Journal of General Virology (1999), **80**, 1357–1365. Printed in Great Britain

Effect of C-terminal deletions in the movement protein of cowpea chlorotic mottle virus on cell-to-cell and long-distance movement

Fatima Osman, Isabelle Schmitz and A. L. N. Rao

Department of Plant Pathology, University of California, Riverside, CA 92521-0122, USA

In order to elucidate the function of the C-terminal region of cowpea chlorotic mottle bromovirus (CCMV) movement protein (MP) in cell-to-cell movement, a set of deletions ranging from 10 to 80 amino acids (ΔMP10, ΔMP20, ΔMP33, ΔMP43, ΔMP60 and ΔMP80) was engineered into the MP gene encoded by the biologically active clone C3/ΔCP-EGFP, a variant of CCMV RNA3 that contained wild-type (wt) MP and the enhanced green fluorescent protein (EGFP) gene in place of the coat protein (CP). The effect of each MP deletion on cell-to-cell movement was examined in three susceptible host plants: *Chenopodium quinoa*, *Nicotiana benthamiana* and cowpea (*Vigna sinensis* cv. Black Eye). The results indicate that, except for mutant ΔMP43, infections resulting from the deletion mutants remained subliminal. Interestingly, infections resulting from inoculating mutant ΔMP43, which lacked the 43 most C-terminal amino acids, spread rapidly between cells and the number of infected cells expressing EGFP approached that of control inoculations made with C3/ΔCP-EGFP. To verify whether the presence of wt CP altered the movement behaviour of these mutants, each MP deletion was also incorporated into the genetic background of wt CCMV RNA3 (pCC3) and inoculated independently to all three hosts. The results suggest that the overall movement process exhibited by each MP mutant is influenced profoundly by the presence of CP and the particular host plant tested.

Introduction

Cowpea chlorotic mottle virus (CCMV) is a multicomponent, single-stranded, positive-sense RNA virus belonging to the *Bromoviridae* (Ahlquist, 1994). Its genome is divided among three RNA components. Replication of CCMV is initiated by two *trans*-acting replicase genes, 1a and 2a, that are encoded by monocistronic genomic RNAs 1 and 2, respectively (Allison *et al.*, 1988). The two gene products encoded by dicistronic genomic RNA3, movement protein (MP) and coat protein (CP), are dispensable for replication but are required for whole plant infections. The 5′-proximal gene, encoding MP, is translated directly from genomic RNA3, whereas the 3′-proximal gene encoding CP is translated from a subgenomic RNA4 that is regenerated from progeny negative-sense RNA3 upon infection (Allison *et al.*, 1988). Dicot-adapted CCMV and monocot-adapted brome mosaic bromovirus (BMV) have been used to investigate the functional roles of MP and CP in bromovirus movement (Mise & Ahlquist, 1995; Rao & Grantham, 1995 *b*; Schmitz & Rao, 1996). Hybrid viruses, constructed by exchanging MP genes precisely between CCMV and BMV, demonstrated that MP plays a crucial role in determining host specificity (Mise *et al.*, 1993). In addition, the MPs of CCMV and BMV have been shown to modulate symptom expression in selected host plants (Fujita *et al.*, 1996; Rao & Grantham, 1995 *a*, *b*).

Molecular and genetic analyses of CCMV and BMV MP and CP genes also showed that these two genes are required for efficient cell-to-cell and long-distance spread in susceptible host plants (Allison *et al.*, 1990; Kasteel *et al.*, 1997; Rao, 1997; Rao & Grantham, 1995 *b*, 1996; Schmitz & Rao, 1996). For example, in BMV, infections resulting from several RNA3 mutants that expressed unaltered MP, but which were incapable of synthesizing wild-type (wt) CP, remained subliminal (Rao & Grantham, 1995 *b*; Rao, 1997; Schmitz & Rao, 1996). Unlike BMV, CP-defective mutants of CCMV are competent for cell-to-cell movement to a limited extent (Rao, 1997). Although the essentiality of bromovirus MP in cell-to-cell movement has been established, the sequence and/or possible structural elements required to mediate this process have not been determined. In an attempt to delineate the

Author for correspondence: A. L. N. Rao.
Fax +1 909 787 4294. e-mail arao@ucrac1.ucr.edu

minimal sequences of MP required to support the movement function, this preliminary study reports the effect of deletions in the C terminus of the CCMV MP on cell-to-cell and long-distance movement.

Methods

■ **Engineering C-terminal deletions into the MP of CCMV.** All MP deletion mutants used in this study (Fig. 1*a*) were created in pCC3WD21, a plasmid containing a full-length cDNA of CCMV RNA3 (De Jong *et al*., 1997). Six deletions were introduced independently by PCR into pCC3WD21 using the desired 5′ and 3′ oligonucleotide primers, as indicated in Table 1. These deletions are designated Δ10, Δ20, Δ33, Δ43, Δ60 and Δ80 (Table 1; Fig. 1*a*). Each PCR product was amplified as a *Bam*HI–*Bgl*II fragment (sites located at the beginning and end of the MP gene, respectively; Fig. 1*a*) and subcloned into pCC3WD21 that had first been digested to completion with *Bam*HI followed by partial digestion with *Bgl*II. The nucleotide sequences of the subcloned fragments were determined to verify the presence of introduced deletions as previously described by Rao *et al*. (1994).

■ **Construction of CCMV MP deletion mutants harbouring enhanced green fluorescent protein (EGFP).** In order to study the effect of the engineered deletions of MP on cell-to-cell movement, a DNA fragment containing the entire EGFP gene was amplified as a *Hpa*I–*Spe*I (positions 1383–1954) fragment from pCC3ΔCP/EGFP (Rao, 1997; Fig. 1*b*) and subcloned into each MP deletion mutant. The mutants were designated Δ10/EGFP, Δ20/EGFP, Δ33/EGFP, Δ43/EGFP, Δ60/EGFP and Δ80/EGFP (Fig. 1*b*).

