of foci contained groups of 21–50 blue cells (Fig. 2, I E, Table 2). Macroscopic blue spots developed at 5 dpi were slightly larger than those observed at 3 dpi (Fig. 2, I C) with 40% of the foci containing more than 50 cells (Fig. 2, I F, Table 2). No increase in the number of cells expressing GUS was observed beyond 5 dpi since samples processed 7 dpi displayed infection foci of the same size as those that appeared at 5 dpi (Table 2). In these assays, because of the heavy indigo precipitation at 3, 5, and 7 dpi, it was difficult to resolve by examining the whole leaf whether infection had spread from epidermal cells to mesophyll or had spread only between epidermal cells (see below).

The size of the infection foci at any given time point in *C. quinoa* was smaller than that observed in *N. benthamiana.* At 1 dpi, each infection focus contained no more than 10 blue cells (Table 2). By 3 dpi, more than 50% of the infection foci contained 11–20 blue cells and by 5 dpi the size of the foci had slightly expanded, covering no more than 30 cells. This number did not change by 7 dpi (Table 2). To further validate the above observations (Fig. 2, I A–F, Table 2) that the cell-to-cell movement exhibited by C3/ΔCP-GUS is not of a nonspecific passive nature and is also not due to the diffusion of GUS but is in fact mediated by CCMV MP alone (Mise and Ahlquist, 1995), leaves of *N. benthamiana* and *C. quinoa* were inoculated with a mixture containing C1 + C2 + C3ΔMP/ΔCP-GUS and processed for observation of GUS activity at 1, 3, and 5 dpi. At 1 dpi, primary leaves of *N. benthamiana* and *C. quinoa* displayed macroscopic pinpoint blue spots (Fig. 2, II G). In contrast to the expanding infection foci resulting from inoculations containing C3/ΔCP-GUS (Fig. 2, I A–C), the size and appearance of these blue spots did not change over time (Fig. 2, II H and I). Microscopic examination at each time point revealed that GUS expression in each infection focus was restricted to a single epidermal cell (Fig. 2, II J–L, Table 2). These obser-

TABLE 1

Symptom Phenotypes and Expression of GUS and EGFP by CCMV and BMV RNA3 Variants

Inoculum[a]	N. benthamiana			C. quinoa			Cowpea		
	Symptoms[b] (I/S)	GUS[c]	EGFP[d]	Symptoms[b] (I/S)	GUS[c]	EGFP[d]	Symptoms[b] (S)	GUS[c]	EGFP[d]
CCMV variants									
wt C3	SL/SL	NT	NT	NL/NI	NT	NT	ChM	NT	NT
C3/ΔCP-GUS	NS/NS	+++	NT	NS/NS	+++	NT	NS	+	NT
C3ΔMP/ΔCP-GUS	NS/NS	+++	NT	NS/NS	+++	NT	NS	+	NT
C3/ΔCP-EGFP	NS/NS	NT	GF	NS/NS	NT	GF	NS	NT	GF
C3ΔMP/ΔCP-EGFP	NS/NS	NT	GF	NS/NS	NT	GF	NS	NT	GF
BMV variants									
wt B3	SL/SL	NT	NT	ChL/SM	NT	NT	NT	NT	NT
B3/ΔCP-GUS	NS/NS	−	NT	NS/NS	+++	NT	NT	NT	NT
B3/ΔCP-EGFP	NS/NS	NT	GF	NS/NS	NT	GF	NT	NT	NT

[a] Each inoculum (150 μg/ml) contained the indicated RNA3 construct and its respective parental wt RNA1 and -2 transcripts.

[b] I/S; inoculated/systemic; SL, symptomless infection; NL; necroctic local lesions; ChL, chlorotic local lesions; SM, systemic mottling; ChM, chlorotic mottling; NS, no symptoms; NT, not tested; NI, not infected. Presence or absence of progeny RNA in each infected plant was confirmed by Northern hybridization using riboprobes of desired specificity.

[c] Presence (+) or absence (−) of GUS activity; (+++) strong macroscopic GUS activity; (+) faint microscopic GUS activity. NT, not tested.

