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A plant virus-encoded protein facilitates long-distance movement of heterologous viral RNA

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ABSTRACT **Transport of plant viruses from cell to cell typically involves one or more viral proteins that supply specific cell-to-cell movement functions. Long-distance transport of viruses through the vascular system is a less well understood process with requirements different from those of cell-to-cell movement. Usually viral coat protein (CP) is required for long-distance movement, but groundnut rosette umbravirus (GRV) does not code for a CP. However, this virus moves efficiently from cell to cell and long distance. We demonstrate here that the protein encoded by *ORF3* of GRV can functionally replace the CP of tobacco mosaic virus (TMV) for long-distance movement. In spite of low levels of virus RNA accumulation in infected cells, chimeric TMV with a replacement of the *CP* gene by GRV *ORF3* was able to move rapidly through the phloem. Moreover, this chimeric virus complemented long-distance movement of another CP-deficient TMV derivative expressing the gene encoding the green fluorescent protein. Thus, the GRV *ORF3*-encoded protein represents a class of trans-acting long-distance movement factors that can facilitate trafficking of an unrelated viral RNA.**

A rapidly growing body of evidence suggests that communications between cells and organs are fundamental for many general biological processes and phenomena in plants such as control of plant growth and development (1, 2), systemic acquired resistance to infection (3), and systemic gene silencing (2, 4, 5). It is believed that not only metabolic substrates but also macromolecules can move from cell to cell through plasmodesmata, the intercellular cytoplasmic channels (6, 7), and via the plant's long-distance transport system, the phloem (2, 4, 5). An example of cell-to-cell trafficking of endogenous plant macromolecules is the recent finding that the maize *knotted1 (kn1)* homeobox gene encodes a nuclear-functional transcriptional regulator, KN1, which moves between cells through plasmodesmata (1). Interestingly, KN1 also facilitates transport of its own mRNA. Endogenous plant macromolecules that are able to move long distances through the phloem have not yet been characterized. However, the sequence specificity of posttranscriptional gene silencing implies that the signals involved in systemic transmission of the silencing state are nucleic acids that, probably in association with some specific plant protein(s), can enter the vasculature of the plant, move long distances, and exit from the phloem (2, 4, 5).

It is suggested that plant viruses move from cell to cell and over long distances by exploiting and modifying these preexisting endogeneous pathways for macromolecular movement (1, 8). During the last 10 years, much information has been obtained on the role of specialized virus-encoded movement proteins (MP) in promoting the cell-to-cell spread of virus infection through plasmodesmata (reviewed in refs. 6–8). Several types of MP have been identified. Some viruses, such as tobacco mosaic virus (TMV), encode single MPs that modify plasmodesmata and facilitate transport of the MPs themselves and of nucleic acids through the modified channel (9–11). Some other groups of viruses encode MPs that form plasmodesmata-associated tubules through which virus particles move (12–14). Yet other viruses, such as potato virus X (PVX), contain a set of movement genes called the "triple gene block," which encodes three proteins that, together with the coat protein (CP), are proposed to function coordinately to transport viral RNA through plasmodesmata (15–17).

Much less is known about the molecular details of long-distance virus movement. It is not clear how viruses enter, move through, or exit the vascular system. Minor veins are generally sheathed by bundle sheath cells and contain various cell types including vascular parenchyma cells, companion cells and enucleate sieve elements (reviewed in ref. 18). Thus, transport of a virus to and within vascular tissue implies movement from mesophyll cells to bundle sheath cells, from bundle sheath cells to vascular parenchyma and companion cells, and entry into sieve elements. The exit from vascular tissue probably occurs in the reverse order. It has been suggested that the plasmodesmata between these types of cells differ from those interconnecting mesophyll cells (18). Analysis of virus–host systems in which systemic virus movement is impaired has provided evidence of the need for specific virus factors, different from the cell-to-cell MP, for trafficking through these types of plasmodesmata (8, 18). With only a few exceptions (19), CP is essential for efficient long-distance transport of plant viruses, because even in the rare cases where the *CP* gene is partially or wholly dispensable for systemic spread, the time required for systemic infection is often increased in its absence (20, 21). Although the precise role of CP in promoting movement via phloem remains to be determined, it may relate to its capacity to form virus particles. Several viruses also encode proteins that provide additional functions needed for systemic spread of infection. Mutations inactivating the p19 protein of tomato bushy stunt virus and the 2b protein of cucumber mosaic virus (CMV) prevented long-distance movement of these viruses in some hosts but not in others (21, 22). A mutation in a central region of the helper component proteinase (HC-Pro) of tobacco etch virus also prevented systemic spread (23). Additionally, some virus-encoded replication proteins appear to have specific roles in long-distance transport (24–26). However, the biochemical roles of these proteins in long-distance movement are not yet known; some may actually have only an indirect function in movement, such as suppressing host response that restricts systemic spread (8, 27, 28).