■ ***In vitro* transcription and transfection of protoplasts.** Full-length cDNA clones pCC1TP1, pCC2TP2 and pCC3TP4 corresponding to CCMV genomic RNAs 1 (C1), 2 (C2) and 3 (C3) and each mutant of pCC3WD21 were linearized with *Xba*I (Allison *et al*., 1988; De Jong *et al*., 1997) prior to *in vitro* transcription with T7 RNA polymerase (Rao & Grantham, 1996). Barley protoplasts were prepared from 6-day-old barley plants (*Hordeum vulgare* cv. Dickson) and transfected with an inoculum mixture containing wt C1 and C2 and either wt C3 or each C3 mutant using a polyethylene glycol procedure (Dreher *et al*., 1989; Rao *et al*., 1994). At 24–48 h post-inoculation (p.i.), total RNA was isolated from transfected protoplasts by using SDS–phenol and subjected to Northern analysis as described previously (Rao *et al*., 1994).

■ **Whole-plant inoculations and progeny analysis.** For whole-plant inoculations, plants of *Nicotiana benthamiana* (3 weeks old), *Chenopodium quinoa* (2 weeks old) and cowpea (*Vigna sinensis* cv. Black Eye; 2 weeks old) were kept in the dark for at least 18 h. The primary leaves of each host plant (two to three leaves) were inoculated mechanically with a desired mixture of *in vitro* transcripts (150 μg/ml), as described previously (Rao *et al*., 1994). Each experiment was repeated at least three times with independently synthesized *in vitro* transcripts. The inoculated plants were kept in the greenhouse at 25 °C and observed for symptom expression over a period of 8 weeks. Progeny RNA was isolated from inoculated and non-inoculated systemic leaves of *N. benthamiana*, *C. quinoa* and cowpea plants and subjected to Northern analysis (Rao *et al*., 1994). ^{32}P-labelled riboprobes specific for CCMV RNA3 were synthesized as described previously (Osman *et al*., 1997). For Western analysis, symptomatic or asymptomatic leaf tissue was ground in SDS–PAGE sample buffer [final concentrations 125 mM Tris–HCl, pH 6·8, 10% (w/v) glycerol, 2·5% (w/v) DTT, 2% SDS, 0·01% bromophenol blue], denatured at 100 °C for 5 min and separated on a 16% SDS–PAGE gel according to Laemmli (1970). Fractionated proteins were transferred electrophoretically to a nitrocellulose membrane and detected with CCMV CP antibody (1 : 3000 dilution) by using an enhanced chemoluminescence (ECL) kit (Amersham). For sequence analysis, all progeny viral RNAs extracted from systemic leaves were suspended in sterile distilled water and subjected to RT–PCR as described previously (Rao *et al*., 1994; Rao & Grantham, 1995*b*). PCR products were sequenced directly by using an *fmol* sequencing kit (Promega), to verify the conservation of introduced deletions.

■ **Detection of EGFP.** Leaves of *N. benthamiana*, *C. quinoa* and cowpea were inoculated with a mixture containing wt C1 and C2 and either C3/ΔCP-EGFP or each MP mutant harbouring EGFP and analysed at 1, 3 and 5 days p.i. Inoculated leaves were viewed under a Nikon Labophot microscope equipped with a super-high-pressure mercury lamp as a source of blue light (Nikon HB 10101 AF), an HFX-IIA epifluorescence attachment and an FITC filter set XF23 (Omega Optical) containing a 488 DF22 excitation filter, a 505 DRL PO2 dichroic filter and a 535 DF35 barrier filter (Rao, 1997). Photographs were taken on Kodak Ektachrome 400 ASA slide film and arranged graphically by using Adobe Photoshop 4.0 software (Adobe Systems).

Results and Discussion

Analysis of cell-to-cell movement of CCMV MP mutants

Although BMV and CCMV are both members of the genus *Bromovirus* and have many common features, the extent to which the MP and CP genes participate in cell-to-cell movement is different. For example, cell-to-cell movement of BMV is dependent on both MP and CP (Schmitz & Rao, 1996), while CCMV can be transported to a certain degree between cells in the absence of CP (Rao, 1997). Consequently, a variant clone of CCMV RNA3 in which CP was replaced with EGFP (C3/ΔCP-EGFP; Rao, 1997) was competent to move rapidly between cells of selected susceptible hosts (Rao, 1997). Therefore, to examine the role played by the C-terminal amino acids of the CCMV MP in cell-to-cell movement, a total of six independent deletions was engineered into the MP gene of C3/ΔCP-EGFP. The size of the deletions ranged from 10 to 80 C-terminal amino acids (Fig. 1*b*; Δ10–Δ80/EGFP). *In vitro* transcripts of each of these mutant clones were inoculated independently together with wt C1 and wt C2 to *N. benthamiana*, *C. quinoa* and cowpea plants. Plants inoculated with transcripts of C3/ΔCP-EGFP (Rao, 1997) served as positive controls. None of the inoculated plants displayed any visible symptoms, even 3 weeks p.i. To examine whether any of the MP deletion mutants were able to move from cell to cell, primary inoculated leaves were harvested at 1, 3 and 5 days p.i. and observed for EGFP expression under an epifluorescence microscope. Results are shown in Table 2 and Fig. 2. Consistent with previous observations (Rao, 1997), examination of primary leaves of *N. benthamiana* (Fig. 2 A, *a–c*) and *C. quinoa* (Fig. 2 B, *a–c*) inoculated with a control inoculum containing C3/ΔCP-EGFP revealed the presence of several infection foci expanding with time and encompassing more than 50 cells per infection site by 5 days p.i. (Table 2). When primary leaves of

