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Intracellular Location of Two Groundnut Rosette Umbravirus Proteins Delivered by PVX and TMV Vectors

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The proteins encoded by open reading frames (ORF) 3 and 4 of groundnut rosette umbravirus (GRV) were expressed in *Nicotiana benthamiana* as fusions with green fluorescent protein (GFP) from modified potato virus X (PVX) and tobacco mosaic virus (TMV) vectors. Regardless of which plant virus vector was used, GFP fused to the ORF3 protein accumulated in large cytoplasmic inclusion bodies and in nucleoli, whereas GFP fused to the ORF4 protein was found in cell walls close to plasmodesmata. Cell-to-cell movement of PVX requires three proteins encoded by the triple gene block (TGB) and also the coat protein (CP). However, when GRV ORF4 was substituted for the PVX CP gene, the hybrid virus was able to move normally in inoculated leaves but not into noninoculated leaves. In contrast, when GRV ORF4 was substituted for the TGB, or for both the TGB and the CP gene, movement of the hybrid viruses was limited to a few epidermal cells neighboring the infection site. Thus, the GRV ORF4 protein can replace the movement proteins of PVX for some of their functions.

INTRODUCTION

To establish a systemic infection, plant viruses must move from sites of initial infection to the rest of the plant. The spread of the infection proceeds in two distinct modes: (i) cell-to-cell movement through plasmodesmata and (ii) long-distance transport through vascular tissues. Because virus movement is a crucial event in plant–virus interactions, its mechanisms have received much attention recently (for reviews see Citovsky, 1993; Leisner and Turgeon, 1993; Lucas and Gilbertson, 1994; Lucas, 1995; Carrington *et al.,* 1996).

It is generally accepted that cell-to-cell movement involves both virus-encoded "movement proteins" (MPs) and host-encoded components (Atabekov and Taliansky, 1990). Although the host factors remain unidentified, viral MPs have been identified for a number of plant viruses. It has also been shown that different MPs may facilitate cell-to-cell movement by different mechanisms. For some viruses, for example, tobacco mosaic tobamovirus (TMV), the MP interacts with plasmodesmata, increasing their permeability, and facilitates trafficking of the viral RNA in nonvirion form (Atkins *et al.,* 1991; Wolf *et al.,* 1989; Citovsky and Zambryski, 1991). TMV does not require coat protein (CP) for cell-to-cell movement but does require it for long-distance movement *via* vascular tissues (Hilf and Dawson, 1993). For other viruses, such as cowpea mosaic comovirus (CPMV), the MP forms tubular structures extending through plasmodesmata of infected cells, which are believed to facilitate cell-to-cell spread of CPMV in the form of virions (Van Lent *et al.,* 1990, 1991; Wellink and Van Kammen, 1989). Unlike viruses that produce a single MP, the genomes of potex-, carla-, hordei-, and some furoviruses contain the so-called triple gene block (TGB) encoding three proteins required for movement (Beck *et al.,* 1991; Petty and Jackson, 1990; Angell *et al.,* 1996). The potexviruses also require CP for cell-to-cell movement (Oparka *et al.,* 1996a; Chapman *et al.,* 1992a; Forster *et al.,* 1992; Sit and AbouHaidar, 1993).

Despite differences in mechanisms of movement, spread of a virus in a plant may be facilitated by the MP of another unrelated virus; i.e., the MPs coded by different plant viruses are often (although not always) functionally interchangeable (Atabekov and Taliansky, 1990; Ziegler-Graff *et al.,* 1991; De Jong and Ahlquist, 1992; Giesman-Cookmeyer *et al.,* 1995; Cooper *et al.,* 1996; Solovyev *et al.,* 1996; Morozov *et al.,* 1997; De Jong *et al.,* 1997).

It is suggested that viruses move from cell to cell and over long distances by exploiting and modifying preexisting pathways for macromolecular movement between cells and throughout the plant (Lucas *et al.,* 1995; Carrington *et al.,* 1996). Studies of different mechanisms for virus movement are likely to shed light on the general phenomenon of intercellular communication.