Members of the genus *Umbravirus*, such as groundnut rosette virus (GRV), represent a special situation because they

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Abbreviations: TMV, tobacco mosaic virus; GRV, groundnut rosette virus; CMV, cucumber mosaic virus; PV, potato virus; MP, movement protein; CP, coat protein; GFP, green fluorescent protein; DPI, days postinoculation; HC-Pro, helper component proteinase.

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do not code for a CP, but nonetheless accumulate and spread systemically very efficiently within infected 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 their systemic spread (29, 30). The RNA genome of GRV contains four ORFs. The two ORFs at the 5′ end of the RNA are expressed by a −1 frameshift to give a single protein that appears to be an RNA-dependent RNA polymerase (30). The other two ORFs overlap each other in different reading frames. *ORF4* encodes the 28-kDa cell-to-cell MP that contains stretches of similarity with several other viral MPs and accumulates in plasmodesmata (30, 31). Database searches with the sequence of the 27-kDa ORF3 protein revealed no significant similarity with any other viral or nonviral proteins, except the corresponding proteins encoded by other umbraviruses (30). In epidermal and mesophyll cells this protein targets nucleoli (31). Functional analysis of the GRV ORF3 protein described here suggests that it is a trans-acting, long-distance movement factor that can facilitate systemic transport of unrelated viral RNA in a nonvirion form.

MATERIALS AND METHODS

Plasmids, Generation of Chimeric cDNA Constructs, and Mutants. 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) (Fig. 1; see also ref. 31). This vector contains multiple cloning sites and an additional copy of the subgenomic promoter for the CP mRNA inserted between the genes for the MP (30-kDa protein) and the CP (Fig. 1). Plasmid pTXS.GFP (32) containing a cDNA insert encoding the jellyfish green fluorescent protein (GFP) was used as a template for PCR amplification of the *GFP* gene sequence. GRV cDNA clone grmp2 (30) was used for PCR amplification of GRV *ORF3* sequences. By using standard DNA manipulation techniques (33), the following constructs were generated. For pTMV-(ORF3) (Fig. 1), a single nucleotide substitution (T → C) was introduced into the plasmid grmp2 to change the initiation codon (A*U*G) of the *ORF4* located inside the GRV *ORF3* to

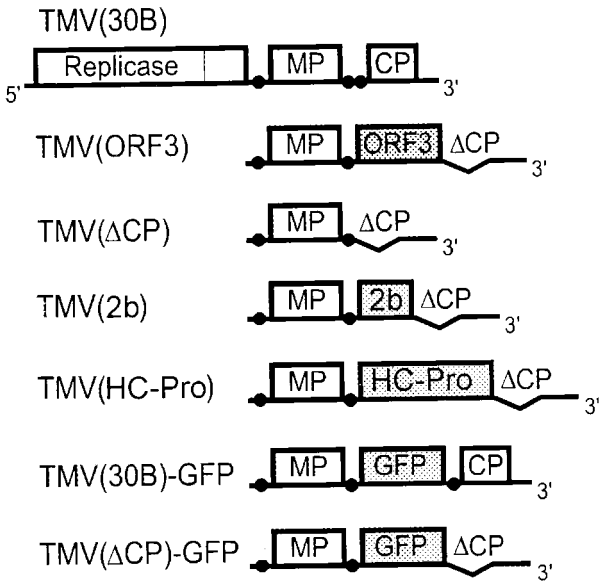

FIG. 1. Schematic representation of TMV-based vector TMV(30B) and its derivatives expressing GRV *ORF3*, CMV *2b* gene, PVY^O *HC-Pro* gene, or *GFP* gene with and without deletion of the *CP* gene. Boxes represent ORFs, lines represent untranslated sequences. MP, TMV movement protein; CP, TMV coat protein; ●, subgenomic promoters. Deleted sequences are indicated.