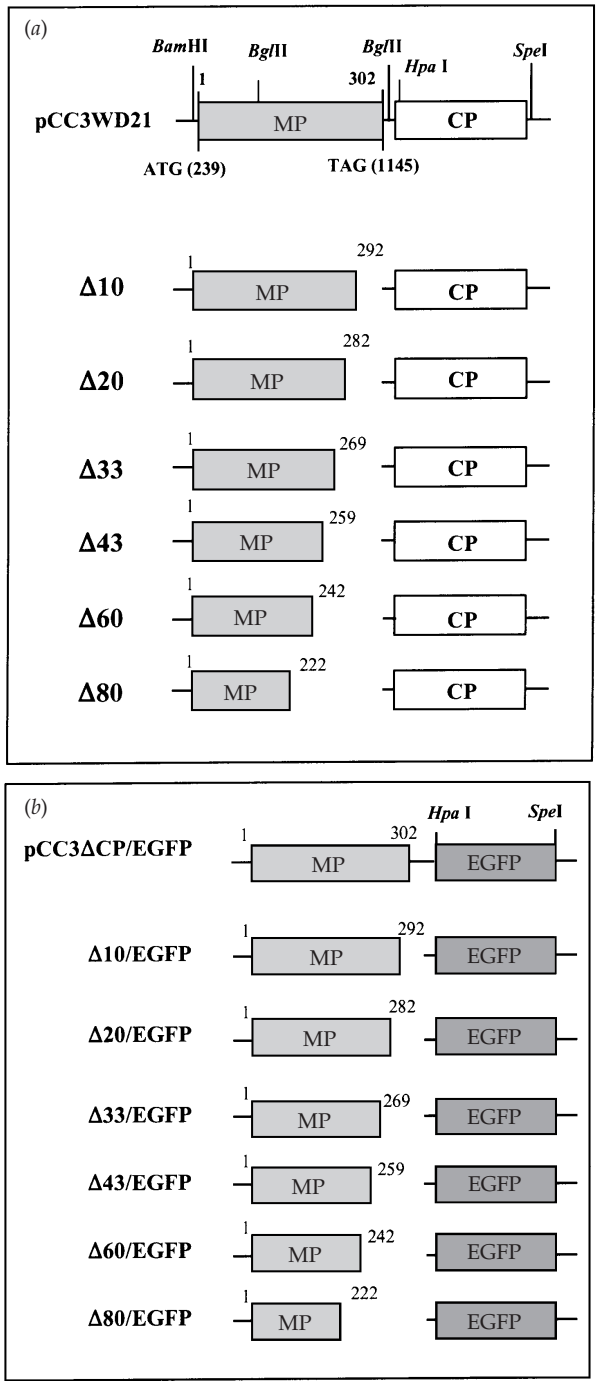

Fig. 1. Characteristics of CCMV MP deletion mutants. (*a*) The structure of pCC3WD21 representing wt CCMV RNA3 is shown, with non-coding sequences represented as a single line and CP as an open box. The light-grey box represents MP. The positions of the CCMV MP start codon (ATG at position 239) and the stop codon (TAG at position 1145) are shown. The extent of the engineered deletions for mutants Δ10 to Δ80 are shown by the decreased size of the MP. The numbers to the top right of the each MP box represent the terminal amino acid of each MP deletion mutant. (*b*) Characteristics of CCMV/EGFP mutants. pCC3ΔCP/EGFP has the EGFP gene, represented by the dark-grey box, in the place of CP (Rao, 1997). The EGFP gene was amplified from CC3ΔCP/EGFP by PCR with 5′ and 3′ oligonucleotide primers containing *Hpa*I and *Spe*I restriction sites, respectively. The resulting PCR product was digested with *Hpa*I and *Spe*I and subcloned into each similarly treated MP deletion mutant shown in (*a*).

N. benthamiana and *C. quinoa* inoculated with MP mutants were examined for the expression of EGFP, the fluorescent signal exhibited by five of the six mutants, Δ10/EGFP, Δ20/EGFP, Δ33/EGFP, Δ60/EGFP and Δ80/EGFP, was confined to one to three cells even at 5 days p.i. (Table 2; Fig. 2 A, *d–i*; B, *d–e*, *g–i*), suggesting that the infection remained subliminal. However, in a few instances a fluorescent signal was observed in sites containing up to five cells (Fig. 2 B, *f*; Table 2), perhaps due to multiple infections during mechanical inoculation, as previously reported for bromo- (Rao, 1997) and potexviruses (Oparka *et al.*, 1995). An unexpected result was the movement behaviour of mutant Δ43/EGFP. In spite of the large deletion, mutant Δ43/EGFP moved rapidly between cells in the inoculated leaves of each host and the number of cells in the infection foci expressing EGFP approached those resulting from control inoculations (Fig. 2 A, *j–l*; B, *j–l*; Table 2). Although the reason for the unanticipated movement behaviour of Δ43/EGFP is unclear, we speculate that specific structural changes induced by deleting the 43 C-terminal amino acids rendered the altered MP more likely to interact favourably with virion RNA than did the other engineered MP deletions. Similar observations were recently reported by Nagano *et al.* (1997). These authors observed that, although a chimeric BMV RNA3 encoding full-length cucumber mosaic virus MP failed to move between cells, a spontaneous deletion of 33 C-terminal amino acids that occurred in the cucumber mosaic virus MP during replication *in vivo* enabled the BMV chimera to move from cell to cell.