Groundnut rosette umbravirus (GRV) is one of a few plant viruses that do not form conventional virus particles but nonetheless accumulates and spreads very efficiently within infected plants (Reddy *et al.,* 1985; Taliansky *et al.,* 1996). GRV RNA contains four large open reading frames (ORFs) (see Fig. 1A). The two ORFs at the

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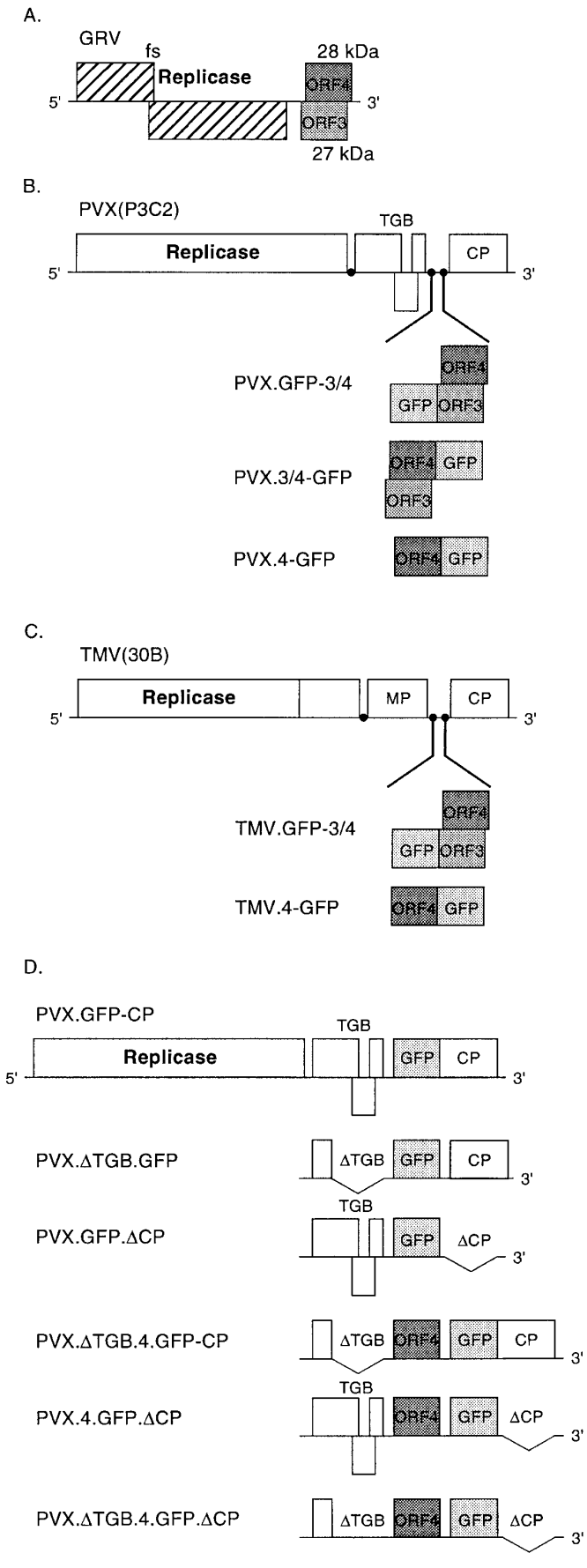


FIG. 1 Schematic representation of GRV genome (A), PVX expressing GRV genes with (D) and without (B) deletions of the TGB and CP genes (corresponding plasmids are designated by substitution of the prefix pTXS for the prefix PVX) and TMV expressing GRV genes (C). Boxes represent open reading frames, lines represent untranslated sequences. The position of frameshift is marked "fs"; TGB, triple gene block; CP, coat protein; MP, movement protein; GFP, gene for green fluorescent protein; ●, subgenomic promoters. Proteins encoded by ORF3 and ORF4 of GRV are marked 27 and 28 kDa, respectively. The sequences containing GRV ORF3 and ORF4 fusions with GFP were inserted into a cloning site located downstream from an additional subgenomic promoter. In PVX.GFP–CP and PVX.ΔTGB.4.GFP–CP, the GFP and CP genes are connected by a linker coding for 2A self-cleavable peptide sequence (Santa Cruz *et al.,* 1996).

5′ end of the RNA are expressed by a -1 frameshift to give a single protein, which appears to be an RNA-dependent RNA polymerase. The other two ORFs overlap each other in different reading frames. The function of the ORF3 27-kDa protein is uncertain, and it shows no significant similarity with any other viral or nonviral proteins (Taliansky *et al.,* 1996). The sequence of the ORF4 28-kDa protein contains stretches of similarity with those of MPs of several other plant viruses, in particular, with the sequence of the 3a MP of cucumber mosaic cucumovirus (CMV) (Taliansky *et al.,* 1996). However, the mechanism of movement of GRV differs from that of CMV because whereas CP plays an essential role in the systemic spread of CMV (Suzuki *et al.,* 1991; Boccard and Baulcombe, 1993; Taliansky and Garcia-Arenal, 1995), GRV encodes no CP. It cannot be excluded that the ORF3 27-kDa protein of GRV is also involved in viral movement.

