Defective Movement of Viruses in the Family *Bromoviridae* Is Differentially Complemented in *Nicotiana benthamiana* Expressing Tobamovirus or Dianthovirus Movement Proteins

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ABSTRACT

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Taxonomically distinct tobacco mosaic tobamovirus (TMV), red clover necrotic mosaic dianthovirus (RCNMV), cucumber mosaic cucumovirus (CMV), brome mosaic bromovirus (BMV), and cowpea chlorotic mottle bromovirus (CCMV) exhibit differences in their host range. Each of these viruses encodes a functionally similar nonstructural movement protein (MP) that is essential for cell-to-cell movement of a progeny virus. Despite the lack of significant amino acid identity among the MPs of CMV, TMV, and RCNMV, movement-defective CMV (CMVFnyΔMP-ΔKPN) was able to move locally and systemically in transgenic *Nicotiana benthamiana* expressing either TMV MP (NB-TMV-MP(+)) or RCNMV MP (NB-RCNMV-MP(+)). These observa-

tions contrast with those of previous studies in which transgenic *N. tabacum* cv. Xanthi plants expressing TMV MP supported only the cell-to-cell movement of CMVFnyΔMP-ΔKPN. To verify whether similar complementation could be observed for movement-defective bromoviruses, NB-TMV-MP(+) and NB-RCNMV-MP(+) plants were inoculated independently with movement-defective variants of BMV (B3ΔMP) and CCMV (CC3ΔMP). Neither NB-TMV-MP(+) nor NB-RCNMV-MP(+) was able to rescue the defective cell-to-cell and long-distance movement of B3ΔMP. In contrast, NB-RCNMV-MP(+) complemented the cell-to-cell, but not the long-distance, movement of CC3ΔMP. Taken together, these studies suggest that virus movement is a complex process and that, in some cases, the host species plays a major role in determining the long-distance movement function of a virus.

Addition keywords: host factor, viral movement.

With few exceptions, plant viruses initially enter host cells during mechanical or biological (vector) damage to the cell wall and plasma membrane. Following entry, the establishment of a successful infection depends on the spread of a virus from the initial infection site to neighboring cells, a process known as cell-to-cell movement. With many viruses, this active process is often mediated by a viral-encoded movement protein(s) (MP) (11,21). In the absence of such cell-to-cell movement, the infection remains subliminal (8,28,36).

The type members of the cucumovirus group, cucumber mosaic virus (CMV), and the bromovirus group, brome mosaic virus (BMV), are genetically very similar and collectively classified in the family *Bromoviridae*. Virions of BMV and CMV exhibit icosahedral symmetry and contain tripartite, single-stranded, positive-sense RNA genomes (1,27). Genomic RNA1 and RNA2 of each virus are monocistronic and encode the nonstructural proteins 1a and 2a, respectively, which are required for viral replication (1,16, 27). Genomic RNA3 of CMV and BMV are dicistronic and encode the 3a protein, a 30-kDa nonstructural protein translated from the 5'-proximal open reading frame. The 3'-proximal gene, which encodes the coat protein (CP), is translated from subgenomic RNA4 generated in vivo from negative-sense RNA3 progeny (6,24). The 3a protein of each virus is functionally analogous to the tobacco mosaic tobamovirus (TMV) MP and is required for cell-to-cell movement (12,25,27,36).

Red clover necrotic mosaic dianthovirus (RCNMV) is an icosahedral virus containing a bipartite, single-stranded, positive-sense RNA genome (20). The largest RNA, RNA1, encodes a polymerase-associated protein and the CP, which is translated from a subgenomic RNA (20). The smaller monocistronic RNA, RNA2, encodes the MP (20). In contrast to BMV, CMV, and RCNMV, TMV is a rod-shaped virus containing a monopartite, single-stranded, positive-sense RNA genome. The genomic RNA is translated into two polymerase-associated proteins. The MP and CP are translated from two subgenomic RNAs (9).