(A*C*G) by overlap-extension PCR (34) using a pair of complementary mutagenic primers, one of which was 5′-GTCAAGTGTAATAAACGTCTTCGCAAGTG-3′. This mutation is predicted to eliminate *ORF4*, but does not change the amino acid sequence encoded by *ORF3*. The fragment containing GRV *ORF3* was then amplified by using oligonucleotides 5′-CATGATCGATATGGACACCACCC-3′ and 5′-CATGCTCGAGTTACGTCGCTTTGC-3′ and cloned between the *Pme*I and *Xho*I sites of pTMV(30B). The *Pml*I-*Hpa*I fragment [nucleotides 5833–6465 of the pTMV(30B) sequence] carrying the native subgenomic promoter for the *CP* gene and the 5′ part of the *CP* gene was excised from the resulting plasmid to give pTMV(ORF3) (Fig. 1). The same fragment was excised from pTMV(30B) to give pTMV(ΔCP) (Fig. 1). For *pTMV(30B)-GFP* (Fig. 1), the *GFP* gene was amplified by using oligonucleotides 5′-GATCGTCGACATGAGTAAAGGAGAAG-3′ and 5′-GATCCTCGAGTTACGTCGCTTTGC-3′ and cloned into the *Xho*I site of pTMV(30B) to give pTMV(30B)-GFP. For pTMV(CP)-GFP, the *Xho*I-*Hpa*I fragment [nucleotides 5782–6465 of the pTMV(30B) sequence] of pTMV(30B)-GFP, carrying the subgenomic promoter and 5′ part of the *CP* gene, was excised to give pTMV(ΔCP)-GFP. For pTMV(noORF3), two point mutations predicted to eliminate an expression of the *ORF3* [nucleotide substitutions (T → G) in the initiation codon (A*U*G) and (T → C) in the 16th (methionine) codon (A*U*G)] were introduced into the plasmid pTMV(ORF3) by overlap-extension PCR using a pair of complementary mutagenic primers, one of which was 5′-GGTGGGTATCACGTCAAGTGTAATAAACGTCTTCG-3′. For pTMV(2b) (Fig. 1), the *2b* gene of CMV (strain Fny) was amplified from the plasmid pFny209 (35) by using oligonucleotides 5′-GGCCTTAATTAATGGAATTGAACGAAGGTG-3′ and 5′-GCATCTCGAGTTTCAGAAAGCACCTTCC-3′ and cloned between the *Pac*I and *Xho*I sites of pTMV(ΔCP). For pTMV(HC-Pro) (Fig. 1), the *HC-Pro* gene of potato virus Y (PVY^O) was obtained by reverse transcription–PCR on total RNA extracted from tobacco plants inoculated with PVY^O by using oligonucleotide 5′-GCATCTCGAGTTACTAACCAACCCTATAATG-3′ with a *Xho*I site preceding sequence complementary to that of a stop codon (UAA) and 18 nt of the 3′ end of the *HC-Pro* gene for first-strand cDNA synthesis and as a reverse primer. The oligonucleotide 5′-GGCCTTAATTAATGTCGAATGCTGATAATTTTTGG-3′ with a *Pac*I site and initiation codon (ATG) preceding 21 nt identical to those of the 5′-end of the *HC-Pro* gene was used as a forward primer. The amplified product was cloned between the *Pac*I and *Xho*I sites of pTMV(ΔCP).

All of the viruses derived from these constructs, designated by eliminating the prefix p in the names of the progenitor plasmids, were tested in *Nicotiana benthamiana* protoplasts. All replicated, but in agreement with previous reports (36, 37), the viruses lacking CP accumulated to significantly lower levels (data not shown).

***In Vitro* Transcription, Inoculation of Plants, and Isolation of Protoplasts.** Plasmids were linearized by digestion with *Kpn*I, and *in vitro* transcripts were synthesized with T7 RNA polymerase by using a mCAP RNA capping kit (Stratagene). The transcripts were inoculated directly to leaves of 3- to 4-week-old *N. benthamiana* plants by rubbing corundum-dusted leaves with the transcription products derived from 0.2 μg of plasmid template.

Biological assays of total nucleic acid extracts from inoculated and uninoculated leaves of *N. benthamiana* were conducted on *Nicotiana tabacum* L. cv. Xanthi nc, a local lesion host of TMV. Viral infectivity was determined as the average number of local lesions per half leaf.

Mesophyll protoplasts were isolated from fully expanded mature uninoculated leaves of plants infected with TMV-(ORF3) and TMV(30B) as described (38).