Another interesting observation was made with controls and the MP mutants harbouring EGFP when they were inoculated to cowpea plants. Of the three hosts examined here, cowpea is considered to be the natural host for CCMV and therefore we envisaged that the ability of each mutant to move between cells in that host would exceed that in the other two hosts. However, in contrast to the rapid cell-to-cell movement of the MP mutants observed in *N. benthamiana* and *C. quinoa* (Fig. 2 A *a–c*; B, *a–c*), in cowpea leaves, the cell-to-cell movement of C3/ΔCP-EGFP, as manifested by a green fluorescent signal, was limited to no more than 10 cells per infection focus even at 5 days p.i. (Table 2). Occasionally a few foci containing 10–15 cells, as previously reported (Rao, 1997), were also observed at 5 days p.i. (Table 2). Thereafter, the fluorescence faded rapidly and disappeared completely by 7 days p.i. In this host, with the exception of mutant Δ43/EGFP, the infections resulting from inoculation with the MP deletion mutants (Δ10/EGFP, Δ20/EGFP, Δ33/EGFP, Δ60/EGFP and Δ80/EGFP) were restricted to single cells (Table 2). As observed for control inoculations with C3/ΔCP-EGFP (Table 2), cowpea leaves inoculated with mutant Δ43/EGFP revealed only a limited number of infection foci expressing EGFP and the number of cells in each focus was restricted to no more than 10 cells even by 5 days p.i. (Table 2). Although the reasons for the fading of the EGFP signal with time in cowpea is not known, the restricted movement of C3/ΔCP-EGFP

Table 1. Primers used in PCR to create C-terminal deletions in the CCMV MP

Primer 5′UUg was used as the 5′ primer in all reactions. Primers Δ10–Δ80 were designed to delete the first 10–80 amino acids, as indicated, from the C terminus of CCMV MP. The amino acid positions deleted are indicated in Fig. 1.

Oligonucleotide	Sequence (5′–3′)
5′ primers	
5′UUg	GGAATAGTTCGATATCATAATTCCTCGTTCTTTGCTGTTTTGGATCCCGATGTCT
3′ primers	
Δ10	TGTTAGCAATAGATCTAGCTAAAGCCCCGCAAC
Δ20	TGTTAGCAATAGATCTAGCTAGATTTTATCCTT
Δ33	TGTTAGCAATAGATCTAGCTATAGTGATTCCTCCTCTAA
Δ43	TGTTAGCAATAGATCTAGCTATGGATCTTGCTTGCTCGTC
Δ60	TGTTAGCAATAGATCTAGCTAATTTACAGATTG
Δ80	TGTTAGCAATAGATCTAGCTATTTCAACCTGTC

Table 2. Analysis of cell-to-cell movement of CCMV MP deletion mutants expressing EGFP in *N. benthamiana*, *C. quinoa* and cowpea

Each inoculum (150 μg/ml) contained wt C1 and C2 and either ΔCP/EGFP or the indicated C3 MP mutant. The data represent the mean number of infection foci containing the number of cells indicated after three independent trials.

No. of cells expressing EGFP per infection focus	ΔCP/EGFP			Δ10/EGFP			Δ20/EGFP			Δ33/EGFP			Δ43/EGFP			Δ60/EGFP			Δ80/EGFP		
Days p.i.	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5
N. benthamiana																					
1–3	1	0	0	14	10	3	11	7	3	6	4	2	3	0	0	5	2	1	0	0	0
4–10	1	1	0	5	0	0	0	0	0	0	1	0	8	1	0	0	0	0	0	0	0
11–30	14	6	1	0	0	0	0	0	0	0	0	0	12	9	10	0	0	0	0	0	0
31–50	3	6	4	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	0	0	0
> 50	1	2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. quinoa																					
1–3	1	0	0	8	7	6	6	5	2	4	4	2	3	0	0	3	1	1	1	0	0
4–10	7	0	0	2	1	0	0	0	0	0	1	0	6	3	1	0	0	0	0	0	0
11–30	14	13	12	0	0	0	0	0	0	0	0	0	8	11	12	0	0	0	0	0	0
31–50	3	6	4	0	0	0	0	0	0	0	0	0	2	1	3	0	0	0	0	0	0
> 50	0	1	7	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Cowpea																					
1–3	3	3	1	1	1	1	0	1	0	1	1	0	3	3	1	0	0	0	0	0	0
4–10	2	1	2	0	0	0	0	0	0	0	1	0	1	2	2	0	0	0	0	0	0
11–30	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31–50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
> 50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

and Δ43/EGFP, as compared to that observed in *N. benthamiana* and *C. quinoa*, could be attributed to the onset of a host defence response in the absence of CP, as hypothesized previously (Rao, 1997; Osman *et al.*, 1997).

The *in vitro* binding activity of bromovirus MP was recently examined (Fujita *et al.*, 1998; Jansen *et al.*, 1998). The binding of BMV MP is cooperative and it bound preferentially to single-stranded nucleic acids (RNA and DNA; Jansen *et al.*, 1998). Further characterization of BMV MP revealed that deletions encompassing the 60 C-terminal amino acids of BMV MP did not effect RNA binding *in vitro* (Fujita *et al.*, 1998). Based on these observations, we expected the CCMV MP mutants constructed in this study to be transported between the cells. A possible explanation for the lack of cell-to-cell spread of Δ10/EGFP, Δ20/EGFP, Δ33/EGFP, Δ60/EGFP and Δ80/EGFP is that the interaction between the MP and