[d] Presence of green fluorescence (GF) in epidermal cells of inoculated leaves as evidenced by epifluorescence microscopy. NT, not tested.

vations agree with the findings of Mise and Ahlquist (1995) that cell-to-cell movement of CCMV is mediated by virus-encoded MP gene and show that initial infection spread of CCMV is independent of the CP gene.

CCMV is capable of long-distance movement in *N. benthamiana* (Mise *et al.*, 1993; Rao and Grantham, 1995a; Table 1). However, no indigo precipitate indicative of GUS activity was observed in noninoculated leaves from plants inoculated with C1 + C2 + C3/ΔCP-GUS at 14 dpi (data not shown), suggesting that C3/ΔCP-GUS is not competent for long-distance spread and therefore was not transported to distal parts of the plant. Although inoculation of C1 + C2 + C3/ΔCP-GUS resulted in a heavy indigo-colored precipitate on the primary leaves of *N. benthamiana* and *C. quinoa* (Table 1), irrespective of the time postinoculation, indigo precipitation in cowpea leaves was not intense (Table 1), complicating the quatitative analysis of cell-to-cell movement in cowpea. This was reproducibly observed in three additional experiments with independently transcribed RNAs. Since the reasons for poor indigo precipitation in cowpea following inoculation with C1 + C2 + C3/ΔCP-GUS are currently unknown, this host was not used in additional experiments involving GUS assays. Taken together, results presented in Fig. 2 and Table 2 clearly demonstrate that, unlike BMV (Schmitz and Rao, 1996), CCMV can be transported from initially infected cells to neighboring healthy cells without CP but the virus is unable to systemically infect these experimental plants without the gene.

## Characteristics of C3/ΔCP-EGFP and C3ΔMP/ΔCP-EGFP variants

Although GUS is a highly sensitive marker for studying subliminal infections (Schmitz and Rao, 1996) and effi-

cient viral spread (Dolja *et al.*, 1992; Scholthof *et al.*, 1993), its application is limited by: (i) its large gene size, (ii) instability of inserted GUS during prolonged infections (Dolja *et al.*, 1992), (iii) the difficulty in resolving various cell types involved in viral movement by visualizing whole leaves due to heavy indigo precipitation (see above), and (iv) the destructive nature of the assay hindering the study of a progressing virus infection in live tissue. The capacity to observe gene expression in living cells using the green fluorescent protein (GFP) of the jellyfish (*Aequorea victoria*; Chalfie *et al.*, 1994; Prasher *et al.*, 1992) has triggered an enormous upsurge in recent research efforts entailing plant virus movement (Baulcombe *et al.*, 1995; Heinlein *et al.*, 1995; Oparka *et al.*, 1995; Padgett *et al.*, 1996). The small gene size (730-bp sequence encoding a 27-kDa protein; Prasher *et al.*, 1992) and the ability to follow virus infection in live tissue using epifluorescence and confocal laser scanning microscopy are some of the attractive traits associated with GFP (Baulcombe *et al.*, 1995). Furthermore, unlike the colorimetric detection of GUS, green fluorescence is an inherent property of GFP and does not require the addition of exogenous substrates or cofactors (Cody *et al.*, 1993) and therefore its detection is dependent only on the accumulation of GFP.