Analysis of RNA. Total RNA was isolated from leaf tissue or protoplasts as described (39). For Northern blot analysis, total RNA preparations were denatured with formaldehyde and formamide. Electrophoresis was performed in 1.5% agarose gels (33). RNA was transferred to Hybond N membrane and immobilized by UV crosslinking. For dot blot hybridization analysis, samples of RNA were spotted onto Hybond N nylon membrane. Hybridization was done as described (33) with ^{32}P-labeled RNA probes complementary to sequences of the TMV replicase gene [nucleotides 445-2675 of pTMV(30B)]. Quantitative analysis of dot blots was done by densitometry of the autoradiographic images using a BioImage (Ann Arbor, MI) Intelligent Quantifier Version 2.5.0. A dilution series of TMV RNA was used as concentration standard.

Detection of GFP Fluorescence in Plants. Plants were illuminated with long-wavelength UV light and photographed as described (32, 40). GFP fluorescence in plant tissues was viewed with a Bio-Rad MRC 1000 confocal laser scanning microscope. The methods used were as described (32, 40).

RESULTS

Symptom Induction by TMV(ORF3), a Hybrid TMV in Which GRV ORF3 Replaced the *CP* Gene. Full-length infectious clones of GRV are not yet available to carry out reverse-genetics analysis of GRV functions. Therefore, in this work we employed a gene-replacement strategy to generate hybrids between TMV and GRV. CP is not required for cell-to-cell movement of TMV but is essential for its long-distance movement (reviewed in ref. 8). The *CP* gene of TMV was deleted and replaced by *ORF3* of GRV in a TMV-based vector, TMV(30B), to give the hybrid TMV(ORF3) (Fig. 1). TMV(30B) and TMV(30B) with a deleted *CP* gene [TMV(ΔCP)] were used as controls (Fig. 1).

TMV(ΔCP) induced pale chlorotic spots on inoculated *N. benthamiana* leaves by 5 days postinoculation (DPI), but no systemic symptoms were observed in these plants even 5 weeks after inoculation. In contrast, TMV(30B) induced very severe systemic symptoms, first observed at 5 DPI. The infected plants were stunted, and showed strong mosaic and deformation of leaves. TMV(ORF3) also induced systemic symptoms on *N. benthamiana* plants. At approximately 7 DPI, expanding leaves at the top of the plant began to show some deformation followed by mild mosaic and rugosity at 10–12 DPI. These results suggest that despite lacking the *CP* gene, TMV(ORF3) had spread systemically.

Accumulation of TMV(ORF3) RNA in Inoculated and Systemically Infected Leaves. To verify that TMV(ORF3) RNA moves systemically, inoculated and upper uninoculated leaves were harvested and analyzed by inoculation of total nucleic acid extracts onto the hypersensitive host, *N. tabacum* L. cv. Xanthi nc. As expected, TMV(30B) RNA accumulated both in inoculated and in uninoculated systemically infected leaves (Table 1). Both TMV(ΔCP) and TMV (ORF3) RNAs also accumulated in inoculated leaves, but only TMV(ORF3) spread systemically (Table 1). It should be noted, however, that levels of accumulation of both viruses lacking CP [TMV(ΔCP) and TMV(ORF3)] were significantly lower compared with those of TMV(30B), probably because of the reduced stability of unprotected RNA. However, in spite of the low level of accumulation, TMV(ORF3) was first detected in uninoculated leaves 4 DPI, the same time as for TMV(30B) (Table 1), implying that both viruses move long distances equally rapidly. TMV(ΔCP) was not detected in uninoculated leaves even 30 DPI.

Northern blot analysis of RNA samples isolated from the inoculated and uninoculated leaves confirmed the results of the biological assays, indicating that despite poor accumulation, TMV(ORF3) RNA spread systemically in *N. benthamiana* plants (Fig. 2). To test directly whether TMV(ORF3) is able not only to move rapidly to uninoculated leaves but also to exit from the vascular system and spread into mesophyll tissues, mesophyll protoplasts from uninoculated systemically infected leaves were isolated. RNA extracted from these protoplasts was analyzed by dot-blot hybridization. As shown in Table 2, viral RNA was detected in protoplasts isolated from leaves systemically infected with either TMV(30B) or TMV(ORF3). However, the amount of the TMV(ORF3) RNA was approximately 1/11 that of TMV(30B) RNA. Quantitation of viral RNA isolated from intact systemically infected leaves revealed a similar ratio (about 1:13) between the levels of accumulation of TMV(ORF3) RNA and TMV(30B) RNA. These results suggest that TMV(ORF3) is able not only to move from inoculated to uninoculated leaves but also to exit from the vascular system.