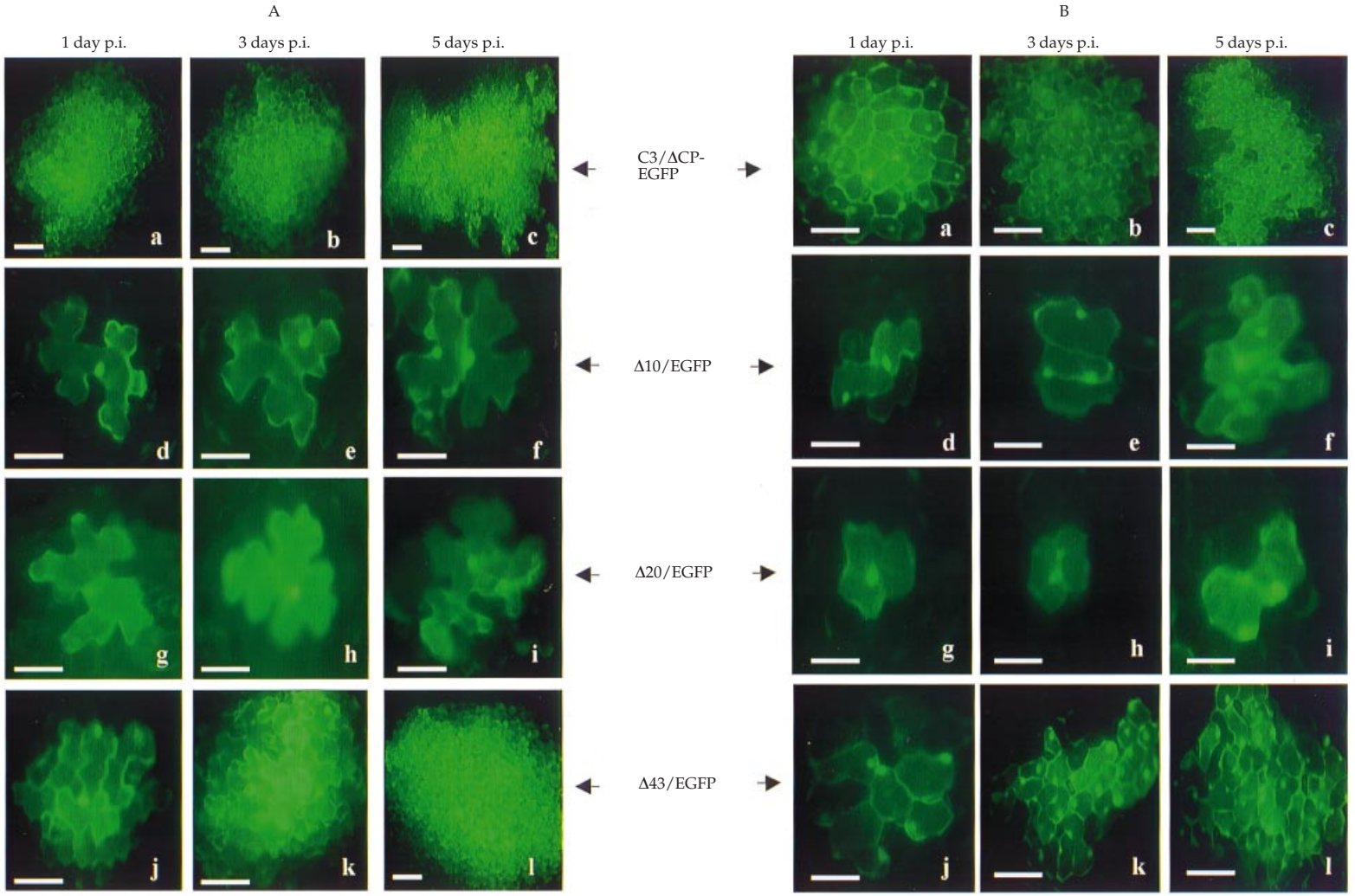

Fig. 2. (A) Epifluorescent microscopic analysis of *Nicotiana benthamiana* leaves inoculated with CCMV MP deletion mutants expressing EGFP. (*a*)–(*c*) Cell-to-cell movement resulting from inoculation with a control containing a mixture of wt C1, wt C2 and C3/ΔCP-EGFP. (*d*)–(*i*) Examples of subliminal infections (single epidermal cells) following inoculation with wt C1, wt C2 and either Δ10/EGFP (*d–f*) or Δ20/EGFP (*g–i*). Bars, 150 μm (*a–c* and *l*) and 75 μm (*d–k*). (B) (*a*)–(*c*) Cell-to-cell movement resulting from the inoculation of primary leaves of *C. quinoa* leaves with a control containing a mixture of wt C1, wt C2 and C3/ΔCP-EGFP. (*d*)–(*i*) Subliminal infections mediated by wt C1, wt C2 and either Δ10/EGFP (*d–f*) or Δ20/EGFP (*g–i*). Bars, 150 μm (*c*) and 75 μm (*a–b*, *d–l*). In both (A) and (B), images (*j*)–(*l*) represent cell-to-cell movement mediated by inoculum containing wt C1, wt C2 and Δ43/EGFP. See Methods for description of photography.

Table 3. Characteristics and progeny analysis of CCMV MP deletion mutants

Each inoculum (150 μg/ml) contained a mixture of *in vitro* transcripts of wt C1 and C2 and either wt C3 or the indicated C3 MP mutant. Symptoms of infection are described as: CM, chlorotic mottling; MSM, mild systemic mottling; NLL, necrotic local lesions; SNLL, small necrotic local lesions; SL, symptomless. CCMV RNA was detected by Northern blotting and CP was detected by Western blotting. L, Local; S, systemic.

	C. quinoa			*N. benthamiana*						Cowpea				
	Symptoms	RNA	CP	Symptoms		RNA		CP		Symptoms	RNA		CP	
Inoculum	L	L	L	L	S	L	S	L	S	S	L	S	L	S
wt C3	NLL	+	+	SL	SL	+	+	+	+	CM	+	+	+	+
Δ10	SNLL	+	+	SL	SL	+	+	+	+	SL	−	−	−	−
Δ20	SL	+	+	SL	SL	+	+	+	+	MSM*	+	+†	+	+†
Δ33	SL	+	+	SL	SL	+	−	+	−	SL	−	−	−	−
Δ43	SNLL	+	+	SL	SL	+	+	+	+	MSM*	+	+†	+	+†
Δ60	SL	+	+	SL	SL	+	−	+	−	SL	−	−	−	−
Δ80	SL	−	−	SL	SL	−	−	−	−	SL	−	−	−	−

* Symptoms appeared 60 days p.i.
† Progeny RNA and CP were detected 60 days p.i.

viral RNA could be different *in vivo* than *in vitro*, and therefore the deletions engineered in MP might lead to altered RNA-binding abilities *in vivo*, thereby affecting cell-to-cell movement. Since the active domain required for RNA binding is located between amino acid residues 189 and 234 for BMV MP (Fujita *et al.*, 1998), we speculate that the deletion of a similar region in the CCMV MP of Δ80/EGFP rendered the mutant incapable of any movement (Table 2). It is also likely that, in addition to the RNA-binding domain, the MP probably contains additional functional domains that are specifically required to promote cell-to-cell spread.