To study the functions of GRV ORF 3 and 4 proteins in virus movement, we have employed a strategy in which a foreign gene is expressed from a heterologous plant virus vector. Using this approach, GRV ORF3 and ORF4 have been expressed from potato virus X (PVX) or TMV, which are suitable vectors for gene expression in plants (Chapman *et al.,* 1992b; W. O. Dawson, unpublished results). To detect the GRV-encoded proteins, we used fusions between these proteins and the jellyfish green fluorescent protein (GFP) (Prasher *et al.,* 1992). GFP has been introduced as a noninvasive reporter in a range of systems including plant viruses (Baulcombe *et al.,* 1995; Oparka *et al.,* 1996b). We also studied the functional compatibility between the MPs of GRV and PVX.

RESULTS

Expression of GRV ORFs from the PVX vector

In the first series of experiments, the GFP gene was fused to the GRV sequence coding for both ORF3 and ORF4, in two different configurations: (i) to the 5′ end of ORF3 to give a fusion of GFP with the 27-kDa protein and (ii) to the 3′ end of ORF4 to give a fusion of GFP with the 28-kDa protein (Fig. 1B). The recombinant genes ob-

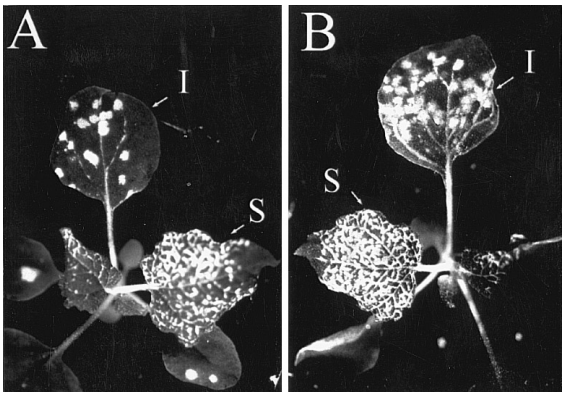


FIG. 2. *Nicotiana benthamiana* plants photographed under long-wavelength UV light 9 days after infection with PVX.GFP-3/4 (A) and PVX.4-GFP (B) showing fluorescent foci in inoculated leaves (I) and fluorescence associated with veins in systemically infected leaves (S).

tained were cloned into the PVX-based expression vector pTXS.P3C2 (Baulcombe *et al.,* 1995) to give pTXS.GFP-3/4 and pTXS.3/4-GFP, respectively (Fig. 1B).

In vitro runoff transcripts synthesized from these plasmids were infective when inoculated to *N. benthamiana* and *N. tabacum* plants. Viruses derived from such infections are subsequently indicated by substitution of the prefix PVX for the prefix pTXS in the name of the progenitor plasmid. All the plants infected with PVX.GFP-3/4 and PVX.3/4-GFP showed typical PVX symptoms in the inoculated and systemically infected leaves. Moreover, PVX.GFP-3/4 caused the development in inoculated leaves of green fluorescent foci, which were clearly visible under long-wavelength UV light starting on the third day postinoculation (d.p.i.). Subsequent systemic infection led to the appearance of green fluorescence in the noninoculated leaves (Fig. 2A). The rate of enlargement of the fluorescent foci in inoculated leaves and the time of appearance of fluorescence in systemic leaves were comparable with those previously described for infection with the GFP expression vector PVX.GFP (Baulcombe *et al.,* 1995). In contrast, green fluorescence was not detected in plants inoculated with PVX.3/4-GFP, although this virus was highly infective. One possibility was that ORF4 was not expressed from the subgenomic RNA, in which the initiation codon of GRV ORF4 was preceded by 61 nucleotides of GRV ORF3. Indeed, Western blot analysis, using antibodies raised against the ORF4 28-kDa protein produced in *Escherichia coli,* did not detect this protein in plants inoculated with PVX.3/4-GFP or PVX.GFP-3/4 (data not shown). To overcome this difficulty, we designed plasmid pTXS.4-GFP containing the ORF4 region fused to the 5′ end of the GFP gene, but lacking the ORF3 fragment preceding the initiation codon of ORF4 (Fig. 1B). PVX.4-GFP, obtained from this plasmid, was infective when inoculated to *N. benthamiana* and *N. tabacum.* The development of green fluorescence in the inoculated and non-inoculated leaves (Fig. 2B) was comparable with that produced by PVX.GFP-3/4 (Fig. 2A).