To determine the role of the ORF3 protein product in the long-distance movement of the hybrid virus [TMV(ORF3)], TMV(noORF3) was generated carrying the same GRV sequences as TMV(ORF3) (Fig. 1), except that the two potential translation start sites were mutated from AUG to AGG and ACG respectively. TMV(noORF3) was able to multiply in inoculated leaves to the levels of TMV(ORF3) but did not induce symptoms or accumulate in uninoculated leaves (Table 1). The failure of TMV(noORF3) to spread systemically

Table 1. Accumulation of viral RNA in *N. benthamiana* plants inoculated with chimeric TMV-based viruses

	DPI					
	3		4		14	
Inoculum	i	u	i	u	i	u
Series 1						
TMV (30B)	46 ± 11	0	128 ± 7	59 ± 4	111 ± 19	189 ± 31
TMV (ΔCP)	9 ± 4	0	24 ± 5	0	31 ± 7	0
TMV (ORF3)	8 ± 3	0	12 ± 6	12 ± 3	22 ± 4	24 ± 5
TMV (30B)-GFP	42 ± 6	nt	62 ± 4	nt	75 ± 13	nt
TMV (ΔCP)-GFP	7 ± 2	nt	12 ± 5	nt	15 ± 8	nt
Series 2						
TMV (noORF3)	12 ± 3	0	14 ± 3	0	25 ± 4	0
TMV (ORF3)	14 ± 2	0	15 ± 3	17 ± 4	27 ± 6	18 ± 4

Data are infectivities as average number of lesions per half-leaf of *N. tabacum* cv. Xanthi nc ± SD from three independent experiments with three replicate plants in each. Total nucleic acid extracts, obtained after different intervals postinoculation (3 DPI, 4 DPI, 14 DPI) from 0.1 g of tissue from *N. benthamiana* plants infected with chimeric viruses, were used as inocula. i, total nucleic acid extracts were obtained from inoculated leaves. u, total nucleic acid extracts were obtained from uninoculated leaves. nt, not tested.

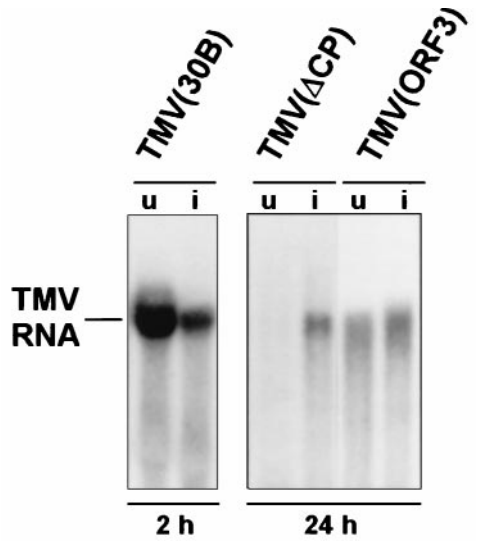

FIG. 2. Representative Northern blot analysis of viral RNAs isolated from inoculated (i) and uninoculated (u) leaves of *N. benthamiana* plants infected with TMV(30B), TMV(ΔCP), and TMV-(ORF3) (9 DPI), as indicated. Exposure time for autoradiography is indicated, and the position of TMV genomic RNA is marked.

indicates that expression of the ORF3 product, rather than the RNA sequence itself, is required for long-distance movement of TMV(ORF3).

In a separate series of experiments, the effects of CMV *2b* and PVY^O *HC-Pro* genes on systemic spread of TMV(ΔCP) were tested. In contrast with the results on TMV(ORF3), TMV(2b) and TMV(HC-Pro) expressing the *2b* gene of CMV or the *HC-Pro* gene of PVY^O, respectively (Fig. 1), multiplied efficiently in inoculated leaves, but could not be detected in uninoculated leaves even 30 DPI, indicating that they were unable to spread systemically (data not shown).

Cell-to-Cell and Long-Distance Movement of TMV(30B)-GFP and TMV(ΔCP)-GFP. GFP is often used as a noninvasive reporter to monitor virus infections (32, 40, 41). The *GFP* gene was inserted into the genomes of TMV(30B) and TMV(ΔCP) to give TMV(30B)-GFP and TMV(ΔCP)-GFP, respectively (Fig. 1). In inoculated leaves of *N. benthamiana,* TMV(ΔCP)-GFP caused the development of green fluorescent foci, which were clearly visible under long-wavelength UV light, starting at 3 DPI. Similar foci appeared at the same time in leaves inoculated with TMV(30B)-GFP. However, the rate of enlargement of fluorescent foci induced by TMV(ΔCP)-GFP (Fig. 3*B*) was significantly higher compared with those induced by TMV(30B)-GFP (Fig. 3*A*). In contrast, biological assays conducted on total nucleic acid extracts from inoculated leaves showed that TMV(30B)-GFP RNA accumulated to much higher levels than TMV(ΔCP)-GFP RNA (Table 1). Thus, it seems that, in spite of the low rates of RNA accumulation, TMV(ΔCP)-GFP moves from cell to cell in inoculated leaves more efficiently than TMV(30B)-GFP. One explanation for this difference may be that the gene encoding the cell-to-cell MP (30-kDa protein) is less highly expressed in TMV(30B)-

