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# Umbravirus-Encoded Proteins both Stabilize Heterologous Viral RNA and Mediate Its Systemic Movement in Some Plant Species

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The proteins encoded by open reading frame 3 (ORF3) of the umbraviruses pea enation mosaic virus-2 and tobacco mottle virus, like that of groundnut rosette virus, mediated the movement of viral RNA through the phloem of infected *Nicotiana benthamiana* or *N. clevelandii* plants when they were expressed from chimeric tobacco mosaic virus in place of the coat protein. However, these chimeras did not move systemically in *N. tabacum.* In lysates of *N. benthamiana* or *N. tabacum* protoplasts, the chimeric RNAs were more stable than was RNA of tobacco mosaic virus lacking the coat protein gene. The chimeric viruses also protected the latter *in trans,* suggesting that the ORF3 proteins can increase the stability of heterologous viral RNA. Umbraviral ORF3 proteins contain a conserved arginine-rich domain, and the possible roles of this motif in the functions of the proteins are discussed. © 2001 Academic Press

*Key Words:* plant virus; umbraviruses; virus movement; stabilization of viral RNA.

## INTRODUCTION

The coat protein (CP) encoded by most plant viruses fulfills various biological functions, primarily the protection of viral RNA by encapsidating it to form virus nucleoprotein particles. The CP is also usually involved in the transmission of viruses by their biological vectors and in the systemic spread of viruses in infected plants (see for reviews, Carrington *et al.,* 1996; Harrison and Murant, 1984). Some plant viruses, such as potato virus X (PVX; Chapman *et al.,* 1992), cucumber mosaic virus (CMV; Canto *et al.,* 1997), and cowpea mosaic virus (Wellink and van Kammen, 1989), require the CP for both cell-to-cell and long-distance movement. Others, such as tobacco mosaic virus (TMV), do not depend on CP for cell-to-cell movement but do require it for long-distance movement (Siegel *et al.,* 1962; Takamatsu *et al.,* 1987; Saito *et al.,* 1990; Osbourn *et al.,* 1990; Holt and Beachy, 1991; Hilf and Dawson, 1993; Spitsin *et al.,* 1999). The genomes of umbraviruses (genus *Umbravirus*) differ from those of all other plant viruses in that they do not encode a CP, and thus no virus particles are formed in infected plants (see for review, Robinson and Murant, 1999). Nevertheless, umbraviruses can accumulate and spread very efficiently within plants. Although umbraviruses depend on the assistance of a luteovirus for aphid transmission, the presence or absence of the luteovirus and its CP does not affect the accumulation and systemic spread of the umbraviruses (Demler *et al.,* 1994). Moreover, unlike most single-stranded viral RNAs, umbraviral RNA remains infective in crude sap extracts for several hours (Murant *et al.,* 1969; Reddy *et al.,* 1985). These observations suggest that umbraviruses can exploit one or more alternative mechanisms for RNA protection and systemic spread, different from those provided by a CP.

The RNA genomes of umbraviruses contain four open reading frames (ORFs) (Fig. 1A). The two ORFs at the 5′ end of the RNA are expressed by a −1 frameshift to yield a single protein that appears to be an RNA-dependent RNA polymerase (Demler *et al.,* 1993; Taliansky *et al.,* 1996; Gibbs *et al.,* 1996). The other two ORFs overlap each other in different reading frames. ORF4 encodes a 27- to 29-kDa protein, which contains stretches of similarity with several plant virus-encoded movement proteins (MPs) that control virus movement from cell-to-cell (Taliansky *et al.,* 1996; Gibbs *et al.,* 1996). In gene replacement experiments, the ORF4 protein encoded by one of the umbraviruses, groundnut rosette virus (GRV), was shown to be able to functionally replace the MPs of PVX or CMV (Ryabov *et al.,* 1998, 1999a), confirming that the GRV ORF4 protein is a cell-to-cell MP. The ORF4 protein enabled cell-to-cell movement of PVX and CMV regardless of the presence or absence of their CPs, but the CPs were still required for long-distance movement.

Database searches with the sequence of the umbraviral 26- to 29-kDa ORF3 proteins revealed no significant similarity with any other viral or nonviral proteins, except the corresponding proteins encoded by different umbraviruses (Taliansky *et al.,* 1996). In epidermal and mesophyll cells, the GRV ORF3 protein is located in small

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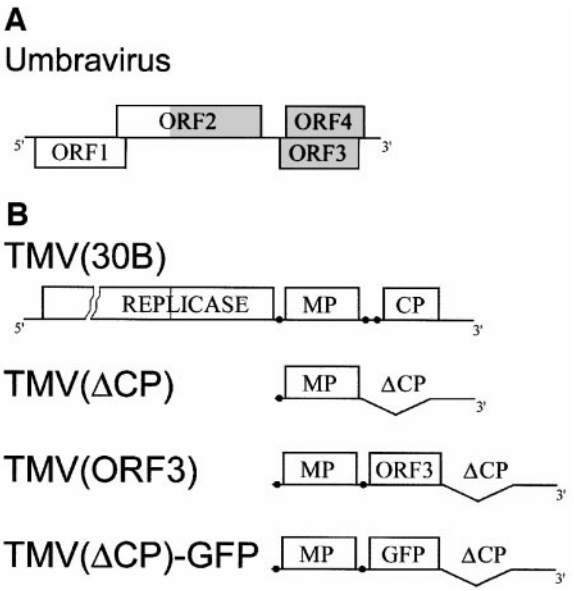