Despite extensive variation in morphology, host range, and genome organization, many taxonomically distinct plant viruses exhibit complementary movement functions that may be a result of MP cross-compatibility (5). For example, the MPs of TMV and RCNMV are functionally homologous, since the cell-to-cell spread of movement-defective variants of TMV and RCNMV can be complemented in transgenic *Nicotiana benthamiana* plants expressing heterologous MP (15). Similarly, while examining the cross-compatibility of MPs of cowpea chlorotic mottle bromovirus (CCMV) and cowpea adapted sunn-hemp mosaic tobamovirus (SHMV), De Jong and Ahlquist (10) found that, when the MP of CCMV was replaced with that of SHMV, the hybrid was competent for systemic infection in cowpea. However, while examining the cross-compatibility between MPs of TMV and CMV, Cooper et al. (8) observed that transgenic *N. tabacum* cv. Xanthi (tobacco) plants expressing the TMV-MP gene supported cell-to-cell movement, but not the systemic movement, of a movement-defective CMV. In this paper, we show that the ability of the TMV and RCNMV MPs to complement long-distance movement of a movement-defective CMV is host dependent. We also show that the extent to which TMV and RCNMV MPs

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support movement of movement-defective mutants of BMV and CCMV is different.

MATERIALS AND METHODS

Construction of BMV and CCMV RNA3 mutants. A MP-defective clone of BMV RNA3 (B3), referred to as B3ΔMP (Fig. 1), was constructed by digesting pT7B3 with *Cla*I (position 601) (Fig. 1) followed by treatment with Bal 31 (35). The resulting digestion products were ligated and transformed. A MP-defective clone of CCMV RNA3 (CC3), referred to as CC3ΔMP, was constructed by deleting 720 bases (as a *Bgl*II fragment) (Fig. 1) from the MP gene sequence of pCC3TP4 (3).

Replication and encapsidation assays in protoplasts. Full-length cDNA clones were transcribed in vitro in the presence of 7-methylguanosine (5') triphospho (5') guanosine cap with T7 RNA polymerase using a MEGAscript T7 kit (Ambion Inc., Austin, TX). Isolation and transfection of barley (*Hordeum vulgare* cv. Dickson) protoplasts with wild-type (wt) and mutant RNAs and in vivo encapsidation assays were performed as described previously (29,32,33).

Plant material and inoculation. Plasmids pT7B1, pT7B2, and pT7B3, when linearized with *Bam*HI and transcribed in vitro with T7 RNA polymerase, collectively produce infectious transcripts corresponding to BMV RNA1 (B1), RNA2 (B2), and RNA3 (B3), respectively (13,26,31). Similarly, full-length cDNA clones corresponding to the three genomic RNAs of CCMV, pCC1TP1, pCC2TP2, and pCC3TP4, from which wt infectious RNA1 (CC1), RNA2 (CC2), and RNA3 (CC3), respectively, can be transcribed in vitro, have been described previously (3). Transgenic *N. benthamiana* plants expressing TMV MP and RCNMV MP (hereafter referred to as NB-TMV-MP(+) and NB-RCNMV-MP(+) plants, respectively) have been described previously (15,40). Transgenic or nontransgenic *N. benthamiana* plants were kept in the dark for at least 18 h prior to inoculation.

Infectious transcripts of wt CMV (CMV-Fny) were synthesized by transcribing cDNA clones of the genomic RNA. Plasmids pFny109, pFny209, and pFny309 were linearized with *Pst*I and transcribed with T7 RNA polymerase to yield infectious transcripts of CMV RNA1 (F1), RNA2 (F2), and RNA3 (F3), respectively (34). A mixture containing all three wt RNA transcripts (150 µg/ml) was used to inoculate *N. benthamiana*. CMVFnyΔMP-ΔKPN, a movement-defective RNA3 mutant of CMV-Fny (serogroup I) (18), was obtained from dried leaf tissue. Leaf homogenate prepared in 50 mM sodium phosphate buffer, pH 7.2, from either CMV-infected *N. benthamiana* plants or CMVFnyΔMP-ΔKPN-infected transgenic *N. tabacum* cv. Samsun plants expressing the CMV-Fny 3a protein (18) were used as sources of inocula. Thus, the source of inoculum for bromoviruses was in vitro transcripts, whereas for that of CMV, it was sap from infected plant tissue. Each experiment was repeated at least three times using four to six plants.

