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Tobamovirus and Dianthovirus Movement Proteins Are Functionally Homologous

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The movement proteins (MPs) of tobacco mosaic tobamovirus (TMV) and red clover necrotic mosaic dianthovirus (RCNMV) enlarge plasmodesmata size exclusion limits, transport RNA from cell to cell, and bind nucleic acids *in vitro*. Despite these functional similarities, they have no sequence homology. However, they do appear to have similar secondary structures. We have used transgenic plants expressing either the TMV MP or the RCNMV MP, and a chimeric TMV that encodes the RCNMV MP as its only functional MP gene, to demonstrate that the MPs of TMV and RCNMV are functionally homologous. Further, both TMV and RCNMV can act as helper viruses to allow the cell-to-cell movement of the heterologous movement-defective viruses. These data support the conclusion that, despite other differences, such as particle morphology, host range, and sequence, TMV and RCNMV share a common mechanism for cell-to-cell movement.

INTRODUCTION

The initial entry and replication of a plant virus in a susceptible host are followed by movement of progeny virus into adjacent uninfected cells, an active process that is necessary for spread of infection. Movement of virus progeny from cell to cell occurs through plasmodesmata, membrane-lined channels that traverse the plant cell wall, and requires virus-encoded movement proteins (MPs) (reviewed by Hull, 1989; Atabekov and Taliansky, 1990; Maule, 1991; Deom *et al.*, 1992). Although MPs have been identified in a number of different RNA and DNA plant viruses, the mechanisms involved in cell-to-cell movement are still largely unknown. Several functions have been attributed to viral MPs, most notably those encoded by the single-stranded, positive-sense RNA viruses, tobacco mosaic *tobamovirus* (TMV) and red clover necrotic mosaic *dianthovirus* (RCNMV). Both the 30-kDa TMV MP and the 35-kDa RCNMV MP have been shown to increase plasmodesmatal size exclusion limits, to transport RNA from cell to cell through plasmodesmata, and to bind single-stranded nucleic acids *in vitro* (Wolf *et al.*, 1989; Citovsky *et al.*, 1990, 1992; Osman *et al.*, 1992; Giesman-Cookmeyer and Lommel, 1993; Fujiwara *et al.*, 1993). These two MPs appear to function in a similar manner, but they share little sequence homology. However, secondary structural analysis suggests that they do share several structural features, including a turn domain, flanked by two hydrophobic regions, near the amino-termini, and a hydrophilic domain at the carboxy-termini (Kendall and Lommel, 1992).

At this time, there appear to be at least two distinct mechanisms for cell-to-cell movement: one that is dependent on the viral capsid protein (CP) and one that is not (Maule, 1991; Deom *et al.*, 1992). Though these two mechanisms appear to possess distinct features, the ability of distantly related viruses to complement one another's cell-to-cell movement function in nonhost plants suggests that their MPs may also share certain mechanistic features, such as interacting with host proteins and/or viral nucleic acids (Atabekov and Taliansky, 1990). Neither TMV nor RCNMV requires CP for cell-to-cell movement, but these two viruses do differ in terms of particle morphology, host range, genetic organization, and gene expression strategies.

The RCNMV genome is bipartite, composed of 3.9- and 1.5-kb RNAs. The larger of the two RNAs, RNA-1, encodes the viral polymerase and capsid proteins and replicates independently in protoplasts (Osman and Buck, 1987; Paje-Manalo and Lommel, 1989). The smaller RNA, RNA-2, is monocistronic and encodes the viral MP. TMV, in contrast, has a single RNA genome of approximately 6.4 kb that encodes the polymerase, movement, and capsid proteins (Fig. 1).

We were interested in determining whether the MPs of TMV and RCNMV are functionally homologous, despite their lack of sequence homology. We have used several approaches, including complementation of movement-defective viruses by MP genes expressed in transgenic plants, the creation of a chimeric virus in which the TMV MP gene is replaced by the RCNMV MP gene, and, finally, helper virus complementation of movement-defective viruses. In all of these experiments, the MPs of TMV and RCNMV were able to provide cell-to-cell movement function to the heterologous (but movement-defective)

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virus, demonstrating their functional equivalence. These data support the findings of secondary structural analyses (Kendall and Lommel, 1992) and also suggest that despite other differences, such as particle morphology and host range, TMV and RCNMV share a common mechanism for cell-to-cell movement.

MATERIALS AND METHODS

Plant transformations

The TMV and RCNMV MP genes were introduced into *Nicotiana benthamiana* plants by *Agrobacterium tumefaciens*-mediated gene transfer as previously described (Horsch *et al.*, 1985; Deom *et al.*, 1987). Transgenic plants expressing the MP genes were selfed and the resulting R_2 seeds were germinated and used in all experiments. For transgenic plants expressing the TMV MP gene, TMV MP(+) plants, expression levels were determined by Western blot analysis (see below).