FIG. 1. Schematic representation of the genome of umbraviruses (A) and of the TMV-based vector TMV(30B) and its derivatives expressing umbraviral ORF3s or the GFP gene (B). Boxes represent ORFs, lines represent untranslated sequences. MP, TMV movement protein; CP, TMV coat protein; (•) subgenomic promoter. Deleted sequences are indicated. The part of the umbraviral genome corresponding to the nucleotide sequences of TMoV RNA that were determined is highlighted in A.

membrane-associated cytoplasmic vesicles and in the nuclei, preferentially targeting nucleoli (Ryabov *et al.,* 1998). Recently, we showed that the GRV ORF3 protein facilitated the long-distance movement of TMV RNA *in cis* and *in trans,* replacing the TMV CP, which is normally essential for the long-distance movement of TMV RNA (Ryabov *et al.,* 1999b). In this work, we show that the ORF3 proteins encoded by two other umbraviruses, pea enation mosaic virus-2 (PEMV-2) and tobacco mottle virus (TMoV), are also able to mediate long-distance movement of heterologous TMV RNA. However, the facilitation of long-distance movement of RNA by the umbraviral ORF3 proteins is host-specific. We also show that the ORF3 proteins can stabilize TMV RNA in lysates of infected protoplasts.

## RESULTS

### PEMV-2 and TMoV ORF3 and generation of chimeric TMV

cDNA clones corresponding to TMoV RNA were generated from virus specific dsRNA isolated from TMoV-infected *Nicotiana benthamiana* plants. The partial genome sequence obtained (GeneBank Accession No. AY007231; see also Fig. 1A) is similar to the genome sequences of other umbraviruses. At the 5′ end is an incomplete ORF capable of coding for a polypeptide consisting of 361 amino acid residues, with 66.5 to 67.5% amino acid sequence identity to the corresponding parts of the polypeptides encoded by ORF 2 of GRV (Taliansky *et al.,* 1996), carrot mottle mimic virus (CMoMV; Gibbs *et al.,* 1996), and PEMV-2 (Demler *et al.,* 1993). At the 3′ end of the partial sequence are two overlapping ORFs, which are arranged in the same way as ORF3 and ORF4 of other umbraviruses (Fig. 1A) and which will be referred to as ORF3 and ORF4 of TMoV. ORF4 encompasses 765 nucleotides and codes for a protein of 254 amino acid residues and a molecular mass of 28 kDa, with 46 to 63% amino acid sequence identity to the ORF4 proteins encoded by other umbraviruses.

The sequence of TMoV ORF3 contains only one potential AUG translation start codon and comprises 705 nucleotides. The polypeptide it encodes consists of 234 amino acid residues, with a molecular mass of 26 kDa and with 29 to 35% amino acid sequence identity to the ORF3 proteins of other umbraviruses (Fig. 2). The ORF3 proteins are rich in arginine, proline, serine, and glycine residues. Measurement of local compositional complexity, using the SEG program (Wootton and Federhen, 1996) indicates that 85–95% of residues in each protein fall within predicted low-complexity, nonglobular regions. The amino terminal halves of the sequences (positions 25–145 of the alignment; Fig. 2) are the most hydrophilic and include a highly basic arginine-rich domain (positions 109–123; Fig. 2) with four conserved arginine residues. The ORF3 protein sequences also contain a hydrophobic element (positions 146–169 of the alignment, Fig. 2) which includes three invariant leucine residues.

Plants infected with the pea enation mosaic virus complex consisting of pea enation mosaic virus-1 (PEMV-1), a luteovirus, and PEMV-2, an umbravirus, produce virus particles containing both PEMV-1 RNA and PEMV-2 RNA encapsidated by the PEMV-1 CP. Thus, to generate cDNA clones corresponding to the PEMV-2 ORF3, viral RNA isolated from such particles was used for reverse transcription–PCR. The primers were designed according to the PEMV-2 sequence published by Demler *et al.* (1993, 1994). The sequence of the PEMV-2 ORF3 fragment generated (GeneBank Accession No. AY007257; Fig. 2) was essentially similar to that published by Demler *et al.* (1993, 1994) and encoded a protein with only six amino acid differences, all in nonconserved regions. Like GRV ORF3 and in contrast to TMoV ORF3 and CMoMV ORF3, PEMV-2 ORF3 contains at the 5′ end two in-frame AUG codons. However, mutational analysis (Demler *et al.,* 1994) has shown that the upstream AUG initiation codon is essential for PEMV-2 infectivity.

The CP gene of TMV was deleted and replaced by ORF3 of either PEMV-2 or TMoV in a TMV-based vector, TMV(30B) (Shivprasad *et al.,* 1999), to give the hybrids TMV(ORF3P) and TMV(ORF3T), respectively, as described previously for the hybrid containing GRV ORF3 (Ryabov *et al.,* 1999b; Fig. 1B), referred to here as