Progeny RNA analysis. BMV was purified from symptomatic leaves as described previously (29). CMV virions were purified from infected leaves using a micropurification procedure (30,32), except that the purification buffer contained 0.5 M sodium citrate, 0.01 M EDTA, and 0.5% thioglycolic acid. RNA was isolated from purified virions using sodium dodecyl sulfate-phenol (29). Total RNA was isolated by grinding a 2.5-cm-square leaf piece excised from either inoculated or uninoculated upper leaves of transgenic and nontransgenic plants according to Rao et al. (29). The RNAs were denatured with glyoxal, electrophoresed in 1% agarose gels, transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH), and hybridized with ³²P-labeled riboprobes of desired specificity (32). For synthesizing riboprobes complementary to the BMV CP region, plasmid pT7B3 (13) was digested with *Bgl*II and *Sac*I, and the resulting 261-base pair fragment was subcloned into the pT7T3 vector (Amersham Pharmacia

Biotech, Piscataway, NJ) to yield pT73/BCP. Riboprobes transcribed with T7 RNA polymerase from *Hind*III-linearized pT73/BCP are of negative-sense polarity and will detect B3 and B4 progeny positive-sense RNA. For synthesizing riboprobes specific for CCMV CP gene sequence, a polymerase chain reaction product encompassing the entire CCMV CP gene was amplified from pCCTP4 using a 5' oligonucleotide primer (dTACAGTCGGATCCGGGAAGTT; *Bam*HI site is underlined), and a 3' oligonucleotide primer (dAGCGGGCACGAATTCACCGGAGT; *Eco*RI site is underlined), and the resulting product was digested with *Bam*HI and *Eco*RI and subcloned into a similarly treated pT7T3 Lac Z (Amersham Pharmacia Biotech) to yield pT73CCCP. Riboprobes transcribed with T7 RNA polymerase from *Hind*III-linearized pT73CCCP are of negative-sense polarity and will detect CC3 and CC4 progeny positive-sense RNA. For synthesizing riboprobes complementary to the CMV CP region, pFny309 (a cDNA clone of CMV-Fny RNA3) (34) was digested with *Sac*I and *Hind*III, and the resulting 266-base pair product was subcloned into pT7T3 to yield pT73/FCP.

RESULTS

Complementation of CMVFnyΔMP-ΔKPN in NB-TMV-MP(+) plants. Previous studies demonstrated that transgenic *N. tabacum* cv. Xanthi expressing the TMV MP gene complemented cell-to-cell movement, but not long-distance spread, of CMVFnyΔMP-ΔKPN (7). To examine whether TMV MP expressed in transgenic *N. benthamiana* plants could complement local and systemic spread of CMVFnyΔMP-ΔKPN, NB-TMV-MP(+) plants were mechanically inoculated with CMVFnyΔMP-ΔKPN (18). Nontransformed *N. benthamiana* were inoculated to serve as a control. As expected, control plants inoculated with CMVFnyΔMP-ΔKPN did not develop symptoms throughout the examination period. However, NB-TMV-MP(+) plants inoculated with CMVFnyΔMP-ΔKPN displayed symptoms of infection on uninoculated upper leaves (Table 1). The time of appearance and characteristics of the symptoms were similar to those induced by wt CMV-Fny (Table 1). To confirm that the symptom phenotypes elicited on NB-TMV-MP(+) plants did not result from contamination by wt CMV-Fny, the following experiments were performed. (i) Total RNA was

Fig. 1. Characteristics of wild-type (wt) brome mosaic bromovirus (BMV) RNA3 (B3) and cowpea chlorotic mottle bromovirus (CCMV) RNA3 (CC3) and their movement-defective mutants. Schematic representation of alterations introduced into biologically active clones of B3 and CC3. The organization of B3 and CC3 is shown with noncoding sequences represented as single lines, BMV movement protein sequence (BMP) as open boxes, CCMV movement protein (CCMP) as cross-hatched boxes, BMV coat protein sequence (BCP) as stippled boxes, and CCMV coat protein (CCCP) as open boxes. The position of selected restriction sites in B3 and CC3 used to create desired deletions in movement protein genes are shown. The bent arrow represents the start of the coat protein-subgenomic RNA initiation site. Expected transcript sizes from wt and each RNA3 mutant are shown at right.