GFP originally isolated from the jellyfish was used in plant virus movement studies (Baulcombe *et al.*, 1995; Heinlein *et al.*, 1996; Oparka *et al.*, 1996). However, in this study, a mutant of GFP, referred to as EGFP (Cormack *et al.*, 1996) was used. Unlike wt GFP, EGFP has a single, strong, red-shifted excitation peak at 488 nm and therefore can be visualized with blue light and standard FITC optics (Cormack *et al.*, 1996). Since the fluorescence
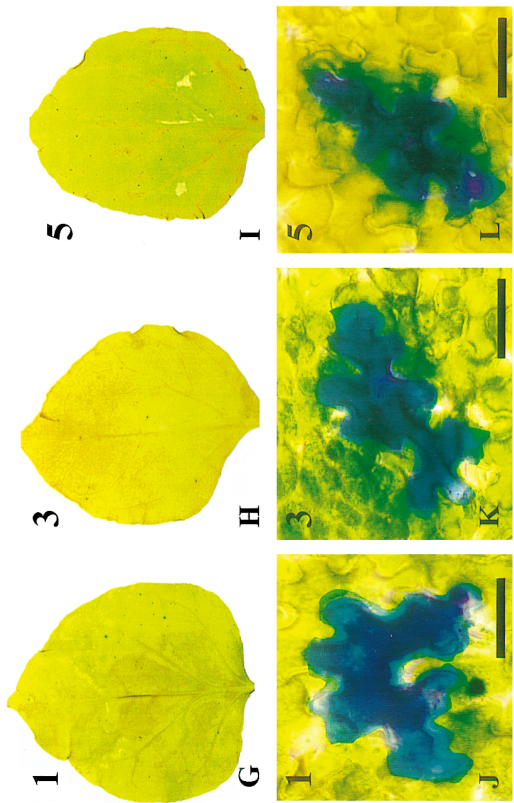
3

I. C1+C2+C3/ΔCP-EGFP

II. C1+C2+C3ΔMP/ΔCP-EGFP

III. B1+B2+B3/ΔCP-EGFP

2
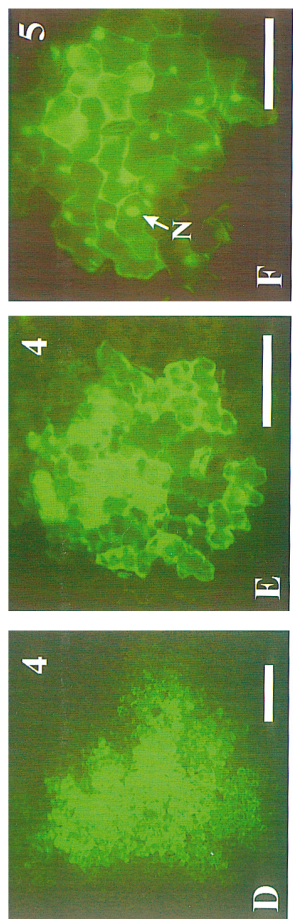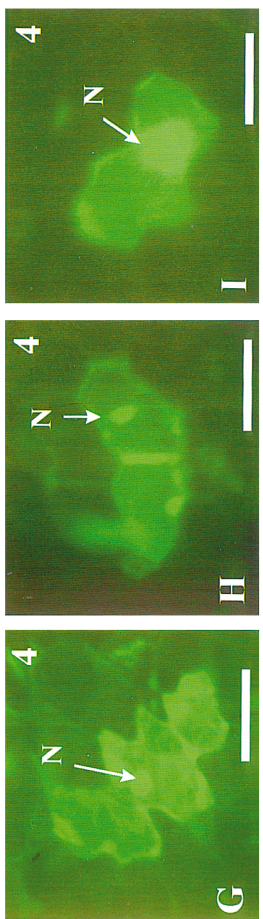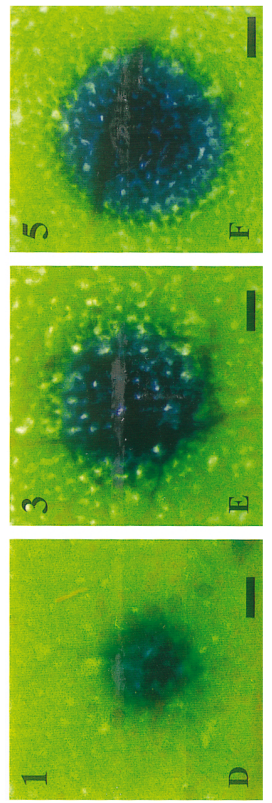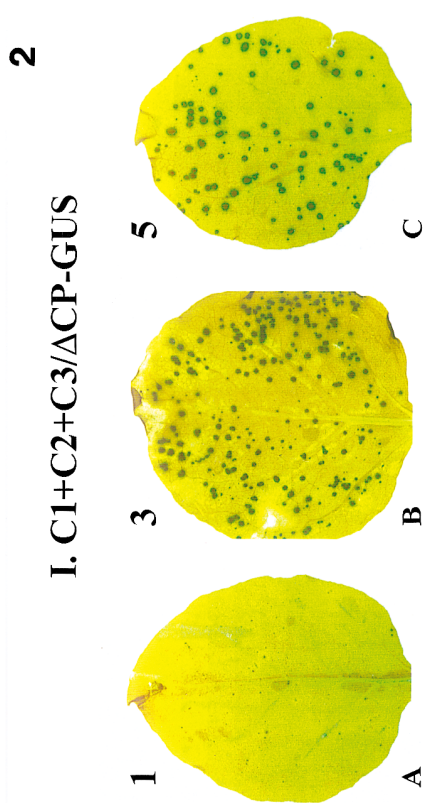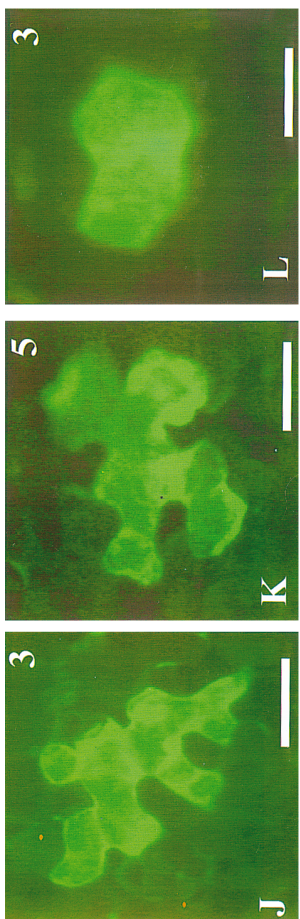
I. C1+C2+C3/ΔCP-GUS