Table 2. The presence of viral RNA in mesophyll cells of leaves systemically infected with TMV(ORF3)

	Source of viral RNA	
Inoculum	Leaf tissues, μg/gram of leaf	Mesophyll protoplasts, ng per 10^6 protoplasts
TMV (ORF3)	3 ± 0.2	28 ± 6
TMV (30B)	38 ± 4	320 ± 30

Viral RNA was quantitated by dot blot hybridization using a dilution series of TMV RNA as concentration standard. Data are mean ± SD from three independent experiments with three replicate plants in each.

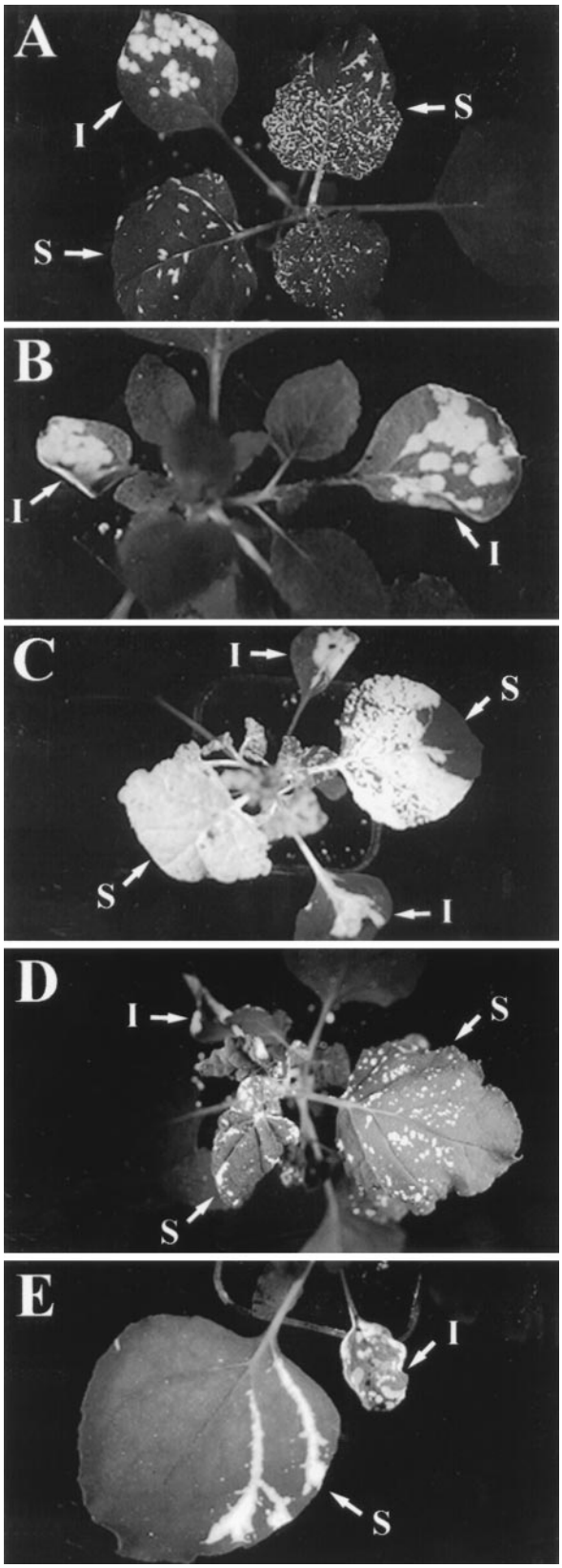

FIG. 3. *N. benthamiana* plants photographed under long-wavelength UV light 8 days (*A* and *B*) and 12 days (*C–E*) after infection with TMV(30B)-GFP (*A* and *C*), TMV(ΔCP)-GFP (*B*), or TMV(ΔCP)-GFP + TMV(ORF3) (*D* and *E*). Inoculated (I) and systemically infected (S) leaves are indicated.