extracted from inoculated and symptomatic systemically infected leaves of each plant and analyzed by Northern hybridization with a radioactive riboprobe complementary to the CP gene of CMV-Fny. A representative example of a Northern blot is shown in Figure 2A. The RNA profile obtained from these Northern blots revealed that, in each transgenic plant inoculated with CMVFnyΔMP-ΔKPN, a RNA3 migrating faster than wt RNA3 was consistently observed, indicating that the introduced deletion in the MP gene of CMV RNA3 was retained (Fig. 2A). (ii) Extracts from symptomatic leaves of each transgenic plant were back-inoculated onto *Chenopodium quinoa* (a local lesion host for CMV), NB-TMV-MP(+), and nontransformed *N. benthamiana* plants. All back-

inoculated NB-TMV-MP(+) plants displayed symptoms, whereas neither *C. quinoa* nor nontransformed plants developed symptoms (Table 1), indicating that NB-TMV-MP(+) had fully complemented CMVFnyΔMP-ΔKPN.

Complementation of CMVFnyΔMP-ΔKPN in NB-RCNMV-MP(+) plants. CMV infects many *Nicotiana* spp. systemically (27). Although RCNMV causes localized infections in *N. tabacum*, it can systemically infect *N. clevelandii* and *N. benthamiana* (15). In *N. benthamiana*, RCNMV induces severe mosaic symptoms, but unlike plants infected with CMV, they are not stunted in their growth. Despite the lack of detectable homology between the MPs of TMV and RCNMV, transgenic *N. benthamiana* plants

TABLE 1. Complementation of cell-to-cell and long-distance movement of the movement-defective RNA3 mutant of wild-type (wt) cucumber mosaic virus (CMV) (CMVFnyΔMP-ΔKPN) in transgenic *Nicotiana benthamiana* plants expressing heterologous movement protein

Plant lines	CMV-Fny ^a		CMVFnyΔMP-ΔKPN ^b		Symptoms of progeny from ^c	
	Symptoms	Northern blot ^d I/S	Symptoms	Northern blot ^d I/S	NB-TMV-MP(+)	NB-RCNMV-MP(+)
1. <i>N. benthamiana</i>						
(a) Nontransformed	M/LD/S ^e	+/+	NI	-/-	NI	NI
(b) NB-TMV-MP(+)	M/LD/S	+/+	M/LD/S	+/+	M/LD/S	M/LD/S
(c) NB-RCNMV-MP(+)	M/LD/S	+/+	M/LD/S	+/+	M/LD/S	M/LD/S
2. <i>Chenopodium quinoa</i>	NLL	+/-	NI	-/-	NI	NI

^a wt CMV (CMV-Fny) inoculum was obtained from nontransgenic *N. benthamiana* previously infected with a mixture containing in vitro transcripts representing all three wt genomic RNAs.

^b CMVFnyΔMP-ΔKPN inoculum was obtained as dried leaves of *N. tabacum* cv. Samsun (18).

^c Symptomatic leaves of either transgenic *N. benthamiana* plants expressing tobacco mosaic tobamovirus (TMV) movement protein (MP) or red clover necrotic mosaic dianthovirus (RCNMV) MP (NB-TMV-MP(+) or NB-RCNMV-MP(+), respectively) infected with CMVFnyΔMP-ΔKPN were used for back inoculations onto indicated host plants.

^d CMV RNA3 and RNA4 were detected by Northern hybridization using CMV coat protein-specific riboprobes. I/S = inoculated leaves/systemic leaves. (+) indicates presence of CMV RNA3 and RNA4; and (-) indicates absence of CMV RNA3 and RNA4.

^e M = mosaic, LD = leaf distortion, S = stunting, NLL = necrotic local lesions, and NI = not infected.