II. C1+C2+C3ΔMP/ΔCP-GUS

emitted by the EGFP is 35× brighter than that of wt GFP (Cormack *et al.,* 1996), viral constructs expressing EGFP at low levels can be viewed more easily. Therefore, a CCMV RNA3 variant capable of efficient EGFP expression was constructed, with characteristics similar to those of pCC3/ΔCP-GUS (Fig. 1). Thus, C3/ΔCP-EGFP RNA transcriptions are 147 nt longer than those of wt C3 (Fig. 1). As a control, a variant of pCC3/ΔCP-EGFP, referred to as pCC3ΔMP/ΔCP-EGFP, was also constructed (Fig. 1). C3ΔMP/ΔCP-EGFP RNA transcripts are 573 nt shorter than those of wt C3 (Fig. 1). The replicative competence of C3/ΔCP-EGFP and C3ΔMP/ΔCP-EGFP and the expression of EGFP from the respective CP subgenomic RNAs were confirmed in *N. benthamiana* protoplast assays when cotransfected in the presence of C1 + C2 (data not shown). More than 90% of transfected protoplasts exhibited green fluorescence when viewed under an epifluorescence microscope and a representative example is shown in Fig. 3 (I A).

Visualization of EGFP in whole leaves infected with C3/ΔCP-EGFP and C3ΔMP/ΔCP-EGFP

To monitor the cell-to-cell movement characteristics of C3/ΔCP-EGFP, *N. benthamiana, C. quinoa,* and cowpea plants were inoculated with a mixture containing *in vitro* transcripts of C1 + C2 + C3/ΔCP-EGFP. None of these plants displayed any phenotypic symptoms throughout the examination period (Table 1). To verify whether inoculation of C1 + C2 + C3/ΔCP-EGFP has resulted in cell-to-cell spread without causing symptoms, primary inoculated leaves from each host plant were harvested at desired time points and observed under an epifluorescence microscope equipped with a FITC filter set (optimal for viewing EGFP; Cormack *et al.,* 1996). Examination of leaves inoculated with variants of CCMV RNA3 containing EGFP exhibited the following nonspecific characteristics: (i) uninfected areas displayed background dark green autofluorescence in all three host plants examined (Fig. 3, I B) and (ii) areas damaged due to mechanical inoculation exhibited bright green autofluorescence consisting of ruptured cells with ragged edges which had a nebular appearance (Fig. 3, I C). However, the specific green fluorescence of EGFP expressed by CCMV CP variants used in this study is strikingly different and distinct from that of the above-mentioned autofluorescence (Fig. 3, compare green fluorescence in I B to that in I D–L). Furthermore, cells contained in infection zones emitting green fluorescence of EGFP had defined boundaries and displayed a distinct green fluorescencing signal from the cytoplasm and as well as in the nuclei (Baulcombe *et al.,* 1995; Fig. 3, compare I C to I D–L).