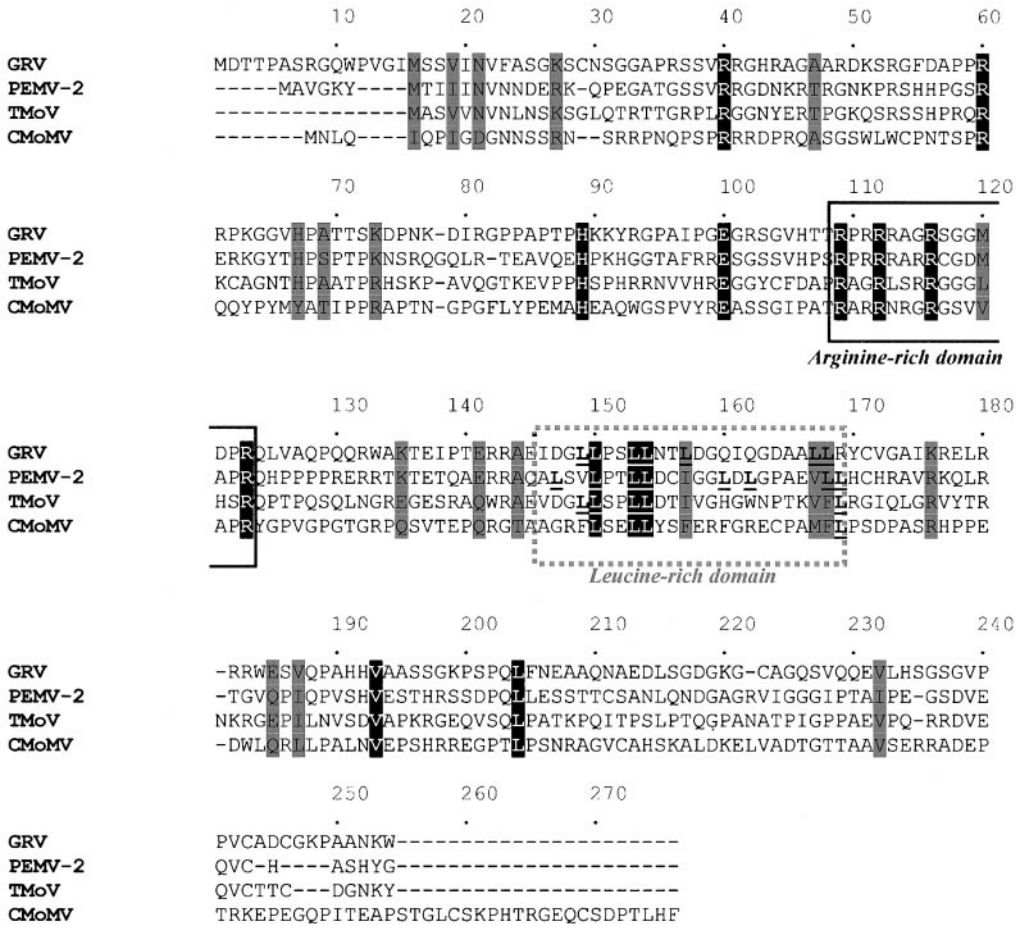


FIG. 2. Comparison between the ORF3 proteins encoded by GRV (Taliansky *et al.,* 1996), PEMV-2 (Demler *et al.,* 1993, 1994), and CMoMV (Gibbs *et al.,* 1996) and TMoV. The amino acids that are identical among all four sequences are highlighted in black, and similar amino acids are highlighted in gray. Arginine-rich and leucine-rich domains are indicated.

TMV(ORF3G). TMV(30B) and TMV(30B) with a deleted CP gene [TMV(ΔCP)] were used as controls (Fig. 1B).

### Effect of the ORF3 proteins on TMV RNA stability

It is generally accepted that the CP protects plant viral RNA from degradation by encapsidating it into virus particles. Mutants of different plant viruses with defective or deleted CP usually accumulate to lower levels in infected tissues or inoculated protoplasts (Boccard and Baulcombe, 1993; Chapman *et al.,* 1992). A similar pattern of RNA accumulation was observed for TMV mutants unable to produce virus particles (Holt and Beachy, 1991; Ishikawa *et al.,* 1991; Saito *et al.,* 1990). Moreover, RNA from CP null mutants is completely destroyed in water or buffer extracts.

Dot-blot hybridization analysis of RNA samples, isolated from *N. benthamiana* protoplasts immediately after collection, showed that both TMV(30B) and TMV(ΔCP) RNAs accumulated in infected protoplasts (Fig. 3A). The time course of accumulation of these viral RNAs was similar at the early stages of infection, but after 8 h postinoculation, the accumulation of TMV(ΔCP) RNA virtually ceased. By 24 h postinoculation, the level of TMV(ΔCP) RNA was approximately 50% of that of TMV(30B) RNA. This difference may be due to the instability of unencapsidated viral RNA in protoplasts. Alternatively, it is possible that the CP (or some *cis*-acting RNA sequences within the CP gene) may play a regulatory role in the later stages of TMV RNA synthesis. However, when the CP gene of TMV was replaced by GRV ORF3, viral RNA accumulated to higher levels than in the case of TMV (ΔCP) RNA and continued to accumulate after 8 h postinoculation, although at a slower rate than TMV(30B) RNA (Fig. 3A). Apparently, expression of the ORF3 product, rather than the ORF3 RNA sequence itself, is required for this effect because mutations in the AUG initiation codon of the ORF3 [TMV(noORF3G)] resulted in diminished levels of RNA accumulation (Fig. 3A), comparable to those of TMV(ΔCP) RNA.