Fig. 2. Progeny RNA analysis from transgenic and nontransformed *Nicotiana benthamiana* plants. **A**, Northern blot hybridization of total RNA isolated from inoculated or uninoculated symptomatic systemic leaves of nontransformed and transgenic *N. benthamiana* plants expressing either tobacco mosaic tobamovirus (TMV) movement protein (MP) or red clover necrotic mosaic dianthovirus (RCNMV) MP (NB-TMV-MP(+) or NB-RCNMV-MP(+), respectively) infected with movement-defective wild-type cucumber mosaic cucumovirus (CMV-Fny) (CMVFnyΔMP-ΔKPN). Northern blots were hybridized with ³²P-labeled negative-sense riboprobes specific for CMV-Fny coat protein (CP) gene. Lanes: Wt = total RNA isolated from symptomatic systemic (S) leaves of nontransformed *N. benthamiana* plants inoculated with CMV-Fny, NT = total RNA isolated from inoculated (I) and uninoculated symptomatic systemic (S) leaves of nontransformed *N. benthamiana* plants inoculated with CMVFnyΔMP-ΔKPN, T = total RNA isolated from inoculated (I) and uninoculated symptomatic systemic (S) leaves of NB-TMV-MP(+) infected with CMVFnyΔMP-ΔKPN, and R = total RNA isolated from inoculated (I) and uninoculated symptomatic systemic (S) leaves of NB-RCNMV-MP(+) infected with CMVFnyΔMP-ΔKPN. **B**, Northern blot analysis of virion RNA. Approximately 100 ng of viral RNA isolated from purified virions was analyzed as described above. Lanes: Wt = RNA isolated from purified virions obtained from nontransformed *N. benthamiana* infected with a mixture of all three wt RNA transcripts of CMV-Fny, T = RNA isolated from purified virions obtained from NB-TMV-MP(+) infected with CMVFnyΔMP-ΔKPN, and R = RNA isolated from purified virions obtained from NB-RCNMV-MP(+) infected with CMVFnyΔMP-ΔKPN. The positions of wt progeny RNA3, subgenomic RNA4, and RNA3ΔMP (i.e., CMVFnyΔMP-ΔKPN) detected by CMV CP-specific probes are shown on the left.

expressing either TMV MP or RCNMV MP are competent for complementing the movement-defective heterologous viruses (15). Therefore, we examined whether CMVFny Δ MP- Δ KPN could be complemented by RCNMV MP. Transgenic *N. benthamiana* plants expressing the RCNMV MP gene were inoculated with CMVFny Δ MP- Δ KPN as described above. While nontransformed control plants did not show signs of infection, NB-RCNMV-MP(+) plants displayed systemic infection and stunting, characteristic of CMV-Fny infection (Table 1). When total RNA isolated from inoculated and symptomatic systemic leaves was subjected to Northern blot analysis, similar to that described for NB-TMV-MP(+) plants, the presence of a faster migrating RNA3 (diagnostic of CMVFny Δ MP- Δ KPN) was clearly evident (Fig. 2A).

The yield of the virus purified from systemically infected leaves of NB-TMV-MP(+) and NB-RCNMV-MP(+) plants infected with CMVFny Δ MP- Δ KPN was similar to the levels obtained from CMV-Fny-infected nontransformed plants (data not shown). Northern blot analysis of RNA extracted from purified CMVFny Δ MP- Δ KPN virions (Fig. 2B) revealed a RNA profile identical to total RNA extracted from CMVFny Δ MP- Δ KPN-infected transgenic plants (Fig. 2A), confirming that systemic symptoms were elicited by virions containing the progeny of CMVFny Δ MP- Δ KPN. These results demonstrate that MPs of TMV and RCNMV, when expressed in transgenic *N. benthamiana* plants, fully complement cell-to-cell and long-distance spread of CMVFny Δ MP- Δ KPN.