Examination of inoculated primary leaves of *N. benthamiana, C. quinoa,* and cowpea harvested at 1–7 dpi with a hand-held UV light did not reveal any fluorescent zones visible to the naked eye (data not shown). However, epifluorescence microscopy revealed the presence of several infection foci with green fluorescent cells and revealed that the spread of infection in each of the three hosts examined had moved into neighboring cells over time (Fig. 3, I D–F). In every case examined, fluorescence was confined to the cytoplasm and nuclei of the epidermal cells, and preliminary results from CLSM optical sections of leaves infected with C3/ΔCP-EGFP revealed that green fluorescence was absent in mesophyll cells (data not shown). Fluorescence was also absent in uninoculated upper leaves at 14 dpi, even by inspection with epifluorescence microscopy. As observed with C3ΔMP/ΔCP-GUS (Fig. 2, II J–L), inoculation of C1 + C2 + C3ΔMP/ΔCP-EGFP to *N. benthamiana, C. quinoa,* and cowpea resulted in subliminal infections encompassing one or two fluorescent epidermal cells (Fig. 3, II G–I) and the fluorescence expressed by C3ΔMP/ΔCP-EGFP did not spread over time beyond these initially infected cells. These observations confirm that cell-to-cell move-

FIG. 2. Visualization of GUS activity in the primary leaves of *N. benthamiana* inoculated with C3 variants. Plants were inoculated with a desired mixture of RNA transcripts at a concentration of 150 μg/ml and whole leaves were vacuum-infiltrated with the histochemical GUS substrate X-glucuronidase. Numbers in the upper left-hand corner indicate days postinoculation. In panel I (top) the photographs represent macroscopic (A–C) and microscopic (D–F) images of infiltrated *N. benthamiana* leaves following inoculation with C1 + C2 + C3/ΔCP-GUS. Cell-to-cell movement is evident due to an increase in the size of the blue infection zones over time. Representative examples of microscopic images shown in E and F contained approximately 30 and 50 blue cells, respectively. Bar, 100 μm. In panel II (bottom), the photographs represent macroscopic images of infiltrated *N. benthamiana* leaves (G–I) inoculated with C1 + C2 + C3ΔMP/ΔCP-GUS. Note that there was no increase in the size of blue infection zones over time. Photographs shown in J through L are microscopic images of subliminal infections respectively elicited on leaves shown in G through I. Note that, irrespective of days postinoculation, GUS expression was confined to single epidermal cells. Bar, 50 μm.

FIG. 3. Epifluorescence microscopic analysis of green fluorescence emitted by EGFP. In panel I (top) (A), a representative example of *N. benthamiana* protoplasts expressing green fluorescence at 24 hr postinoculation (hpi) with C1 + C2 + C3/ΔCP-EGFP is shown. In panel I (B), autofluorescence exhibited by a portion of healthy *N. benthamiana* leaf is shown. In panel I (C), autofluorescence exhibited by an area injured due to mechanical inoculation in *N. benthamiana* is shown. Photographic images demonstrating epidermal cell-to-cell movement in *N. benthamiana* (D), cowpea (E), and *C. quinoa* (F) following inoculation with C1 + C2 + C3/ΔCP-EGFP are shown. Photographic images shown in panel II (middle) demonstrate one (G and I) or two (H) epidermal cells of *N. benthamiana* (G), cowpea (H), and *C. quinoa* (I) infected with C1 + C2 + C3ΔMP/ΔCP-EGFP. In panel III, representative examples of single epidermal cells of *N. benthamiana* (J and K) and *C. quinoa* (L) infected with B1 + B2 + B3/ΔCP-EGFP are shown. Fluorescence microscopy was performed with a Nikon Labophot microscope equipped with HFX-IIA episcopic-fluorescence attachment, super-high-pressure mercury lamp (Nikon HB 10101AF), and FITC filter set XF23 (Omega optical) containing a 488DF22 excitation filter, 505DRLP02 dichroic filter, and barrier filter 535DF35. Images were photographed with a Kodak Ektachrome film (400 ASA). Bar, 100 μm for image shown in B and D and 50 μm for all other images. Numbers in the upper right-hand corner indicate days postinoculation. N, nucleus.