RNA and virus inoculations

TMV-U1 (common strain) and U3/12MPfs, a mutant strain of TMV that expresses a dysfunctional MP (Holt and Beachy, 1991), were propagated in *Nicotiana tabacum* cv. Xanthi and TMV MP(+) *N. tabacum* cv. Xanthi, respectively (Deom *et al.*, 1994). Tobamoviruses were purified according to Gooding and Hiebert (1967). U3/12MPfs, hereafter designated TMV-MPfs, expresses a truncated MP 62 amino acids in length and fails to move from cell to cell (Holt and Beachy, 1991). Where indicated, TMV or TMV-MPfs were diluted to 20 μg/ml in 20 m*M* sodium phosphate buffer, pH 7.0, and inoculated onto Carborundum-dusted leaves (Takahashi, 1956). Infectious transcripts from full-length TMV or chimeric TMV cDNA clones were synthesized *in vitro* as described (Deom *et al.*, 1994).

RCNMV-infected tissue was ground in a mortar and pestle in 20 m*M* sodium phosphate buffer, pH 7.0, containing Celite and used as inoculum either alone or in the presence of 20 μg/ml of TMV-MPfs. RCNMV RNA-1 was transcribed from a *Sma*I linearized template as previously described (Xiong and Lommel, 1991; Giesman-Cookmeyer and Lommel, 1993). RNA from a 300-μl reaction mix was diluted in GKP buffer (50 m*M* glycine, 30 m*M* K_2HPO_4, pH 9.2, 1% Bentonite, 1% Celite) and used to inoculate a total of four leaves on two *N. benthamiana* or *N. tabacum* cv. Xanthi plants.

Northern blot analyses

Total RNA from infected tissue was prepared for Northern blot analysis as described previously (Giesman-Cookmeyer and Lommel, 1993). Blots were hybridized with DNA fragments nick translated in the presence of [α-^{32}P]dCTP (Maniatis *et al.*, 1982). To detect the presence of the TMV MP gene, a 0.6-kb *Hin*dIII–*Cla*I fragment or a 0.3-kb *Bgl*II–*Pvu*II fragment was isolated from a clone of the 3′ half of TMV (pT3NA; Deom *et al.*, 1994). Similarly, a 0.5-kb *Cla*I–*Sac*I fragment was isolated from pT3NA and used to detect the TMV CP gene. For RCNMV, a 0.9-kb *Nco*I–*Xba*I fragment isolated from pRC2IG-5′Nco (Giesman-Cookmeyer and Lommel, 1993) was used to detect the RCNMV MP gene and a 1.4-kb *Bam*HI fragment from pRC1IG (Xiong and Lommel, 1991) was used to detect the RCNMV CP gene. Before electrophoresis, RNAs were denatured for 1 min at 65° in RNA buffer (0.5 ml deionized formamide, 0.17 ml 37% formaldehyde, 0.5 ml 8 *M* urea, 0.15 ml 0.5 *M* EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol). Agarose gels (1%) were subjected to electrophoresis at 40 mA for 1.5 hr. Gels were blotted onto nylon membranes (MSI, Westboro, MA) in 20× SSC overnight and dried *in vacuo* for 2 hr at 80°.

Western blot analyses

Fully expanded leaf tissue (200 μg fresh weight) was ground in 1.5-ml microfuge tubes with fitted pestles in 2 vol of ice-cold grinding buffer (100 m*M* Tris–HCl, pH 8.0, 10 m*M* EDTA, pH 8.0, 5 m*M* DTT). The homogenates were centrifuged at 15,000 *g* for 5 min to obtain a crude cell-wall pellet. The crude cell-wall fraction was suspended in 2 vol of sample buffer (Laemmli, 1970), boiled for 3 min, and centrifuged at 15,000 *g* for 5 min. The supernatant was analyzed for TMV MP. For detection of TMV CP in transgenic *N. benthamiana* plants expressing the RCNMV-MP and inoculated with TMV-MPfs or TMV, leaf tissue (200–300 μg fresh weight) was ground in 1 vol of sample buffer and the homogenates were centrifuged at 10,000 *g* for 5 min. Supernatants were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis in 12.5% gels and blotted onto nitrocellulose (Deom *et al.*, 1987). The TMV MP, TMV CP, and RCNMV MP were detected using rabbit anti-TMV MP, rabbit anti-TMV CP, or rabbit anti-RCNMV MP polyclonal antisera, respectively. Goat anti-rabbit polyclonal antiserum, conjugated with alkaline phosphatase, was used as the secondary antibody for visualization (Promega, Madison, WI, and Sigma Immunochemicals, St. Louis, MO).