Complementation of B3 Δ MP and CC3 Δ MP in NB-TMV-MP(+) and NB-RCNMV-MP(+) plants. The characteristics of

Fig. 3. Progeny RNA analysis from transgenic and nontransformed *Nicotiana benthamiana* plants. **A**, Northern hybridization of total RNA isolated from inoculated (I) or uninoculated systemic (S) leaves of nontransformed (NT) transgenic *N. benthamiana* plants expressing either tobacco mosaic tobamovirus (TMV) movement protein (MP) or red clover necrotic mosaic dianthovirus (RCNMV) MP (NB-TMV-MP(+) [T] or NB-RCNMV-MP(+) [R], respectively) infected with either wild-type (wt) brome mosaic bromovirus (BMV) RNA3 (B3) or movement-defective variants of BMV (B3 Δ MP) along with wt BMV RNA1 and RNA2. **B**, Northern hybridization of total RNA isolated from inoculated (I) or uninoculated systemic (S) leaves of nontransformed (NT) NB-TMV-MP(+) (T) or NB-RCNMV-MP(+) (R) plants infected with either cowpea chlorotic mottle bromovirus (CCMV) RNA3 (CC3) or movement-defective variants of CCMV (CC3 Δ MP) along with wt CCMV RNA1 and RNA2. Preparation of total RNA samples and conditions of electrophoresis are described in Figure 2. **A and B**, Blots were hybridized with riboprobes specific for the BMV coat protein (CP) and CCMV CP genes, respectively. **A**, The left panel (B3) was exposed for 12 h, whereas the right panel (B3 Δ MP) was exposed for 72 h. **B**, CC3 and CC3 Δ MP were exposed for 72 h. **A and B**, The position of wt B3 and CC3 and their respective subgenomic RNA are shown on the left. The position of B3 Δ MP, CC3 Δ MP, and their respective subgenomic RNA4 is shown on the right.

the B3ΔMP and CC3ΔMP used in this study are illustrated in Figure 1. Both mutants, B3ΔMP and CC3ΔMP, replicated efficiently in the presence of their respective wt RNA1 and RNA2 in barley protoplasts and synthesized proteins of expected sizes (analyzed according to Laemmli [19]; data not shown). Although the genome organization of bromoviruses is distinct from that of RCNMV, the type member of the dianthoviruses, some identity exists between RCNMV MP and amino acids 181 to 234 of bromovirus MP (3, 20). In contrast, direct sequence comparisons between bromovirus and tobamovirus MPs failed to reveal any identity (23,38). Despite the lack of identity between the MPs of SHMV and CCMV, hybrid CCMV RNA3 harboring the MP of SHMV was competent for establishing systemic infection in cowpea (10). Because of the cross-compatibility between bromovirus and tobamovirus MPs, the sequence identity conserved between the MPs of bromoviruses and RCNMV (3,20), and the fact that *N. benthamiana* is a common permissive host for members of tobamo-, bromo-, and dianthoviruses, we sought to examine whether the defective movement exhibited by B3ΔMP and CC3ΔMP could be complemented in NB-TMV-MP(+) and NB-RCNMV-MP(+) plants. Therefore, NB-TMV-MP(+) and NB-RCNMV-MP(+) plants were independently inoculated with either B1+B2+B3ΔMP or CC1+CC2+CC3ΔMP. As a control, nontransformed *N. benthamiana* plants were inoculated with the same inoculum.

Inoculation of nontransformed *N. benthamiana* with either wt BMV or wt CCMV inoculum resulted in symptomless infections, and progeny accumulated to detectable levels in inoculated and systemically infected leaves (Fig. 3, Table 2). Likewise, nontransformed *N. benthamiana*, NB-TMV-MP(+), and NB-RCNMV-MP(+) plants inoculated independently with either B1+B2+B3ΔMP or CC1+CC2+CC3ΔMP also remained symptomless throughout the examination period. To verify whether defective movement exhibited by B3ΔMP and CC3ΔMP was rescued by the MP transgenes in these symptomless plants, total progeny RNA was isolated from inoculated and uninoculated symptomless systemic leaves and subjected to Northern hybridization using riboprobes specific for CP genes of BMV and CCMV. The results are summarized in Figure 3 and Table 2.