The spread of MP deletion mutants in the presence of CP is modulated differentially in each of the three hosts

From the above data (Table 2 and Fig. 2), it is apparent that the C-terminal amino acids of CCMV MP are critical for cell-to-cell movement, since infections resulting from mutants Δ10/EGFP, Δ20/EGFP, Δ33/EGFP and Δ60/EGFP remained subliminal. However, since both MP and CP are required for efficient local spread, the movement behaviour of these MP deletion mutants could possibly be influenced by the presence of CP, which is required for efficient cell-to-cell movement (Allison *et al.*, 1990; Rao, 1997; Osman *et al.*, 1997). To test this possibility, each MP deletion was incorporated into the genetic background of pCC3WD21 as a *Bam*HI–*Bgl*II fragment (Fig. 1*a*) and *in vitro* transcripts were inoculated independently together with wt C1 and C2 to all three hosts. A mixture containing all three wt transcripts of CCMV was inoculated to serve as a positive control. Symptom phenotypes induced by the control inoculum and each MP mutant and their progeny analysis are summarized in Table 3. As anticipated, control inoculations induced characteristic necrotic local lesions in *C. quinoa*, while *N. benthamiana* plants showed no symptoms (Table 3). In cowpea, characteristic chlorotic mottling was evident 15 days p.i. for the wt control (Table 3). Northern and Western blot analysis of progeny recovered from each of these symptomatic leaves confirmed that the observed symptoms were due to CCMV infection (Table 3; Fig. 3).

Inoculation of each CCMV MP deletion mutant expressing unaltered CP to *N. benthamiana*, *C. quinoa* and cowpea revealed that each mutant showed distinct movement behaviour and symptom phenotypes in each of the three hosts. For example, in *C. quinoa*, mutants Δ10 and Δ43 were able to induce faint, necrotic local lesions (Table 3), suggesting that efficient cell-to-cell movement had occurred. However, the onset of lesions by mutant Δ10 was delayed by 1 week. Although no local lesions were induced by mutants Δ20, Δ33 and Δ60, even 30 days p.i., progeny analysis (Table 3; Fig. 3*a*, *b*) revealed that these mutants were nonetheless transported between cells, though not to an extent that induced visible local lesions. No progeny were recovered from primary leaves of any hosts inoculated with mutant Δ80 (Table 3; Fig. 3).

To test the effect of engineered MP deletions on long-distance spread, *N. benthamiana* and cowpea were inoculated independently with wt C1 and C2 and either wt C3 or the desired C3 MP mutant. Progeny analysis of mutants obtained from asymptomatic inoculated and non-inoculated systemic leaves of *N. benthamiana* revealed that mutants Δ10 and Δ20 were able to move locally with the same efficiency as did the wt control. However, their ability to move systemically was reduced, since progeny from non-inoculated systemic leaves could not be recovered until 30 days p.i., as opposed to 15 days p.i. for the wt control (Table 3; Fig. 3*c*, *d*). The spread of