The role of the ORF3 protein in protecting viral RNA was tested by allowing nucleases of lysed protoplasts to degrade nonencapsidated viral RNA (Mohan *et al.,* 1995). When the protoplasts had been destroyed by transferring

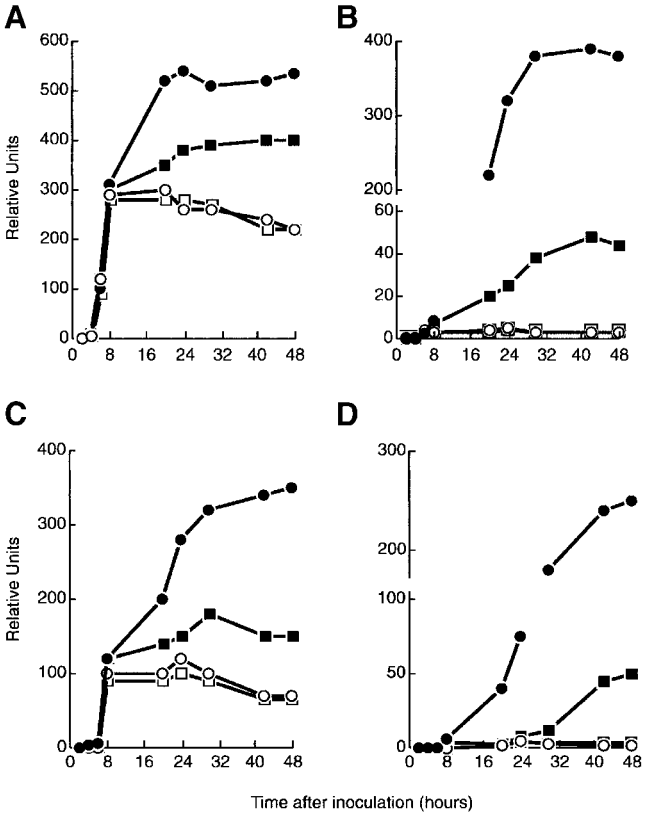


FIG. 3. Time course of accumulation of viral RNA isolated from *N. benthamiana* (A and B) and *N. tabacum* (C and D) protoplasts either immediately after collection (A and C) or 15 min after destroying protoplasts by osmotic shock (B and D). Protoplasts were inoculated with TMV(30B) (●), TMV(△CP) (○), TMV(ORF3G) (■), or TMV(noORF3G) (□). RNA levels are indicated in relative units determined by densitometry of dot-blot hybridization data and are the average of triplicate samples. Each experiment was repeated at least twice, with similar results.

into Tris–HCl buffer, pH 7.5, containing no mannitol 15 min prior to isolation of RNA, the final yield of TMV(30B) RNA was only slightly affected. Degradation of RNA was mainly observed when protoplasts were destroyed at early stages of infection (before 20 h postinoculation) when the RNA was presumably not yet encapsidated (Fig. 3B). In these experiments TMV(ΔCP) RNA was barely detectable, reaching levels less than 1% of those of TMV(30B) (Figs. 3B and 4A, lanes 3, 9 versus 2, 8). However, when the TMV CP gene was replaced by GRV ORF3, PEMV-2 ORF3, or TMoV ORF3, a significant fraction of the RNA was protected from degradation (Figs. 3B and 4A). TMV(noORF3G) RNA was no more stable than TMV(ΔCP) RNA (Fig. 3B), indicating the stabilizing role of the ORF3 product rather than the ORF3 RNA sequence itself.

Experiments in *N. tabacum* protoplasts yielded similar results (Figs. 3C, 3D, and 4B), although RNA levels were slightly lower than those in *N. benthamiana* protoplasts. These results indicate that the ORF3 proteins can increase accumulation of TMV RNA in the absence of CP, and protect it from degradation, not only in *N. benthamiana* but also in *N. tabacum* protoplasts.

To test if the ORF3 protein can protect TMV RNA in protoplast lysates *in trans, N. benthamiana* protoplasts were co-inoculated with TMV(ΔCP)-GFP, which contains the GFP gene in place of the CP gene (Ryabov *et al.,* 1999b; Fig. 1B), and TMV(ORF3P), TMV(ORF3T), or TMV(ORF3G). Northern blot hybridization analysis, using cDNA probes specific to the GFP gene, showed that the level of accumulation of TMV(ΔCP)-GFP RNA increased dramatically as a result of co-inoculation (Fig. 5, lane 2 versus lanes 3, 4, 5, 7). Mixture of protoplasts separately infected with TMV(ORF3G) and with TMV(ΔCP)-GFP before lysis did not lead to protection of TMV(ΔCP)-GFP

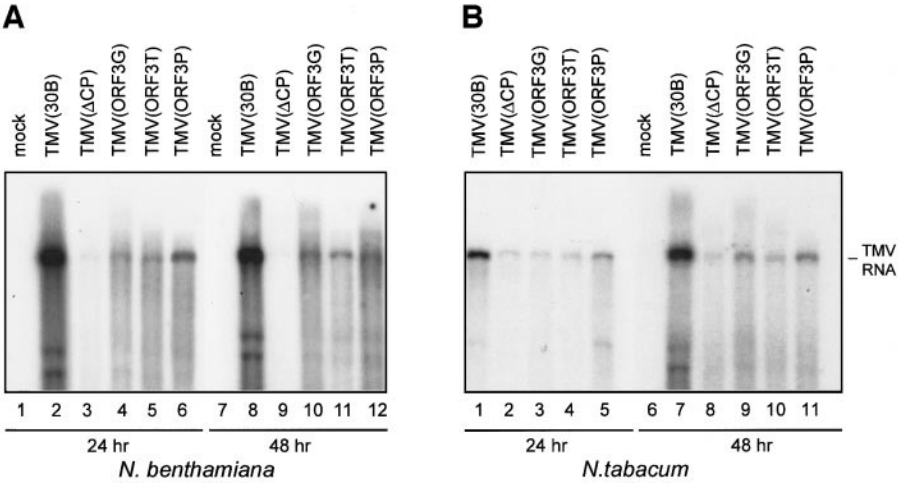


FIG. 4. Northern blot hybridization analysis of total viral RNA isolated from *N. benthamiana* (A) and *N. tabacum* (B) protoplasts inoculated with TMV(30B), TMV(ΔCP), TMV(ORF3G), TMV(ORF3T), or TMV(ORF3P) 24 h and 48 h postinoculation, as indicated. Protoplasts were destroyed by osmotic shock 15 min prior to isolation of RNA (see Materials and Methods). The position of TMV genomic RNA is marked. Hybridization was done with $^{32}$P-labeled cDNA probes corresponding to the TMV replicase gene.