In nontransformed control plants inoculated with B1+B2+B3ΔMP, progeny RNA3 corresponding to B3ΔMP RNA3 was not detected when 1 µg of total RNA was analyzed (data not shown). However, when 15 µg of total RNA was analyzed, progeny containing B3ΔMP of expected size was detected (perhaps recovered from the initially inoculated cells) after prolonged (72 h) autoradiographic exposures (Fig. 3A). In these plants, progeny RNA characteristic of BMV was not detected in the systemic leaves (Fig. 3A). Similarly, when progeny RNAs from B1+B2+B3ΔMP-inoculated NB-TMV-MP(+) and NB-RCNMV-MP(+) were analyzed, detection levels of progeny containing B3ΔMP in these plant lines paralleled those of nontransformed controls (Fig. 3A), suggesting that

the MPs of TMV and RCNMV were not able to complement the cell-to-cell movement of B3ΔMP. As observed for B3ΔMP, inoculation of CC3ΔMP to either nontransgenic *N. benthamiana* or NB-TMV-MP(+) resulted in subliminal infections, and signals corresponding to the progeny were weakly detected only after prolonged autoradiographic exposures (once again presumably due to detection of viral RNA in initially inoculated cells) (Fig. 3B, Table 2). Interestingly, inoculation of NB-RCNMV-MP(+) with CC3ΔMP resulted in efficient accumulation of progeny RNAs of expected electrophoretic mobilities in inoculated, but not in uninoculated, upper leaves (Fig. 3B).

DISCUSSION

The results of this study demonstrate that heterologous MPs of TMV and RCNMV, when expressed in *N. benthamiana*, can complement cell-to-cell and long-distance movement of a taxonomically distinct CMVFnyΔMP-ΔKPN. This contrasts with previous observations by Cooper et al. (8) showing that TMV MP failed to support the long-distance movement of CMVFnyΔMP-ΔKPN in transgenic *N. tabacum* cv. Xanthi. Taken together, the results show that the extent to which virus movement can be complemented by a heterologous MP is regulated by the host species in which the transgene is expressed. For CMV, in addition to MP, CP and viral replicase genes have been shown to participate in cell-to-cell and long-distance movement (6,14,37,39). Since the CP gene remained unaltered in CMVFnyΔMP-ΔKPN, the inability of CMVFnyΔMP-ΔKPN to move long distance in transgenic *N. tabacum* cv. Xanthi expressing the TMV MP gene is not due to a defective CP. Thus, the blockage of long-distance movement of CMVFnyΔMP-ΔKPN, after initial spread in inoculated leaves of transgenic *N. tabacum* cv. Xanthi expressing TMV MP, could be due to the absence of a host factor, or MP may not be required for entry into the vascular system of *N. benthamiana*. This suggestion is further supported by the complementation of CMVFnyΔMP-ΔKPN in transgenic *N. benthamiana* expressing the RCNMV MP gene (Fig. 2A, Table 1) and the lack of reciprocal complementation in *N. tabacum* cv. Samsun transgenic for CMV MP (18).

The reason why a MP would allow the long-distance movement of one virus in a particular host, but not another, may be found at the molecular level. It has been assumed that, in addition to MP, an unidentified host factor(s) is also involved in potentiating the cell-to-cell movement of progeny viruses (11). There could be a host factor(s) in *N. benthamiana* that is absent in *N. tabacum* cv. Xanthi that facilitates the function of the TMV or RCNMV MPs and that is required for long-distance movement. It has been suggested that another viral protein, in addition to the MP, is required to increase the plasmodesmatal size exclusion limit (SEL) between the bundle sheath/phloem parenchyma cell barrier for entry of the virus into the vasculature (12). In this scenario, long-dis-

TABLE 2. Analysis of movement characteristics exhibited by B3ΔMP or CC3ΔMP in NB-TMV-MP(+) and NB-RCNMV-MP(+) plants^a

Plant lines	wt BMV ^b		wt CCMV ^b		B3ΔMP		CC3ΔMP	
	Symptoms ^c	Northern blot ^d I/S	Symptoms ^c	Northern blot ^d I/S	Symptoms ^c	Northern blot ^d I/S	Symptoms ^c	Northern blot ^d I/S
1. <i>N. benthamiana</i>								
NB-TMV-MP(+)	NT	NT	NT	NT	None	+/-	None	+/-
NB-RCNMV-MP(+)	NT	NT	NT	NT	None	+/-	None	+/-
Nontransformed	None	+/+	None	+/+	None	+/-	None	+/-
2. <i>Chenopodium quinoa</i>	CLL/SM	+/+	NLL	+/-	None	-/-	None	-/-

^a B3ΔMP = movement-defective variants of brome mosaic bromovirus (BMV); CC3ΔMP = movement-defective variants of cowpea chlorotic mottle bromovirus (CCMV); and NB-TMV-MP(+) and NB-RCNMV-MP(+) = transgenic *Nicotiana benthamiana* plants expressing tobacco mosaic tobamovirus (TMV) movement protein (MP) or red clover necrotic mosaic dianthovirus (RCNMV) MP, respectively.