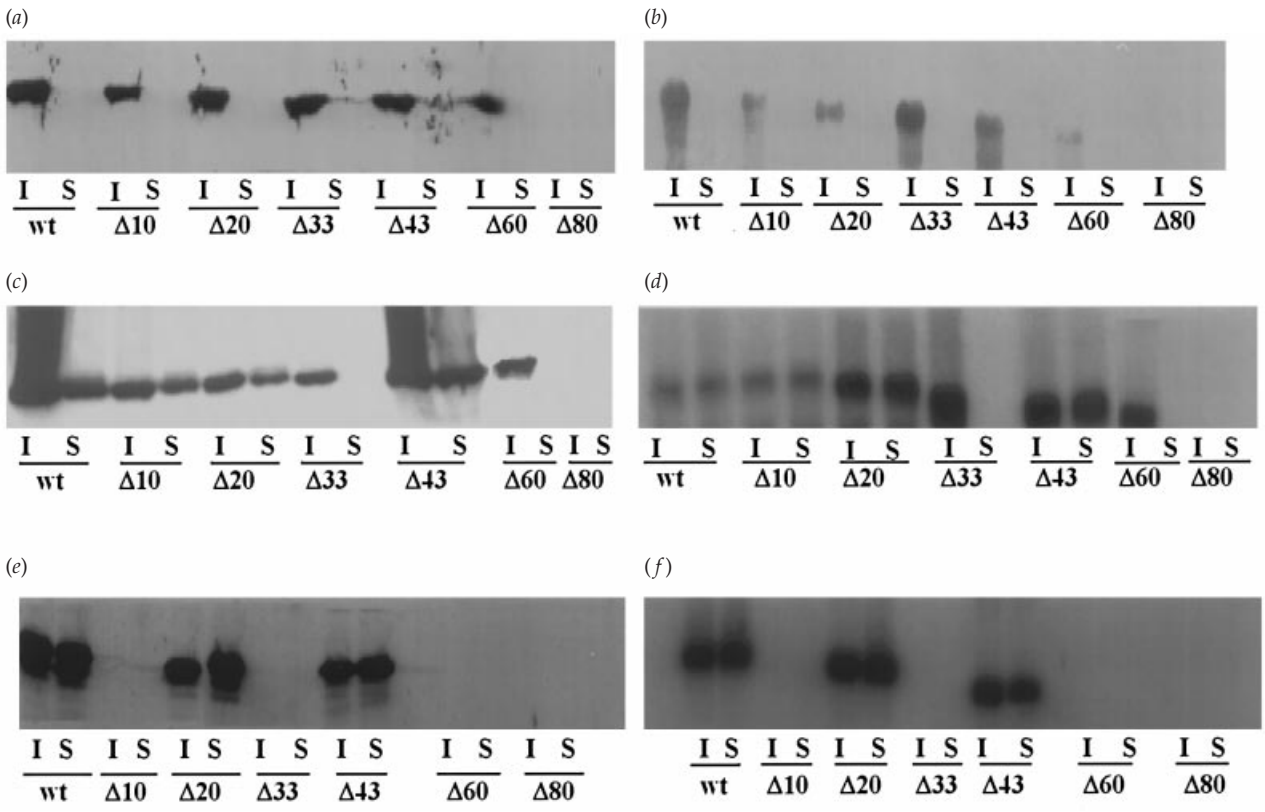

Fig. 3. Progeny analysis of CCMV MP deletion mutants in plants. Western (*a*, *c*, *e*) and Northern (*b*, *d*, *f*) blot analyses of CP accumulation are shown. CP and progeny RNA recovered following inoculation with wt C1 and wt C2 and either wt C3 or the indicated MP deletion mutant from either inoculated (I) or systemic (S) leaves of *C. quinoa* (*a*, *b*), *N. benthamiana* (*c*, *d*) and cowpea (*e*, *f*) were analysed. Protein samples were suspended in SDS–PAGE sample buffer, denatured by boiling for 5 min and subjected to 16% SDS–PAGE. After the proteins were transferred to a nitrocellulose membrane, the blots were probed with antibodies specific for CCMV CP and subjected to ECL detection. Northern blot hybridization was performed as described previously (Rao *et al.*, 1994). Blots were probed with riboprobes specific for CCMV MP sequences.

mutants Δ33 and Δ60 was restricted to inoculated leaves only and no progeny were recovered from non-inoculated systemic leaves, even 60 days p.i. (Table 3; Fig. 3*c*, *d*). No detectable movement was recorded for mutant Δ80 in either inoculated or non-inoculated systemic leaves (Table 3; Fig. 3*c*, *d*). For mutants Δ10, Δ20, Δ33 and Δ60, cDNA from progeny recovered from systemically infected leaves of *N. benthamiana* was sequenced and found to maintain the introduced deletions (data not shown).

In contrast to the observations made with *C. quinoa* and *N. benthamiana*, the behaviour of each MP mutant was distinct when tested in cowpea. For example, mutant Δ10, which was competent for local spread in *C. quinoa* and local as well as long-distance movement in *N. benthamiana*, failed even to move locally in cowpea (Table 3; Fig. 3*e*, *f*). By contrast, two other mutants, Δ20 and Δ43, were able to move from cell to cell and long distances in cowpea, although sufficient accumulation of CP (Fig. 3*e*) or progeny RNA (Fig. 3*f*) in non-inoculated systemic leaves for detection by Western or Northern blot analysis required at least 60 days (Table 3). Nonetheless, mutants Δ20 and Δ43 merely induced a mild systemic mosaic, while chlorotic mottling, characteristic of a wt CCMV infection, could not be observed (Table 3). Like mutant Δ10, three other mutants, Δ33, Δ60 and Δ80, were also non-infectious to cowpea (Fig. 3*e*, *f*).

Taken together, the above data indicate that the presence of CCMV CP can influence the movement process profoundly, since the defective movement exhibited by mutants Δ10/EGFP and Δ20/EGFP in *N. benthamiana* (Fig. 2; Table 2) could be restored by CP (Fig. 3*c*, *d*; Table 3). These observations further reinforce previous observations that bromovirus movement requires both MP and CP (Kasteel *et al.*, 1997; Rao & Grantham, 1995*b*; Schmitz & Rao, 1996). For mutants Δ33 and Δ60, however, CP could promote only cell-to-cell but not long-distance movement (Fig. 3*c*, *d*; Table 3). These observations suggest that movement is a complex process and is regulated by the interaction of viral genes with each other as well as with plant host factors. The differential long-distance movement observed in *N. benthamiana* and cowpea for each MP mutant could be due to varied interactions between MP and unknown host factors. It appears that *N. benthamiana* is more permissive to CCMV movement than is

cowpea (Table 2; Fig. 2). For example, 24 h after inoculation of C3/ΔCP-EGFP to *N. benthamiana*, infection foci encompassing > 100 cells could be observed, whereas the same inoculum applied to cowpea resulted in infection foci consisting of no more than 10 cells (Table 2). This reduced spread in cowpea could be attributed to the onset of a host defence response after initial spread (Mise & Ahlquist, 1995), especially in the absence of CP (Osman *et al*., 1997). Other possible explanations include the suggestion that in addition to MP, an unidentified host factor(s) is also involved in promoting the cell-to-cell movement of a given virus (Carrington *et al*., 1996; Deom *et al*., 1992). Thus, there could be a host factor(s) present in *N. benthamiana* that is absent in cowpea (Rao *et al*., 1998). Another possible reason for the susceptibility of *N. benthamiana* to several plant viruses is that the size exclusion limit of plasmodesmata is inherently larger than it is in other plants.

The mechanism of cell-to-cell movement in bromoviruses is not fully understood and appears to be complex. For example, experimental evidence suggests that BMV requires encapsidation-competent CP for efficient cell-to-cell movement (Rao & Grantham, 1995*b*; Rao, 1997). Kasteel *et al*. (1997) reported the presence of virus-like particles in tubular structures extending from protoplasts infected with BMV, implying that a tubule-guided mechanism is conceivable for BMV movement. This form of virus movement, however, needs to be verified in BMV-infected tissue. At present it is not known whether CCMV induces tubules in infected protoplasts or plants. Although MP and CP are essential for the overall movement of bromoviruses, replicase genes have also been shown to influence movement processes. Traynor *et al*. (1991) reported that selected BMV replicase mutants, capable of efficient replication in protoplasts, failed to support systemic infection in barley. Thus, the effective movement of bromoviruses requires a cooperative interaction between all three virus-encoded gene products and as yet unidentified host factors.

Another factor likely to influence virus movement is host resistance due to incompatible interactions between host components and virus gene products (Carrington *et al*., 1996; Osman *et al*., 1997). Several BMV variants defective for expression of functional CP failed to move from cell to cell, despite the fact that these variants produced wt MP. This defective movement could be due to the absence of a functional CP, which is required to overcome host defence (Rao & Grantham, 1995*b*, 1996).