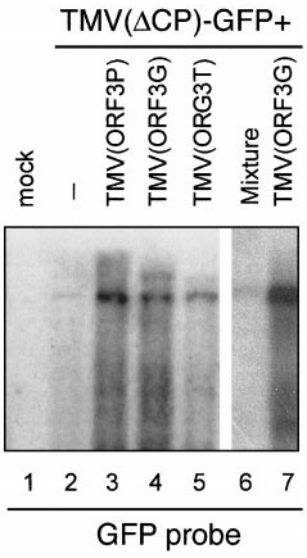


FIG. 5. Northern blot hybridization analysis of total viral RNA isolated from *N. benthamiana* protoplasts inoculated with TMV(ΔCP)-GFP in the absence (2) or the presence of TMV(ORF3P) (3) , TMV(ORF3G) (4, 7), or TMV(ORF3T) (5) 48 h postinoculation. Protoplasts were destroyed by osmotic shock 15 min prior to isolation of RNA (see Materials and Methods). Protoplasts inoculated with TMV(ORF3G) and TMV(ΔCP)-GFP separately were mixed before osmotic shock (6). Hybridization was done with $^{32}$P-labeled cDNA probes corresponding to the GFP gene.

RNA, indicating that the events that resulted in protection took place in the intact protoplasts (Fig. 5, lane 6).

### Accumulation of TMV(ORF3P) and TMV(ORF3T) in infected plants: host specificity of long-distance movement

As described previously (Ryabov *et al.,* 1999b), in contrast to TMV(ΔCP), which is unable to spread systemically, TMV expressing GRV ORF3 in place of the CP [TMV(ORF3G)] induced systemic symptoms and moved systemically in *N. benthamiana* plants. TMV(ORF3P) and TMV(ORF3T) also induced systemic symptoms in *N. benthamiana,* which were more severe and appeared earlier (4–5 days postinoculation) than those induced by TMV(ORF3G). To verify that TMV(ORF3P) and TMV(ORF3T) RNAs had moved systemically, inoculated and upper uninoculated leaves were harvested and analyzed by Northern blotting. As expected TMV(30B) RNA accumulated both in inoculated and uninoculated systemically infected leaves, but TMV(ΔCP) was unable to spread systemically and was detected only in the inoculated leaves (Fig. 6). Using probes specific to the TMV replicase gene and to PEMV-2 ORF3 or TMoV ORF3, it was confirmed that chimeric TMV(ORF3P) and TMV(ORF3T) RNAs were capable of rapidly moving systemically (Fig. 6), indicating that the PEMV-2 and TMoV ORF3 proteins mediate long-distance transport of heterologous viral (TMV) RNA in infected plants. As with TMV(ORF3G) (Ryabov *et al.,* 1999b), mutations in the translational start codons of the PEMV-2 or TMoV ORF3 prevented long-distance movement of the recombinant TMV(ORF3P) and TMV(ORF3T), implicating the ORF3 product rather than the RNA sequence itself in mediating this process.

The capability for systemic movement of TMV(ORF3P), TMV(ORF3T), and TMV(ORF3G) was also tested in two other plant species. As expected, all three chimeric viruses were able to move systemically in *Nicotiana clevelandii,* which is a systemic host for TMV, PEMV-2, TMoV, and GRV (Table 1). However, in *N. tabacum* MD 609, which is a systemic host for TMV, TMoV, and PEMV-2 but not for GRV, TMV(30B) RNA accumulated in both inocu-

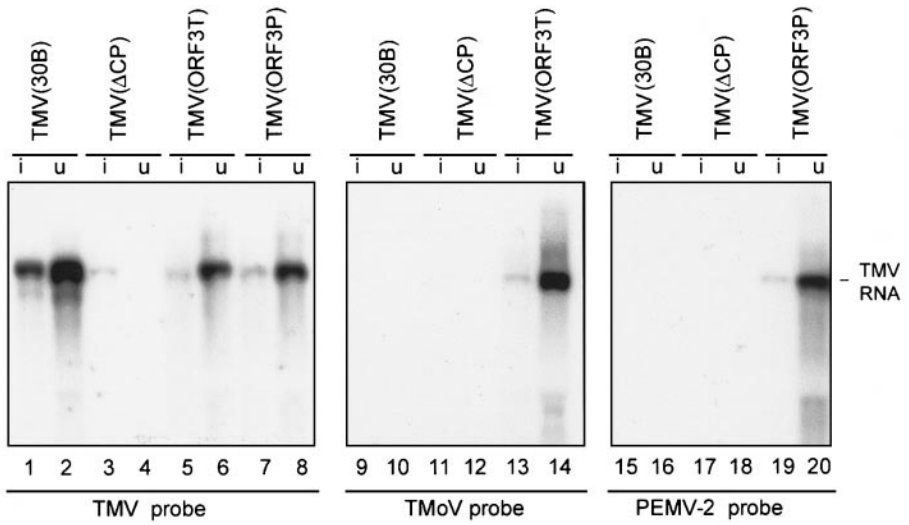


FIG. 6. Northern blot hybridization analysis of total RNA isolated from inoculated (i) and uninoculated (u) leaves of *N. benthamiana* plants inoculated with TMV(30B), TMV(ΔCP), TMV(ORF3T), or TMV(ORF3P), as indicated, 9 days postinoculation. Hybridization was done with $^{32}$P-labeled cDNA probes corresponding to the TMV replicase gene or full-length ORF3 sequences of TMoV or PEMV-2, as indicated. The position of TMV genomic RNA is marked.