^b Wild-type (wt) BMV and CCMV inoculum contained a mixture of all three wt transcripts at 150 µg/ml.

^c NT = not tested, none = no visible symptoms, CLL/SM = chlorotic local lesions/systemic mottling, and NLL = necrotic local lesions.

^d Either BMV RNA3 or RNA4 or CCMV RNA3 or RNA4 were detected by Northern hybridization using coat protein-specific riboprobes. I/S = inoculated leaves/systemic leaves; (+) indicates presence of RNA3 and RNA4 of wt, B3ΔMP, or CC3ΔMP; and (-) indicates absence of RNA3 and RNA4 of wt RNA3, B3ΔMP, or CC3ΔMP.

tance movement of a movement-defective CMV genome would require a compatible interaction between the second viral component (perhaps CP or replicase genes), the heterologous MPs, and the host factor.

Another possibility is that a host factor in *N. tabacum* could interfere with the interaction between the TMV or RCNMV MPs and the CMV genome and restrict long-distance movement. The necessity for proper viral protein/host interactions is brought to light by studies on the modification of plasmodesmatal SEL by viral MPs and the binding of TMV to plant cell cytoskeleton microtubules (17,21,22). Interference of proper interaction between viral components and host proteins during plasmodesmata dilation or microtubule binding within cells that compose barriers to vascular entry may result in restricted long-distance movement (7).

It is well established that in *N. tabacum*, following TMV infection, the MP increases the plasmodesmatal SEL, permitting cell-to-cell movement of progeny virus (21). Currently, the plasmodesmatal SEL in *N. benthamiana* is not known. It is possible that the plasmodesmatal SEL in *N. benthamiana* at the bundle sheath/phloem parenchyma cell barrier is inherently higher than that in *N. tabacum*. This perhaps would explain why *N. benthamiana* is susceptible to a heterologous MP-mediated systemic infection by CMVFnyΔMP-ΔKPN and also to monocot adapted viruses such as BMV.

In contrast to full complementation of CMVFnyΔMP-ΔKPN by NB-TMV-MP(+) and NB-RCNMV-MP(+) plants, similar analysis of B3ΔMP and CC3ΔMP revealed that the movement of bromoviruses is distinct from that of the genetically related cucumoviruses. We envisioned that at least RCNMV MP, if not the TMV MP, would support the cell-to-cell movement of both B3ΔMP and CC3ΔMP because of previously noted similarities between the MP genes of bromoviruses and dianthoviruses (3,20). However, RCNMV MP complemented cell-to-cell movement of movement-defective progeny containing CC3ΔMP, but not B3ΔMP (Fig. 3). These observations further accentuate previous conclusions that the requirements for the movement of BMV are distinct from those of CCMV (28). Although a similarity exists between the MP genes of RCNMV and amino acids 181 to 234 of the BMV and CCMV MPs (2,20), the preceding 70 to 75 residues of the CCMV MP, but not BMV MP, show stronger similarity to the corresponding region of the RCNMV MP. Therefore, it is likely that, because of this extended similarity, RCNMV MP complemented the cell-to-cell movement of CC3ΔMP.

Unlike CMV, another limiting factor for efficient bromovirus movement is the *cis* requirement of both MP and CP. For example, coinoculation of two RNA3 mutants of either BMV or CCMV, each defective in either MP or CP, failed to complement and induce an infection; infection occurred only when a functional wt RNA3 was regenerated by recombination (4,36). These observations accentuate that, in bromoviruses, movement between cells will occur only when both MP and CP gene sequences are present in *cis*. This, however, needs to be demonstrated experimentally.

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