Generation of a chimeric TMV containing the RCNMV MP gene

A chimeric TMV was created by substituting the RCNMV MP gene for a portion of the TMV MP gene. The RCNMV MP gene plus 200 nucleotides of the 3′ untranslated region was excised from pRC2IG-5′Nco by digestion with *Nco*I. The 3′ recessed ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I and deoxynucleotides (Maniatis *et al.*, 1982), and the DNA fragment was ligated into the *Eco*RV

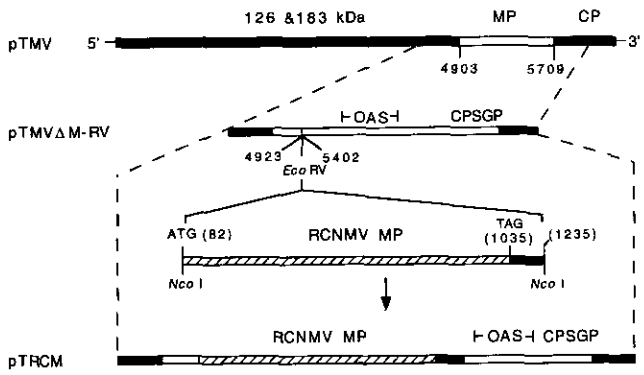

FIG. 1. Schematic diagram showing construction of pTRCM. The full-length cDNA of the TMV genomic RNA, pTMV, and derivatives are under the transcriptional control of the T7 promoter. pTMVΔM-RV has been described (Gafny *et al.*, 1992) and is discussed under Materials and Methods. The modified MP gene of pTMVΔM-RV is shown. pTRCM was constructed by inserting the RCNMV MP gene cDNA, plus 3′ flanking sequences, into the *Eco*RV site in pTMVΔM-RV. Locations of the OAS, origin of assembly site, and the coat protein subgenomic promoter, CPSGP, are indicated. Numbers below the diagrams designate wild-type TMV nucleotide positions, while numbers above the diagrams indicate RCNMV RNA-2 nucleotide positions. Black boxes represent TMV sequences, open boxes represent TMV MP gene sequences, open hatched boxes represent the RCNMV MP gene, and stippled boxes represent RCNMV untranslated nucleotide sequences that flank the 3′ terminus of the RCNMV MP gene.

site of pTMVΔM-RV to create pTRCM (Fig. 1). pTMVΔM-RV is a TMV expression vector that has most of the 5′ half of the MP gene sequence deleted (nucleotides 4924–5401), an *Eco*RV cloning site, and a modified start codon (ATG → ACG) (Fig. 1). Transcripts from pTMVΔM-RV replicate only in initially infected cells and fail to move from cell to cell (Gafny *et al.*, 1992).

RESULTS

Generation and characterization of transgenic *N. benthamiana* plants expressing the TMV MP

Transgenic *N. benthamiana* plants expressing the TMV MP gene, under the transcriptional control of the cauliflower mosaic virus 35S promoter, were regenerated via *Agrobacterium*-mediated leaf disc transformation (Horsch *et al.*, 1985; Deom *et al.*, 1987). Nine transgenic plant lines (R_2 seedlings) were analyzed by Western blot analysis and found to express the TMV MP gene (data not shown). Transgenic plant lines Nb11 and Nb15 expressed high levels of TMV MP, relative to the other seven lines, and were chosen for further study. The segregation ratio of R1 seedlings from line Nb11 was 15 [MP(+)]:1 [MP(−)] and from line Nb15 was 3 [MP(+)]:1 [MP(−)], indicating that the MP gene was expressed from either two loci (Nb11) or a single locus (Nb15). Western blot analysis indicated that the MP synthesized in transgenic TMV MP(+) *N. benthamiana* plants comigrated with the TMV MP produced in transgenic TMV MP(+) tobacco (*N. tabacum* cv. Xanthi line 277; Deom *et al.*, 1987), as well as MP produced in untransformed tobacco and *N. benthamiana* plants infected with TMV (data not shown).