GFP, for example because of its more distant position from the 3′ end of the RNA. Another possibility is that, in the presence of CP, formation of virus particles may diminish cell-to-cell movement (by sequestering RNA) and cause a switch to long-distance transport.

After the development of fluorescent foci in the inoculated leaves, subsequent systemic infection by TMV(30B)-GFP led to the appearance of green fluorescence in the uninoculated leaves at 7 DPI (Fig. 3 *A* and *C*). In contrast, as expected, no systemic infection by TMV(ΔCP)-GFP occurred (Fig. 3*B*), and fluorescence in the uninoculated leaves was never observed.

Complementation of the Long-Distance Movement Defect of the TMV CP-Deletion Mutant by TMV(ORF3). All attempts to generate infectious TMV derivatives producing both GRV ORF3 protein and GFP were unsuccessful. Therefore, complementation of the long-distance movement defect of TMV(ΔCP)-GFP by TMV(ORF3) was tested. TMV(ΔCP)-GFP was coinoculated with TMV(ORF3) onto *N. benthamiana.* The majority (12/16) of the doubly infected plants showed systemic symptoms characteristic of TMV(ORF3) and developed green fluorescent zones generated by TMV(ΔCP)-GFP in both inoculated and uninoculated leaves (Fig. 3 *D* and *E*), implying systemic spread of TMV(ΔCP)-GFP in the presence of TMV(ORF3). In inoculated leaves, fluorescent spots induced by TMV(ΔCP)-GFP in the presence or absence of TMV(ORF3) were practically indistinguishable, but in uninoculated leaves the fluorescence appeared only in the case of mixed TMV(ΔCP)-GFP + TMV(ORF3) infection. In doubly inoculated plants, the first indication of entry of TMV(ΔCP)-GFP into an uninoculated leaf was the appearance of fluorescent flecks along veins on the lamina, indicating that the virus was being unloaded at discrete foci. After the appearance of these fluorescent flecks, some leaf veins became more clearly delineated by fluorescence (Fig. 3*E*), and with time the mesophyll tissues neighboring the flecks also became labeled (Fig. 3 *D* and *E*). Confocal laser scanning microscopy confirmed these observations and showed that up to 90% of mesophyll cells in the fluorescent area were infected with TMV(ΔCP)-GFP. The time of appearance of GFP fluorescence (≈8 DPI) and the pattern of virus unloading in uninoculated leaves observed in mixed TMV(ΔCP)-GFP + TMV(ORF3) infections were similar to those observed for TMV(30B)-GFP (Fig. 3 *A* and *C*) and corresponded to the usual manner of long-distance virus movement associated with the vascular system (41). Because TMV(ΔCP)-GFP was unable to move long distances alone, these results suggest that TMV(ORF3) can complement long-distance movement of TMV(ΔCP)-GFP. However, the number of initial fluorescent flecks in uninoculated leaves generated as a result of complementation of TMV(ΔCP)-GFP by TMV(ORF3), and the extent of their spread, were usually lower than in the case of TMV(30B)-GFP infection and varied significantly from leaf to leaf (Fig. 3*C* vs. 3 *D* and *E*), probably reflecting differences in efficiencies of complementation that may depend on numerous factors including interference between virus variants. TMV(ORF3) does not depend on TMV(ΔCP)-GFP for replication and spread and therefore may sometimes outcompete it, decreasing the efficiency of the complementation.

To confirm that the effect on systemic spread of TMV(ΔCP)-GFP was based on complementation rather than on recombination, the virus RNA progeny that accumulated in the uninoculated leaves of the doubly infected plants was analyzed by back inoculation to a local lesion host of TMV, *N. tabacum* cv. Xanthi nc. Subsequent transfer of virus from individual lesions to a systemic host, *N. benthamiana*, produced one of two phenotypes characteristic of each the original viruses: either systemic symptoms without fluorescence [TMV(ORF3)] or no systemic symptoms and fluorescence in inoculated but not in uninoculated leaves [TMV(ΔCP)-GFP]. No plants displayed fluorescence in uninoculated leaves as would be expected if recombinants containing both GFP and *ORF3* had been generated.

These results clearly show that GRV ORF3 protein expressed from TMV(ORF3) can mediate, in *trans* as well as in *cis*, the long-distance movement of RNA of the unrelated virus, TMV.