TABLE 2

Analysis of Cell-to-Cell Movement in *N. benthamiana* and *C. quinoa* Plants Inoculated with CCMV RNA3 Variants Expressing GUS

Inoculum[a]	Days postinoculation (dpi)	No. of infection sites examined[b]	% infection sites with indicated number of cells[c]					
			1–4	5–10	11–20	21–30	30–50	>50
N. benthamiana								
C3/CP-GUS	1	22	1	49	50	0	0	0
	3	83	0	0	9	45	46	0
	5	126	0	0	6	24	30	40
	7	110	0	0	4	26	31	39
C3ΔMP/CP-GUS	1	1	100	0	0	0	0	0
	3	22	100	0	0	0	0	0
	5	46	100	0	0	0	0	0
C. quinoa								
C3/CP-GUS	1	27	1	99	0	0	0	0
	3	51	1	41	58	0	0	0
	5	66	0	23	44	33	0	0
	7	57	0	20	46	34	0	0
C3ΔMP/CP-GUS	1	16	100	0	0	0	0	0
	3	41	100	0	0	0	0	0
	5	37	100	0	0	0	0	0

[a] Each inoculum (150 μg/ml) contained C1 + C2 and the indicated C3 transcript.
[b] Average number of infection sites from four independent experiments at each time point.
[c] Infection sites were examined by microscopic observation of GUS activity in primary inoculated leaves.

ment of CCMV is mediated only by virus-encoded MP. Importantly, this result was not readily elucidated using the GUS assay.

Infections resulting from B3/ΔCP-EGFP are subliminal

Previous histochemical GUS and fluorescence *in situ* hybridization (FISH) analysis of *C. quinoa* leaves inoculated with several BMV CP defective variants demonstrated that CP is obligatory to mediate cell-to-cell movement of BMV (Schmitz and Rao, 1996). Since CCMV was transported between the epidermal cells of *N. benthamiana* without CP (Figs. 2 and 3, Table 2), it was envisioned that this host may permit mediation of cell-to-cell movement of CP defective variants of BMV even though this did not occur in *C. quinoa* (Schmitz and Rao, 1996). To substantiate this conjecture, an inoculum containing B1 + B2 + B3/ΔCP-GUS, which had previously been shown to cause subliminal infections in *C. quinoa* (Schmitz and Rao, 1996), was inoculated to *N. benthamiana* and processed for GUS activity. Although heavy GUS expression was observed in transfected protoplasts of *N. benthamiana,* irrespective of time postinoculation, no GUS activity was observed in primary inoculated leaves (Table 1), a situation similar to low GUS activity detected in cowpea for C3/ΔCP-GUS (Table 1). Therefore, a CP variant of BMV RNA3 harboring EGFP in place of CP was constructed. Although the presence of a sequence encoding the eight N-terminal amino acids of BMV CP is essential

for efficient expression of foreign genes (Mori *et al.,* 1993), in contrast to the CCMV CP ORF (Fig. 1), no compatible unique restriction site is located within the sequence encoding the first 10 N-proximal amino acids of pT7B3 (Fig. 1). Since the first 25 N-proximal amino acids of BMV and CCMV CP genes are highly conserved (Sacher and Ahlquist, 1989; Rao and Grantham, 1996), this limitation was circumvented by incorporating the EGFP sequence from plasmid pCC3/ΔCP-EGFP into pT7B3 (Fig. 1). As observed with C3/ΔCP-EGFP, transfection of *N. benthamiana* protoplasts with *in vitro* synthesized transcripts of B3/ΔCP-EGFP (210 nt longer than those of wt B3) resulted in the synthesis of EGFP as evidenced by the presence of green fluorescent protoplasts similar to those shown in Fig. 3 (I A).