Experiments were undertaken to determine if transgenic TMV MP(+) Nb15 plants would complement a TMV MP mutant, TMV-MPfs, which contains a frameshift mutation in the MP gene. TMV-MPfs is defective in cell-to-cell movement, but replicates as well as wild-type TMV in infected tobacco leaves (Holt and Beachy, 1991). Similar frameshift mutants in the MP gene of TMV-L have been shown to replicate normally in tobacco protoplasts (Meshi *et al.*, 1987). Seedlings of untransformed *N. benthamiana* and transgenic TMV MP(+) Nb15 were inoculated with TMV or TMV-MPfs. Disease induced by TMV in *N. benthamiana* plants and transgenic TMV MP(+) Nb15 plants was identical both in the rate at which it developed and in symptom severity. Typical systemic symptoms, vein clearing and leaf yellowing, appeared at 4 days postinoculation (dpi) and were severe by 6 to 9 dpi. *N. benthamiana* plants infected with TMV exhibited severe stunting and developed stem necrosis, followed by wilting and death, by 12 to 14 dpi (Fig. 2, plant D). Similarly, systemic symptoms appeared at 4 dpi on transgenic TMV MP(+) Nb15 plants inoculated with TMV-MPfs and were severe at 10 to 14 dpi. Transgenic TMV MP(+) Nb15 plants inoculated with TMV-MPfs were severely stunted and eventually developed stem necrosis, followed by wilting and death at 18 to 21 dpi (Fig. 2, plant E). As expected, no disease symptoms were observed in mock-inoculated *N. benthamiana* (Fig. 2, plant A), in mock-inoculated Nb15 plants (Fig. 2, plant C), in untransformed *N. benthamiana* inoculated with TMV-MPfs (Fig. 2, plant B), or in Nb15 plants that segregated MP(−) inoculated with TMV-MPfs (data not shown). Also, no virus was detected in extracts from inoculated leaves either by Western blot analysis for TMV CP or by infectivity assays on hypersensitive tobacco (*N. tabacum* cv. Xanthi NN) (data not shown). To confirm that disease symptoms observed on transgenic TMV MP(+) Nb15 plants inoculated with TMV-MPfs did not result from contamination with wild-type TMV, extracts from inoculated and uninoculated infected leaves were inoculated onto two hypersensitive tobacco lines, *N. tabacum* cv. Xanthi NN and transgenic TMV MP(+) *N. tabacum* cv. Xanthi NN (Deom *et al.*, 1991). Transgenic TMV MP(+) hypersensitive tobacco expresses functional TMV MP and complements TMV strains lacking functional MP genes. Lesions developed by 2 dpi on transgenic TMV MP(+) hypersensitive tobacco plants, but failed to develop on untransformed hypersensitive tobacco (data not shown), indicating that the MP gene expressed in transgenic TMV MP(+) Nb15 plants complemented the nonfunctional MP gene in TMV-MPfs and that neither contamination by wild-type virus nor recombination with the transgene had occurred.

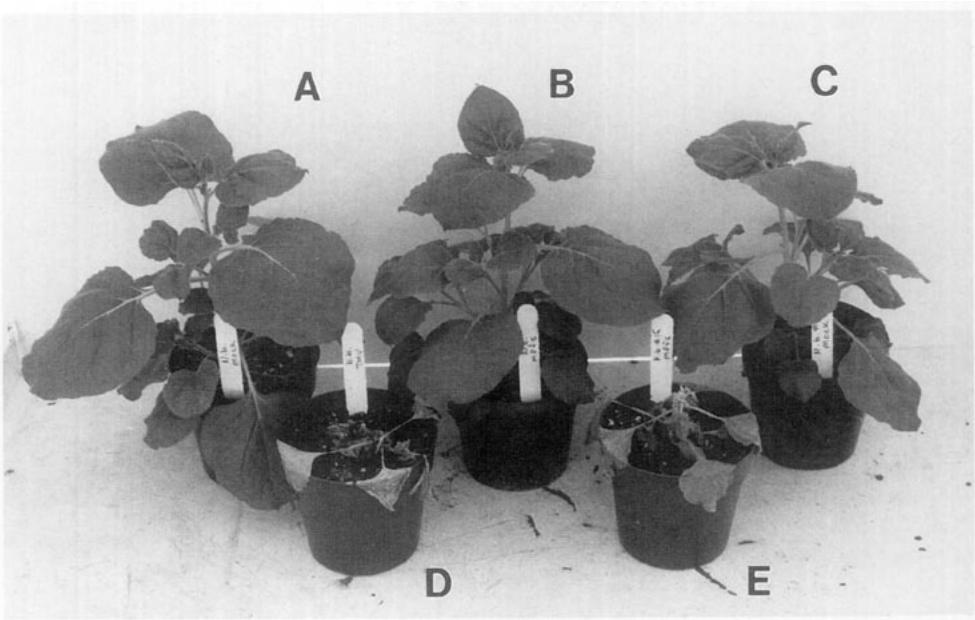

FIG. 2. Transgenic TMV MP(+) *N. benthamiana* plants express functional MP. Transgenic TMV MP(+) and untransformed *N. benthamiana* plants inoculated with either TMV or TMV-MPfs are shown at 21 dpi. (A) Mock-inoculated, untransformed plant; (B) TMV-MPfs-inoculated, untransformed plant; (C) mock-inoculated TMV MP(+) plant; (D) TMV-inoculated untransformed plant; (E) TMV-MPfs-inoculated TMV MP(+) plant.