TABLE 1

Host Specificity of Long-Distance Movement of CP-Deficient TMV Expressing Umbraviral ORF3s

| Host | | Accumulation of viral RNA in inoculated and systemic leaves of plants infected with[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TMV(30B) | TMV(ΔCP) | GRV | TMV(ORF3G) | TMoV | TMV(ORF3T) | PEMV-2 | TMV(ORF3P) |
| *N. benthamiana* | i[b] | + | + | + | + | + | + | + | + |
| | s[b] | + | − | + | + | + | + | + | + |
| *N. clevelandii* | i | + | + | + | + | + | + | + | + |
| | s | + | − | + | + | + | + | + | + |
| *N. tabacum* | i | + | + | − | + | + | + | + | + |
| MD609 | s | + | − | − | − | + | − | + | − |

[a] Data based on dot-blot hybridization assay. Detection of viral RNA is represented by +, indicating the presence of RNA in the specified leaves starting 8 days postinoculation. Leaves in which viral RNA was not detected even 30 days postinoculation are indicated as −.

[b] i, inoculated leaves, s, uninoculated upper leaves.

lated and uninoculated leaves, but the chimeric viruses, like TMV(ΔCP), accumulated only in inoculated leaves and did not spread systemically (Table 1).

## DISCUSSION

TMoV has been classified as a species in the genus *Umbravirus* mainly on the basis of its biological properties. The sequence data reported here support this classification by suggesting that it shows the characteristic genome organization of other members of the genus and by showing that it encodes homologous proteins.

The TMV-encoded CP is a multifunctional protein which forms nucleoprotein particles encapsidating viral RNA and is required for long-distance movement of TMV (see for review Carrington *et al.,* 1996). TMV mutants with defective or deleted CP genes accumulate RNA at reduced levels (Holt and Beachy, 1991; Ishikawa *et al.,* 1991; Saito *et al.,* 1990), and the RNA is unstable in water or buffer extracts of infected leaves, probably because it is unprotected from degradation by RNases. Such mutants are also unable to spread rapidly through the plant's vascular system. These properties of CP-deficient mutants are not unique to TMV and are characteristic of CP-deficient mutants of many other plant viruses, some of which in addition demonstrate a requirement for CP for cell-to-cell movement (see for review, Carrington *et al.,* 1996). However, umbraviruses lack a CP gene and have presumably adapted to surviving without a CP. In nature, umbraviruses are usually associated with luteovirus assistors to give biological complexes. Umbraviral RNA in such complexes is encapsidated by luteovirus-encoded CP and as a result becomes aphid transmissible (see for review, Robinson and Murant, 1999). However, both under experimental conditions and in nature umbraviruses can become separated from the complexes and are able to exist without luteovirus assistors and their CPs (Elnagar and Murant, 1978; Murant, 1990; Naidu *et al.,* 1999). Therefore, lack of a CP must be compensated by another umbravirus-encoded protein(s).

It is well documented that umbraviral RNA accumulates to high levels in infected plants and remains infective even in water extracts of these plants for several hours (Demler *et al.,* 1994; Murant *et al.,* 1969; Reddy *et al.,* 1985). In this work, we demonstrate that the ORF3 protein encoded by GRV, PEMV-2, or TMoV can stabilize TMV RNA in lysates of protoplasts. It is noteworthy that this can occur both *in cis* and *in trans.* An interesting parallel is that with barley stripe mosaic virus (BSMV), another virus that does not require CP for systemic movement (Petty and Jackson, 1990). BSMV-infected barley tissue contains relatively large amounts of unencapsidated viral RNA, apparently stabilized in buffer extracts by interaction with a nonstructural viral protein (Brakke *et al.,* 1988), probably the βb gene product, which is required for virus movement (Petty and Jackson, 1990). Stabilization of brome mosaic virus RNA3 by the 1a protein has recently been demonstrated by Janda and Ahlquist (1998) and Sullivan and Ahlquist (1999).

Previously, we showed that the ORF3 protein encoded by GRV also facilitated long-distance movement of CP-deficient TMV RNA *in cis* as well as *in trans* in their common host *N. benthamiana* (Ryabov *et al.,* 1999b). Results of experiments described in this paper confirm that the ability to facilitate long-distance movement of RNA molecules is not only characteristic of the GRV ORF3 protein but represents a general property of proteins encoded by the ORF3s of umbraviruses. However, we have also found here that this ORF3-mediated long-distance movement function is host-dependent. Although *N. benthamiana, N. clevelandii,* and *N. tabacum* are all permissive hosts for TMoV and PEMV-2 and the ORF3 proteins are therefore apparently capable of mediating systemic movement of homologous viral RNA in these plant species, heterologous TMV RNA can be transported by the same ORF3 proteins in *N. benthamiana* and *N. clevelandii* but not in *N. tabacum* (Table 1). For their movement function the ORF3 proteins may need to interact with some hypothetical host factor, but it is hard

to see how specificity of such an interaction can be invoked to explain the different host dependence of the transport of homologous and heterologous viral RNAs. It is noteworthy that in *N. tabacum* CP–deficient TMV is unable to overcome a barrier to movement between phloem parenchyma cells and the companion cell/sieve element complex (Ding *et al.,* 1996). A possible explanation for the observed host specificity is that to mediate passage through this barrier in *N. tabacum,* the umbraviral ORF3 proteins may require the contribution of another virus-specific factor, such as a compatible cell-to-cell MP. This may be provided by the ORF4 protein in the case of native PEMV-2 or TMoV infections, but CP-deficient TMV may be unable to provide such a factor. In *N. benthamiana* and *N. clevelandii* the barrier to movement of TMV(ΔCP) may be of a different kind or in a different place (for example, between companion cells and sieve elements) and may be overcome by the ORF3 protein on its own.