To examine the cell-to-cell movement of B3/ΔCP-EGFP, a mixture containing B1 + B2 + B3/ΔCP-EGFP was inoculated to *N. benthamiana* and *C. quinoa* plants. As for CCMV, *N. benthamiana* is a symptomless systemic host for BMV (Table 1; Rao and Grantham, 1995a). Unlike CCMV in *C. quinoa,* BMV induces chlorotic local lesions followed by systemic mottling in uninoculated upper leaves (Rao and Grantham, 1996). Control inoculations made with all three wt transcripts of BMV induced characteristic infections in *N. benthamiana* and *C. quinoa* (Table 1). None of the plants inoculated with B1 + B2 + B3/ΔCP-EGFP displayed any visible symptoms (Table 1). To verify whether infections resulting from B3/ΔCP-EGFP, like C3/ΔCP-EGFP, are capable of cell-to-cell movement without causing any visible phenotypic symp-

toms, primary leaves of *N. benthamiana* and *C. quinoa* were examined under an epifluorescence microscope for green fluorescent cells. Irrespective of the time post-inoculation and the type of host plant, green fluorescence characteristic of EGFP was observed in only one or two epidermal cells (Fig. 3, III J–L), suggesting that, unlike C3/ΔCP-EGFP, infection resulting from B3/ΔCP-EGFP did not spread to adjacent epidermal cells over time. The lack of cell-to-cell movement for B3/ΔCP-EGFP is not due to the presence of the first 10 N-terminal amino acids from CCMV CP (which differ in 2 amino acid substitutions at positions 3 and 8 relative to BMV CP; Rao and Grantham, 1996, Fig. 1), since mature BMV CP lacking the first 18 N-terminal amino acids is competent for encapsidation and movement (Rao and Grantham, 1996). These observations further accentuated the lack of cell-to-cell movement exhibited by the CP defective variants of BMV examined in previous studies (Schmitz and Rao, 1996) and in this study (Fig. 3, III J–I) is not due to the type of host plant but is due to the inherent dependence on functional CP (Rao and Grantham, 1995b; Schmitz and Rao, 1996).

DISCUSSION

Previous *in situ* analyses of movement characteristics of several CP defective variants of BMV, a monocot-adapted bromovirus, demonstrated that the synthesis of a functional CP, in addition to MP, is essential for cell-to-cell spread (Schmitz and Rao, 1996). In the current study, the movement of CP deficient variants of CCMV, a dicot-adapted bromovirus, was sensitively monitored using GUS and EGFP reporter genes and we observed that CCMV can be transported between epidermal cells without CP (Figs. 2 and 3, I). For example, the number of epidermal cells infected with C3/ΔCP-GUS (Fig. 2) and C3/ΔCP-EGFP (Table 2, Fig. 3, I D–F) increased over time when assayed by histochemical GUS analysis and epifluorescence microscopy, respectively. In contrast, similar analysis revealed that irrespective of the time postinoculation, the infection of B3/ΔCP-EGFP analyzed by the presence of green fluorescence remained subliminal (Fig. 3, IIIJ–L; Table 2). Even though Flasinski *et al.* (1995) suggested that BMV can move cell-to-cell without CP, we have differing observations (Rao and Grantham, 1995b, 1996; Schmitz and Rao, 1996).