Complementation of cell-to-cell movement of RCNMV RNA-1 by transgenic *N. benthamiana* plant lines expressing the TMV MP gene

To determine whether the TMV MP could functionally substitute for the RCNMV MP, RCNMV RNA-1 was mechanically inoculated onto transgenic TMV MP(+) Nb11 and Nb15 plants, as well as untransformed *N. benthamiana* plants, and observed for the appearance of symptoms on the inoculated and uninoculated leaves. As a positive control, wild-type RCNMV (RNA-1 and RNA-2) was also inoculated onto all three plant lines. RCNMV-1 is capable of independent replication in protoplasts, but in plants cannot move out of the initially inoculated cell and cause an infection in the absence of RNA-2 and the RCNMV MP (Osman and Buck, 1987; Paje-Manalo and Lommel, 1989). The wild-type RCNMV induced ringspots on inoculated leaves after 4 dpi and necrosis and mosaic symptoms on systemic leaves by 6 to 7 dpi. No difference in the rate or severity of symptoms was observed on the transgenic plant lines when compared to the untransformed *N. benthamiana* plants inoculated with RCNMV (data not shown). On transgenic TMV MP(+) Nb11 and Nb15 plants inoculated with RCNMV RNA-1, symptoms were observed on the inoculated leaves by 10 dpi, but were never observed on the uninoculated leaves. The symptoms, three to five slowly expanding ringspots per leaf, were delayed in the rate of appearance and were less severe than those induced by wild-type RCNMV. No symptoms were observed on the untransformed *N. benthamiana* plants. Total RNA was prepared from the inoculated and uninoculated leaves of the infected plants and analyzed by Northern blot analysis for the presence of RCNMV RNA-1 and RNA-2. As can be seen in Fig. 3, RNA-1 was detected in the inoculated leaves from transgenic TMV MP(+) Nb11 and Nb15 plants inoculated with RCNMV RNA-1, but was not detected in the inoculated leaves of untransformed *N. benthamiana* plants. The amount of RNA-1 detected in the transgenic plants inoculated with RNA-1 is approximately 10-fold lower than in plants infected with the wild-type virus (Fig. 3). RNA-1 was not detected in the uninoculated

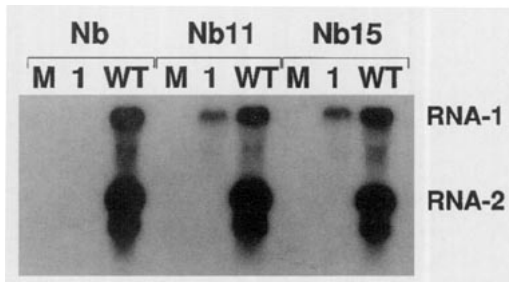

FIG. 3. TMV MP(+) *N. benthamiana* plants facilitate cell-to-cell movement of RCNMV RNA-1. A Northern blot analysis of total RNAs extracted from transgenic TMV MP(+) or untransformed *N. benthamiana* plants inoculated with either RCNMV RNA-1 or wild-type RCNMV (RNAs 1 and 2) is shown. Lanes marked M, mock-inoculated; lanes marked 1, inoculated with RCNMV RNA-1; lanes marked WT, RCNMV-inoculated. The first three lanes contain RNAs isolated from the inoculated leaves of untransformed *N. benthamiana* plants. The middle group of three lanes contain RNAs isolated from the inoculated leaves of transgenic TMV MP(+) Nb11 plants. The three rightmost lanes contain RNAs isolated from the inoculated leaves of transgenic TMV MP(+) Nb15 plants. Total RNA (50 μg) was loaded onto each lane of a 1% agarose gel, except for lanes marked WT, in which only 10 μg was loaded. After being subjected to electrophoresis, the RNAs were transferred to nylon membrane. The blot was probed with the RCNMV CP and MP gene sequences to detect RCNMV RNAs 1 and 2, respectively.

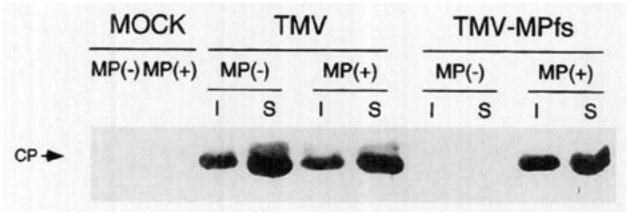

FIG. 4. Transgenic RCNMV MP(+) plants complement the cell-to-cell movement defect of TMV-MPfs. A Western blot analysis of tissue extracted from RCNMV MP(+) and untransformed *N. benthamiana* plants inoculated with either TMV (20 μg/ml) or TMV-MPfs (20 μg/ml) is shown. Homogenates from 50 μg of fresh-weight leaf tissue were centrifuged at 10,000 *g* for 5 min. The supernatants were subjected to SDS–polyacrylamide gel electrophoresis in a 12.5% gel and blotted onto nylon membrane. The blot was probed with polyclonal antiserum against the TMV CP. Lanes marked I, tissue from inoculated leaves; lanes marked S, tissue from uninoculated leaves. Lanes containing tissue from untransformed plants are labeled MP(−) and from transgenic plants expressing the RCNMV MP gene are labeled MP(+). Inoculum used is indicated above the lanes, mock, TMV, or TMV-MPfs.

leaves of any of the three plant lines inoculated with RNA-1 alone (data not shown). This suggests that the TMV MP is able to provide the cell-to-cell movement function for RCNMV RNA-1 *in trans*, but does not facilitate long-distance movement into uninoculated leaves.