GRV and TMV(ORF3G) share the same host-dependence for systemic movement; both infect *N. benthamiana* and *N. clevelandii,* but not *N. tabacum,* systemically. It will be interesting to determine whether the barrier to movement in *N. tabacum* is the same for these two viruses.

The umbravirus-encoded ORF3 proteins represent a novel class of long-distance movement factors (Ryabov *et al.,* 1999b), which are able to transport unrelated viral RNAs and which are different from proteins, such as the 2b protein of CMV and the HC-Pro protein of potyviruses, that enhance systemic spread by blocking an RNA-mediated host defense mechanism (akin to posttranscriptional gene silencing, PTGS). The 2b and HC-Pro proteins are suppressors of PTGS (Anandalakshmi *et al.,* 1998; Brigneti *et al.,* 1998; Kasschau and Carrington, 1998). However, attempts to demonstrate similar suppressor activity by the GRV or PEMV-2 ORF3 proteins using experimental systems designed by Voinnet *et al.* (1999, 2000) were unsuccessful (our unpublished results; O. Voinnet, personal communication).

Two properties of the ORF3 proteins described here, stabilization of viral RNA and mediation of its long-distance movement, suggest that they can interact with viral RNA. This interaction may involve the conserved arginine residues (see Fig. 2); indeed, arginine residues have been shown to play a relevant role in a number of RNA–protein interactions (Draper, 1995). The ORF3 protein alone or in concert with a plant protein(s) may form a specific complex with viral RNA to protect it and transport it into and through the phloem. We cannot rule out the possibility that the ORF3 proteins mediate long-distance movement of TMV RNA merely as a result of slight increase of RNA levels *in vivo,* ensuring an increase of RNA concentration up to some hypothetical threshold level required for systemic spread. However, it is more likely that the ORF3 proteins play a more active role in this process.

The umbravirus-encoded ORF3 proteins possess two functions that are usually characteristic of plant virus-encoded CPs. Moreover, ORF3 and ORF4 of umbraviruses overlap each other in a similar way to the CP and MP genes of luteoviruses, and this suggests that the umbravirus-encoded ORF3 proteins may be either "primitive" coat proteins that protect viral RNA and facilitate its long-distance movement but have not developed the ability to form conventional virus particles or degenerate CPs, no longer able to form virus particles but still able to protect and transport RNA systemically. The ability to package viral RNA could have been lost by this protein when an ancestral umbravirus acquired the ability for its RNA to be encapsidated by assistor luteovirus CP and consequently a novel means of plant-to-plant transmission. It has been demonstrated that deletion of certain amino acid sequences from the CP of CMV (Kaplan *et al.,* 1998) or cowpea chlorotic mottle virus (Schneider *et al.,* 1997) abolishes their ability to encapsidate viral RNA into virus particles, although they retain the ability to protect and transport viral RNA. In this context it is noteworthy that, although no significant amino acid sequence similarity was found between the umbravirus-encoded ORF3 proteins and plant virus CPs in general and CPs encoded by luteoviruses in particular, the arginine-rich part of the ORF3 proteins resembles the disordered positively charged R-domain at the amino terminus of CPs of icosahedral RNA viruses, which is involved in the interaction with RNA (Liljas, 1986). The ORF3 protein may also resemble in function the recently discovered plant protein CmPP16 that can move, together with its own mRNA, into sieve elements, thus delivering the RNA into the long-distance translocation stream (Xoconostle-Cazares *et al.,* 1999). However, no sequence similarity was observed between the sequences of the umbraviral ORF3 proteins and the CmPP16 protein.

## MATERIALS AND METHODS

### Virus cultures

The tobacco rosette disease complex, described by Smith (1945), was maintained in tobacco by aphid or graft transmission. The sap-transmissible component, TMoV, was obtained by manual inoculation of *N. benthamiana.* Pea enation mosaic virus complex was an aphid–transmitted isolate "LANS," originally obtained from Dr. R. Hull. Both viruses were propagated in *N. benthamiana* and stored as ethanol-precipitated nucleic acid extracts.

### Preparation of dsRNA from TMoV-infected plants, synthesis and cloning of cDNA, and sequence determination

DsRNA was prepared from 100-g portions of TMoV-infected *N. benthamiana* leaf tissue essentially as de-

scribed by Taliansky *et al.* (1996). The aqueous phase after phenol extraction was adjusted to 18.5% (v/v) ethanol and chromatographed twice on Whatman CF-11 cellulose. Total ssRNA was isolated as described by Blok *et al.* (1994).