BMV and CCMV exhibit similar genome organization and replication mechanisms (Ahlquist, 1994) and their CPs share 70% identity at the amino acid level (Speir *et al.*, 1995). Despite these similarities, one possible explanation for the observation that CCMV can move between epidermal cells without CP (Figs. 2 and 3 I; Allison *et al.*, 1990) whereas BMV cannot (Fig. 3, III) is that these viruses utilize different mechanisms to cross different cell types. For example, BMV has been shown to produce tubules containing virus-like particles (Kasteel *et al.*,

1996), evidence of a mechanism favored for como- and nepoviruses (Ritzenthaler *et al.*, 1995; Van Lent *et al.*, 1991). Thus, the need for CP in BMV movement is apparent. At present it is not known whether CCMV induces tubules or is transported between epidermal cells in a nonvirion form (i.e., TMV-like movement; Wolf *et al.*, 1989). Evidence that CCMV moves in a nonvirion form comes from the data presented in this study since CCMV CP deficient variants efficiently moved between epidermal cells for up to 5 dpi (Table 2, Figs. 2 and 3). This conjecture is further supported by the observation that a hybrid CCMV RNA3 bearing cowpea-adapted sunhemp mosaic tobamovirus (SHMV) MP can fully support CCMV movement (cell-to-cell and long distance movement) in cowpea (De Jong and Ahlquist, 1992), suggesting that CCMV can move in TMV-like manner. However, it remains to be seen whether MP from a non-cowpea-adapted tobamovirus would also support CCMV movement.

It is also possible that the defective epidermal cell-to-cell movement exhibited by BMV CP variants is due to host resistance responses. A cooperative interaction between viral gene products and the host appears to play a major role in viral movement (Carrington *et al.*, 1996). Thus, incompatible interactions result in a reduced spread and consequently the host attains resistance to infection. One common type of incompatibility that results in a resistance response of plants against pathogens is the hypersensitive response (HR) (Dawson, 1992). We previously demonstrated that deletion of the amino-terminal region from the matured wt BMV CP resulted in the induction of HR due to an incompatible interaction between virus containing modified CP and *C. quinoa* (Rao and Grantham, 1995b, 1996). Therefore it is possible that the subliminal infection induced by the CP defective variants of BMV examined in this study and in previous studies (Fig. 1; Rao and Grantham, 1995b, 1996; Schmitz and Rao, 1996) is due to the inability of the virus to overcome host resistance responses. Other differences between CCMV and BMV CP (such as nonconserved amino acids), and possibly differences between other viral genes as well, may play roles in inducing or overcoming host responses.

Although the results of this study and previous studies accentuate the importance of CP in bromovirus cell-to-cell movement (Allison *et al.*, 1990; Rao and Grantham, 1995b, 1996; Sacher and Ahlquist, 1989; Schmitz and Rao, 1996), it is well established that the nonstructural 3a MP is also essential for virus movement (Mise and Ahlquist, 1993), since several bromovirus RNA3 variants with unaltered CP but defective MP failed to support cell-to-cell movement (Mise and Ahlquist, 1993; Mise *et al.*, 1995; Schmitz and Rao, 1996). In addition to MP and CP, replicase genes have been shown to profoundly influence the bromovirus movement process, since BMV replicase mutants that were capable of efficient replication and packaging in protoplasts failed to systemically infect

barley (Traynor *et al.,* 1991). The participation of virus products other than MP and CP in movement has been reported for several other viral systems. For example, replicase genes of CMV (Gal-On *et al.,* 1995), BSMV (Petty *et al.,* 1990; Weiland and Edwards, 1994), and TMV (Nelson *et al.,* 1993), a nonstructural protein p19 of TBSV (Scholthof *et al.,* 1995), and a helper component protease protein of potyviruses (Cronin *et al.,* 1995) have also been shown to have specific roles in movement.

In conclusion, the results of this study together with several site-directed mutagenesis studies of biologically active bromoviral clones (Allison *et al.,* 1988; Mise and Ahlquist, 1993; Sacher and Ahlquist, 1989; Traynor *et al.,* 1991) reveal that all four bromoviral genes are essential to condition the movement process. The availability of different colored GFPs (Heim and Tsien, 1996; Rizzuto *et al.,* 1996) offers the possibility of tagging individual viral genes with the desired chromophores to study the localization and accumulation of movement-associated viral gene products over time, which should help in delineating the role of each virus-encoded gene in the active movement process.

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