Complementation of cell-to-cell movement of TMV-MPfs by transgenic *N. benthamiana* plant lines expressing the RCNMV MP gene

N. benthamiana plant lines expressing the RCNMV MP gene were generated by *Agrobacterium*-mediated transformation and shown to facilitate the cell-to-cell movement of RCNMV RNA-1 (Vaewhongs and Lommel, 1995). However, the transformed lines failed to facilitate long-distance movement of RCNMV RNA-1.

Transgenic *N. benthamiana* plants expressing the RCNMV MP, designated pGA482-35K (RCNMV MP(+) plants), as well as control RCNMV MP(−) plants, were inoculated with TMV-MPfs to determine whether the RCNMV MP was able to functionally complement the defective TMV MP gene. Wild-type TMV was also inoculated onto untransformed and transgenic RCNMV MP(+) *N. benthamiana* plants as a positive control. Symptoms appeared on plants inoculated with the wild-type TMV as described above, and no differences in disease symptoms were observed between the transgenic and untransformed plant lines. TMV-MPfs-induced symptoms on transgenic RCNMV MP(+) plants were essentially identical to those induced by wild-type TMV on either transgenic RCNMV MP(+) or MP(−) plants. Symptoms were observed on the inoculated leaves at 2 dpi and on the uninoculated leaves at 4 dpi. No symptoms were detected on untransformed plants. The presence of the TMV CP in inoculated and uninoculated leaves of transgenic RCNMV MP(+) plants inoculated with TMV-MPfs was confirmed by Western blot analysis (Fig. 4). As expected, no TMV CP was detected in leaf tissue from untransformed plants inoculated with TMV-MPfs. These data indicate that the RCNMV MP is able to provide the cell-to-cell movement function *in trans* for the movement-defective TMV-MPfs and that the RCNMV MP does not interfere with the encapsidation or long-distance movement of TMV.

Infectivity of a chimeric TMV containing the RCNMV MP gene

To further test the ability of the RCNMV MP to provide the cell-to-cell movement function for TMV, a chimeric TMV, designated TRCM, was constructed that contains the RCNMV MP gene. Infectious transcripts generated *in vitro* from pTRCM and pTMV (Fig. 1) were inoculated onto *N. benthamiana* and tobacco plants. TRCM transcripts systemically infected both *N. benthamiana* and tobacco, although systemic symptoms induced by TRCM on tobacco were both less severe and slower to appear (3- to 4-day delay) than symptoms induced by TMV transcripts (data not shown). *In vitro* transcripts from pTRCM and pTMV were inoculated onto opposite half leaves of hypersensitive tobacco to compare lesion development. Lesions induced by TRCM appeared at 3 dpi, while those induced by TMV appeared at 2 dpi. In addition, lesions induced by TRCM increased in diameter at a slower rate than those induced by TMV. By 6 dpi, lesions induced by TRCM were 58% the diameter of lesions induced by TMV (data not shown), suggesting that the RCNMV MP is less efficient than the TMV MP at facilitating cell-to-cell movement in tobacco. The amount of TRCM virions purified from systemically infected leaves of tobacco at 21 dpi was 0.90 mg/g fresh weight compared to 4.5 mg/g fresh weight for TMV. Total RNA extracted from systemically infected leaves of TRCM-infected tobacco was analyzed by Northern blot analysis. As can be seen in Fig. 5, the plants infected with TRCM were not contaminated with TMV. The TRCM genome is 677 nucleotides larger than the wild-type TMV genome. As expected, the TRCM genomic and MP subgenomic RNAs are larger than those of wild-type TMV (Fig. 5). Following purification of TRCM from systemically infected tobacco leaves, cDNA to the MP gene sequence was obtained by reverse transcription and polymerase chain reaction and sequenced. The initial RCNMV MP construct present in TRCM was stably maintained in the systemically infected tobacco plants. These data demonstrate that the RCNMV MP is able to provide the cell-to-cell movement function for a movement-defective TMV not only *in trans*, but also *in cis* as part of the TMV genome.