Synthesis and cloning of cDNA corresponding to TMoV RNA was carried out as described previously for GRV RNA (Taliansky *et al.,* 1996). To produce the first series of cDNA clones, dsRNA was denatured with 10 mM methylmercuric hydroxide and used as template for synthesis of first-strand cDNA by Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of random deoxyribonucleotide hexamers (Boehringer Mannheim, Mannheim, Germany). Second-strand cDNA synthesis was accomplished with DNA polymerase I (Promega) in the presence of RNase H (Promega). Double-stranded cDNA fragments were blunt-ended by T4 DNA polymerase (Promega) treatment and then cloned into the *Sma*I restriction site of pUC18. Following sequence analysis of clones from this first series, a second series was generated using synthetic oligonucleotide primers designed to match specific sites in the genomic RNA. For the first-strand cDNA synthesis, total ssRNA was used as template, and the products of this reaction were amplified by PCR and cloned into the pGEM-T vector (Promega, Madison, WI).

Cloned cDNAs were sequenced on a ABI Model 373A Stretch DNA sequencer (Perkin–Elmer, Norwalk, CT) using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin–Elmer).

## Plasmids and generation of chimeric cDNA constructs

Chimeric TMV constructs were made by using the TMV-based vector pTMV(30B), kindly provided by W. O. Dawson (Citrus Research and Education Center, Lake Alfred, FL; Shivprasad *et al.,* 1999) and described previously (Ryabov *et al.,* 1999b, see also Fig. 1B). Plasmids pTMV(ΔCP), containing a full-length cDNA copy of TMV RNA with a deleted CP gene, pTMV(ΔCP)-GFP, containing the GFP gene in place of the CP gene, pTMV(ORF3) [referred to here as pTMV(ORF3G)], in which the CP gene was deleted and replaced by GRV ORF3, and pTMV-(noORF3) [referred to here as pTMV(noORF3G)], containing point mutations predicted to eliminate expression of the ORF3, were described by Ryabov *et al.* (1999b) (see also Fig. 1B).

The following constructs were generated by using standard DNA manipulation techniques (Sambrook *et al.,* 1989). For pTMV(ORF3T) (Fig. 1B), the ORF3 gene of TMoV was amplified using an ORF3-containing TMoV cDNA clone and oligonucleotides 5′-GGCCTTAATTAATGGGCAAGTGTTGTAAATGTCAAC-3′ and 5′-GCATCTCGAGCTAGTATTTGTTCCCATCACAG-3′ and cloned between the *Pac*I and *Xho*I sites of pTMV(ΔCP). For pTMV(ORF3P) (Fig. 1B), the ORF3 gene of PEMV-2 was obtained by reverse transcription–PCR on RNA extracted from purified pea enation mosaic virus complex using oligonucleotide primers designed according to the PEMV-2 nucleotide sequence determined by Demler *et al.* (1993, 1994). The oligonucleotide 5′-GCATGTCGACATCACCCGTAGTGAGAG-3′ was used for first-strand cDNA synthesis and as a reverse primer, and the oligonucleotide 5′-GGCCTTAATTAAATGGCGGTAGGGAAATATATGAC-3′ as a forward primer. The amplified product was cloned between the *Pac*I and *Xho*I sites of pTMV(ΔCP). The sequence of PEMV-2 ORF3 was determined as described above.

All of the viruses derived from these constructs were designated by eliminating the prefix p in the names of the progenitor plasmids.

## *In vitro* transcription and inoculation of plants and protoplasts

Plasmids were linearized by digestion with *Kpn*I, and *in vitro* transcripts were synthesized with T7 RNA polymerase by using the mMESSAGE mMACHINE T7 Kit (Ambion Inc., Austin, TX). The transcripts derived from 0.2 μg of plasmid template were inoculated directly to corundum-dusted leaves of *N. benthamiana, N. clevelandii,* or *N. tabacum* MD609 plants. Mesophyll protoplasts were prepared from leaves of *N. benthamiana* or *N. tabacum* MD609 as described by Power and Chapman (1985), and $10^6$ protoplasts were electroporated with RNA transcripts, as described by Gal-On *et al.* (1994).

## Analysis of RNA

RNA was isolated either from protoplasts immediately on collection or from protoplasts that had been destroyed by osmotic shock by transfer from mannitol-containing media into 0.1 M Tris–HCl buffer, pH 7.5, and incubated for 15 min. RNA was isolated from 0.2 g of leaf tissue or $10^6$ protoplasts as described by Canto *et al.* (1997). For Northern blot analysis, $\frac{1}{10}$ of each RNA preparation was denatured with formaldehyde and formamide. Electrophoresis was in 1.5% agarose gels, as outlined by Sambrook *et al.* (1989). RNA was transferred to Hybond N membrane by the capillary method with 20× SSC (3 M sodium chloride and 0.3 M sodium citrate, pH 7.0) and immobilized by UV cross-linking. For dot-blot hybridization analysis, samples of RNA were spotted onto Hybond N nylon membrane. Hybridization was done as described by Sambrook *et al.* (1989) with $^{32}$P-labeled cDNA probes corresponding to the TMV replicase gene [nucleotides 442–2675 of pTMV(30B)], the GFP gene, or to full-length ORF3 sequences of TMoV, PEMV-2, or GRV. Quantitative analysis of dot blots was done by densitometry of the autoradiographic images with the Intelligent Quantifier, version 2.5.0 (BioImage, Ann Arbor, MI). A

dilution series of TMV RNA was used as a concentration standard. RNA levels were calculated in relative units.

### Sequence analysis

Sequence data were assembled and analyzed with the aid of the UWGCG programs (Devereux *et al.,* 1984). For sequence comparisons, the GAP and BESTFIT programs were used. CLUSTALV was used to generate multiple alignments of amino acid sequences. Estimation of local compositional complexity of proteins was conducted using the SEG program (Wootton and Federhen, 1996).

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