We thank Paul Ahlquist for providing CCMV cDNA clones and pCC3 WD21, Mark Young for providing CCMV CP antiserum and George Grantham for excellent technical assistance and for helpful comments on the manuscript. Research was supported by a grant from Regents of the University of California.

References

Ahlquist, P. (1994). Bromoviruses. In *Encyclopedia of Virology*, vol. 1, pp. 181–185. Edited by R. G. Webster & A. Granoff. San Diego: Academic Press.

Allison, R. F., Janda, M. & Ahlquist, P. (1988). Infectious *in vitro* transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. *Journal of Virology* **62**, 3581–3588.

Allison, R. F., Thompson, C. & Ahlquist, P. (1990). Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection. *Proceedings of the National Academy of Sciences, USA* **87**, 1820–1824.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K. & Schaad, M. (1996). Cell-to-cell and long distance transport of viruses in plants. *Plant Cell* **8**, 1669–1681.

De Jong, W., Mise, K., Chu, A. & Ahlquist, P. (1997). Effects of coat protein mutations and reduced movement protein expression on infection spread by cowpea chlorotic mottle virus and its hybrid derivatives. *Virology* **232**, 167–173.

Deom, C. M., Lapidot, M. & Beachy, R. N. (1992). Plant virus movement proteins. *Cell* **69**, 221–224.

Dreher, T. W., Rao, A. L. N. & Hall, T. C. (1989). Replication *in vivo* of mutant brome mosaic virus RNAs defective in aminoacylation. *Journal of Molecular Biology* **206**, 425–438.

Fujita, Y., Mise, K., Okuno, T., Ahlquist, P. & Furusawa, I. (1996). A single codon change in a conserved motif of a bromovirus movement protein gene confers compatibility with a new host. *Virology* **223**, 283–291.

Fujita, Y., Mise, K., Kajiura, Y, Dohi, K. & Furusawa, I. (1998). Nucleic acid-binding properties and subcellular localization of the 3a protein of brome mosaic bromovirus. *Journal of General Virology* **79**, 1273–1280.

Jansen, K. A., Wolfs, C. J., Lohuis, H., Goldbach, R. W. & Verduin, B. J. (1998). Characterization of the brome mosaic virus movement protein expressed in *E. coli*. *Virology* **242**, 387–394.

Kasteel, D. T. J., van der Wel, N. N., Jansen, K. A. J., Goldbach, R. W. & van Lent, J. W. M. (1997). Tubule-forming capacity of the movement proteins of alfalfa mosaic virus and brome mosaic virus. *Journal of General Virology* **78**, 2089–2093.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Mise, K. & Ahlquist, P. (1995). Host-specificity restriction by bromovirus cell-to-cell movement protein occurs after initial cell-to-cell spread of infection in nonhost plants. *Virology* **206**, 276–286.

Mise, K., Allison, R. F., Janda, M. & Ahlquist, P. (1993). Bromovirus movement protein genes play a crucial role in host specificity. *Journal of Virology* **67**, 2815–2823.

Nagano, H., Okuno, T., Mise, K. & Furusawa, I. (1997). Deletion of the C-terminal 33 amino acids of cucumber mosaic virus movement protein enables a chimeric brome mosaic virus to move from cell to cell. *Journal of Virology* **71**, 2270–2276.

Oparka, K. J., Roberts, A. G., Prior, D. A. M., Chapman, S., Baulcombe, D. C. & Santa Cruz, S. (1995). Imaging the green fluorescent protein in plants – viruses carry the torch. *Protoplasma* **189**, 133–141.

Osman, F., Grantham, G. L. & Rao, A. L. N. (1997). Molecular studies on bromovirus capsid protein. IV. Coat protein exchanges between brome mosaic and cowpea chlorotic mottle viruses exhibit neutral effects in heterologous hosts. *Virology* **238**, 452–459.

Rao, A. L. N. (1997). Molecular studies on bromovirus capsid protein. III. Analysis of cell-to-cell movement competence of coat protein defective variants of cowpea chlorotic mottle virus. *Virology* **232**, 385–395.

Rao, A. L. N. & Grantham, G. L. (1995*a*). A spontaneous mutation in

the movement protein gene of brome mosaic virus modulates symptom phenotype in *Nicotiana benthamiana*. *Journal of Virology* **69**, 2689–2691.

Rao, A. L. N. & Grantham, G. L. (1995*b*). Biological significance of the seven amino-terminal basic residues of brome mosaic virus coat protein. *Virology* **211**, 42–52.

Rao, A. L. N. & Grantham, G. L. (1996). Molecular studies on bromovirus capsid protein. II. Functional analysis of the amino-terminal arginine-rich motif and its role in encapsidation, movement, and pathology. *Virology* **226**, 294–305.

Rao, A. L. N., Duggal, R., Lahser, F. & Hall, T. C. (1994). Analysis of RNA replication in plant viruses. In *Methods in Molecular Genetics*: *Molecular Virology Techniques*, vol. 4, pp. 216–236. Edited by K. W. Adolph. Orlando: Academic Press.

Rao, A. L. N., Cooper, B. & Deom, C. M (1998). Defective movement of viruses in the family *Bromoviridae* is differentially complemented in *Nicotiana benthamiana* expressing tobamovirus or dianthovirus movement proteins. *Phytopathology* **88**, 666–672.

Schmitz, I. & Rao, A. L. N. (1996). Molecular studies on bromovirus capsid protein. I. Characterization of cell-to-cell movement-defective RNA3 variants of brome mosaic virus. *Virology* **226**, 281–293.

Traynor, P., Young, B. M. & Ahlquist, P. (1991). Deletion analysis of brome mosaic virus 2a protein: effects on RNA replication and systemic spread. *Journal of Virology* **65**, 2807–2815.

Received 18 November 1998; Accepted 2 February 1999