Both TMV and RCNMV can act as helper viruses to facilitate the cell-to-cell spread of movement-defective viruses

Virus spread from cell to cell may depend not only on the presence of a viral MP, but also on interactions

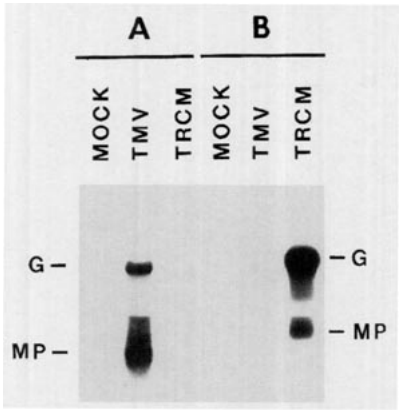

FIG. 5. Northern blot analysis of viral RNA from uninoculated leaves of tobacco plants mock inoculated, inoculated with TMV, or inoculated with TRCM transcripts. Total RNA was extracted from uninoculated leaves at 21 dpi, electrophoresed through 1% agarose containing formaldehyde, and transferred to nitrocellulose (Deom *et al.*, 1987). Total RNA (5 μg) was loaded onto each lane. The inoculum is indicated at the top of each lane. (A) Detection of TMV RNA in uninoculated leaves from TMV-infected tobacco using a nick-translated, ^{32}P-labeled cDNA comprising a portion of the TMV MP gene (nucleotides 4968–5236) not present in TRCM. (B) Detection of TRCM RNA in uninoculated leaves from TRCM-infected tobacco using a nick-translated ^{32}P-labeled cDNA comprising the RCNMV MP gene (not present in TMV). G indicates TMV genomic RNA (left) and TRCM genomic RNA (right). MP indicates TMV-MP mRNA (left) and TRCM-MP mRNA (right).

between other viral gene products, although such interactions have not yet been characterized (DeJong and Ahlquist, 1992; Taliansky *et al.*, 1992; Atabekov and Taliansky, 1990). This complicates much of the data obtained from helper-virus experiments. Nevertheless, as a final test of the abilities of the RCNMV and TMV MPs to functionally substitute for one another, two helper-virus experiments were conducted. Movement-defective TMV-MPfs was coinoculated with wild-type RCNMV onto *N. benthamiana* plants and RCNMV RNA-1 was coinoculated with TMV on both *N. benthamiana* and tobacco plants. After the appearance of symptoms on the inoculated leaves, total RNA was extracted from both inoculated and uninoculated leaf tissue and analyzed by Northern blot analysis for the presence of the movement-defective viruses. As seen in Fig. 6, both TMV-MPfs and RCNMV RNA-1 were detected in the inoculated leaves of the plants coinfected with the respective helper viruses, RCNMV and TMV. This suggests that both RCNMV and TMV can act as helper viruses to provide cell-to-cell movement. However, neither helper virus was able to facilitate long-distance movement of the defective viruses into uninoculated leaves. In contrast, the inability of TMV to facilitate long-distance movement of RCNMV RNA-1 may be due to the requirement of both RCNMV RNAs 1 and 2 for virion formation and long-distance movement (Fig. 6B).

DISCUSSION

We have shown that the MPs of TMV and RCNMV are functionally homologous. The most dramatic example of this was the generation of a chimera between TMV and RCNMV, TRCM, in which the TMV MP was replaced by the RCNMV MP. Interestingly, this chimera systemically infected both *N. benthamiana,* a normal systemic host for both TMV and RCNMV, as well as tobacco, a systemic host for TMV, but not RCNMV. RCNMV moves from cell to cell in the Xanthi cultivar of tobacco and induces symptoms on the inoculated leaves, but the wild-type virus fails to move long distance. Therefore, the blockage to long-distance movement of RCNMV in tobacco is a function of virus sequences and components other than the RCNMV MP. Our results are consistent with the findings of De Jong and Ahlquist (1992), who were able to create a viable hybrid between icosahedral cowpea chlorotic mottle bromovirus (CCMV) and the rod-shaped sunnhemp mosaic tobamovirus (SHMV) by substituting the SHMV MP gene for the CCMV 3a gene.

De Jong and Ahlquist (1992) hypothesized that the viability of the CCMV–SHMV hybrid implies that the movement protein of SHMV does not require any viral-specific interactions to facilitate cell-to-cell movement. This conclusion is especially significant when considering the fact that SHMV cannot complement defects in cell-to-cell movement of CCMV in a common host cowpea (De Jong and Ahlquist, 1992). Similarly, our findings that the chimera, TRCM, is able to move not only cell to cell, but also long distance, to cause a systemic infection in *N. benthamiana* and tobacco, suggests that virus-specific interactions between the TMV MP and other virus-encoded factors during infection are probably not essential.

As a helper virus, RCNMV facilitated cell-to-cell movement of TMV-MPfs rather poorly and did not allow long-distance movement at all (Fig. 6). It is possible that the RCNMV MP does not have as high an affinity for heterologous viral RNA *in vivo,* especially in the presence of the RCNMV genomic RNA and other RCNMV components. Alternatively, the replication of TMV-MPfs may have been down-regulated because of the coincident RCNMV infection. Indeed, the level of TMV-MPfs detected in the inoculated leaves was approximately 2–5% that of the wild-type RCNMV, and this may in part explain the failure to detect TMV-MPfs in the uninoculated leaves (Fig. 6B). When TMV-MPfs is inoculated on transgenic *N. benthamiana* plants expressing the RCNMV MP, the level of cell-to-cell and long-distance movement, based on the levels of TMV CP detected, is indistinguishable from that of the wild-type TMV (Fig. 4). This supports the notion that the presence of the wild-type RCNMV helper virus inhibits either the movement or the replication of TMV-MPfs, but that the RCNMV MP itself does not.