DISCUSSION

Previous investigations revealed that cell-to-cell movement and long-distance transport of plant viruses are distinct processes with different requirements (reviewed in ref. 8). Recently, it has been shown that the ORF4 protein of GRV facilitates cell-to-cell movement (31). Here, we demonstrate that another GRV nonstructural protein, encoded by *ORF3*, provides a specific trans-active function in vascular-associated long distance transport. This protein can functionally replace TMV CP, which is critical for phloem-dependent spread of TMV (42–49). Recently, it has been found that, at least in *N. tabacum*, CP is not required for TMV to penetrate from bundle sheath cells into vascular parenchyma cells, the presumed first step in the process of phloem-dependent movement, but that CP is required for further movement into the companion cell/sieve elements complex (49). Thus, results presented here suggest that the GRV ORF3 protein may control entry into the vascular system at the level of the companion cell/sieve elements complex (49) and perhaps also exit of infective material from phloem to mesophyll cells in systemically infected leaves.

ORF3 has been found in all three (GRV, pea enation mosaic virus 2, and carrot mottle mimic virus; refs. 30, 50, and 51) umbraviruses sequenced to date. The deduced amino acid sequences of the corresponding proteins also are conserved (30). Analysis of the amino acid sequences of the ORF3 proteins by using the programs PILEUP and PEPTIDESTRUCTURE revealed that the most conserved central region consists of a rather basic and highly hydrophilic domain (amino acids 108–130), which seems to be exposed on the protein surface, and a hydrophobic part (amino acids 151–180). One can speculate that the basic hydrophilic domain may possess RNA-binding capacity. However, a database search with the sequences of these proteins has revealed no significant similarity with any other known viral or nonviral protein (30).

Several other plant virus proteins, such as the 2b protein of CMV, the HC-Pro protein of tobacco etch virus and probably of other potyviruses, and the p19 protein of tomato bushy stunt virus, have also been shown to be involved in systemic virus spread (21–23). All of these proteins have been demonstrated to be pathogenicity determinants of the respective viruses (21, 22, 52, 53). They also enhance the accumulation and symptoms of PVX when they are expressed from PVX vectors (28, 52, 53). In contrast to these proteins, the GRV ORF3 protein expressed from a PVX vector has no effect on systemic infection by PVX (unpublished data). Recently, direct evidence has been reported that the 2b and HC-Pro proteins can suppress posttranscriptional gene silencing (27, 28). It has been suggested that they act by blocking a potential host-defense mechanism (akin to gene silencing) that restricts systemic spread (28) rather than by promoting the process of long-distance virus movement itself. In accordance with this suggestion, the CMV 2b and PVY^{O} HC-Pro proteins were unable to replace functionally TMV CP, which is directly involved in phloem-associated long distance movement.

Thus, the GRV ORF3 protein represents another class of trans-acting long-distance RNA movement factors, and is a nonstructural viral protein that can accomplish long-distance movement of an unrelated viral RNA. However, a prerequisite for ORF3 protein-directed long-distance spread is effective cell-to-cell movement of the dependent RNA. Thus, GRV ORF3 protein could not functionally replace CP for long distance

movement of PVX RNA, because PVX CP also is required for cell-to-cell movement (31).

Another interesting feature of the GRV ORF3 protein is that, because of the inability of GRV to form virus particles, this protein must be adapted to transport RNA in nonvirion form. This process may closely resemble long-distance transport of endogenous plant macromolecules. Plant virus evolution might have involved the acquisition of cellular genes (54), and it is possible that the putative plant long-distance movement factors that operate in endogenous plant-transport systems were the progenitors to the GRV ORF3 protein. However, GRV *ORF3* almost completely overlaps *ORF4*, and this arrangement seems typical in umbraviruses (30). The ORF4 protein is a cell-to-cell movement protein that has clear similarities in sequence with the MPs of other plant viruses (30), and all of these MPs probably share a common origin. The *ORF3* sequence, however, seems unique to the umbraviruses and has most likely arisen as a result of "overprinting" (55) on *ORF4* to give a functional, and perhaps structural, analogue of the hypothetical cellular long-distance transport factor. Thus, umbraviruses might have evolved from a virus that had conventional cell-to-cell *MP* and *CP* genes. Once the ancestral umbravirus had developed an *ORF3* and acquired the ability for its RNA to be packaged by helper virus CP and thereby transmitted by the vector of the helper virus (D.J.R., E.V.R., S. K. Raj, I. M. Roberts, and M.E.T., unpublished results), its own CP became expendable.

On a practical level, expression in transgenic plants of the ORF3 protein may constitute a powerful approach to the modulation of plant transport processes. It may also be valuable in the design, environmental containment, and complementation of plant virus vectors to produce pharmaceutical or industrial proteins.

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