Cell-to-cell movement of RCNMV RNA-1, facilitated both by TMV helper virus infection and by the transgenically expressed TMV MP in *N. benthamiana* plants, was less efficient than that seen during a wild-type RCNMV infection (Figs. 3 and 6). This is distinct from the situation described above because the transgenic plants express-

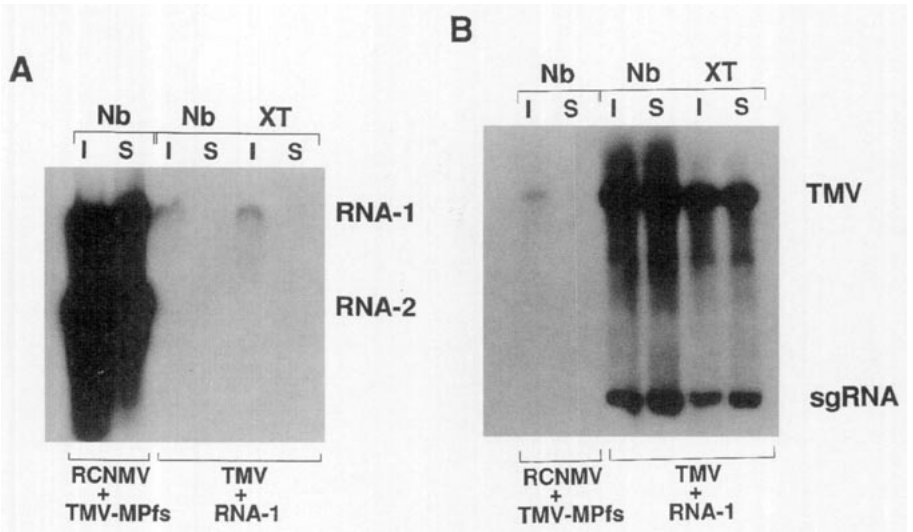

FIG. 6. TMV and RCNMV can act as helper viruses to provide cell-to-cell movement of movement-defective viruses. A Northern blot analysis of tissue from *N. benthamiana* and tobacco plants infected with TMV and RCNMV RNA-1 or RCNMV and TMV-MPfs. Total RNA (20 μg) isolated from the inoculated (I) and uninoculated (S) leaves of infected plants was loaded onto a 1% agarose gel and transferred to a nylon membrane. The blot was probed with either RCNMV CP and MP gene sequences (from RNAs 1 and 2, respectively) (A) or TMV CP sequences (B). Inoculum used is indicated at the bottom of lanes, RCNMV + TMV-MPfs or TMV + RCNMV RNA-1. Host plant, *N. benthamiana* (Nb) or *N. tabacum* cv. Xanthi (XT), is indicated at the top of lanes. In (A), the relative positions of RCNMV RNAs 1 and 2 are indicated. In (B), the positions of TMV genomic and subgenomic (sg) RNAs are indicated.

ing the TMV MP do not fully complement the absence of the RCNMV MP, whereas the transgenic plants expressing the RCNMV MP fully restore cell-to-cell movement and systemic infection to TMV-MPfs. One explanation for this may be that RCNMV has more stringent requirements for cell-to-cell movement than TMV. For example, there may be virus-specific interactions between the RCNMV MP and the viral genome, which are required for movement. It is also possible that the RCNMV MP affects the level of viral replication, perhaps by interacting with the replicase or replication complex. However, this seems unlikely, since it has been reported that RCNMV RNA-1 can replicate to wild-type levels in the absence of RNA-2 in cowpea, *N. benthamiana*, and tobacco protoplasts (Osman and Buck, 1987; Paje-Manalo and Lommel, 1989).

The failure of the TMV helper virus, as well as the transgenically expressed TMV MP, to allow the long-distance movement of RCNMV RNA-1 in *N. benthamiana*, in contrast, is confounded by the findings that RCNMV virion formation and CP accumulation require the presence of RCNMV RNAs 1 and 2. CP neither accumulated nor formed virions in the absence of RNA-2, suggesting that both RCNMV RNA-1 and RNA-2 are required for encapsidation and long-distance movement. When RCNMV RNA-1 was coinoculated with a mutant RNA-2 that does not express a functional MP, long-distance movement of RCNMV was observed in transgenic plants expressing the RCNMV MP, but not in untransformed *N. benthamiana* plants or in control plants transformed with the expression vector alone. This supports the hypotheses that virion formation cannot occur in the absence of RNA-2 and that it is required for long-distance movement (Vaewhongs and Lommel, 1995).

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