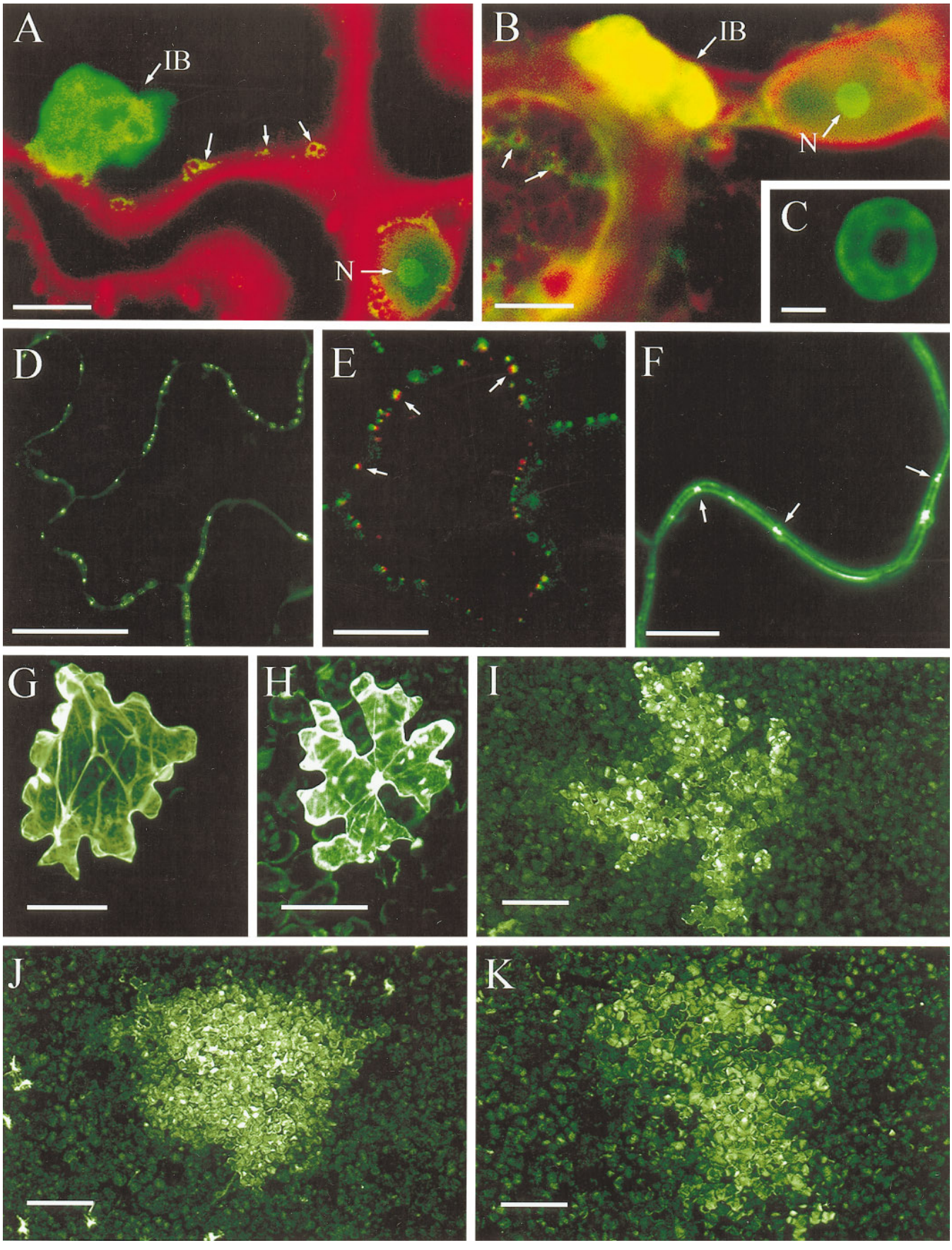
GFP fusion with GRV ORF3 protein forms cytoplasmic granules and accumulates in nucleoli

Confocal laser scanning microscopy of infected *N. benthamiana* and *N. tabacum* leaves was used to monitor the intracellular localization of the GFP-tagged GRV proteins. The green fluorescence in epidermal and mesophyll cells of both *N. benthamiana* and *N. tabacum* infected with PVX.GFP-3/4 was present mainly in large cytoplasmic inclusion bodies (Fig. 3A) frequently associated with nuclei. These structures appeared to be similar to the virus-induced amorphous inclusion bodies characteristic of PVX-infected cells (Shalla and Shepard, 1972; Boevink *et al.,* 1996). Sometimes the inclusion bodies contained membranous vesicles surrounded by GFP-containing granules (Fig. 3A). Similar granules were also located throughout the cytoplasm in association with membranes of the endoplasmic reticulum that were stained with the fluorescent probe rhodamine B hexyl ester (Fig. 3A). Free GFP produced from PVX.GFP (Baulcombe *et al.,* 1995) was shown to accumulate in the cytoplasm diffusely, so the ability to form granular aggregates could be attributed to the fusion with the GRV ORF3 protein. Interestingly, green fluorescence attributable to the GFP-tagged ORF3 protein was also detected in nucleoli (Fig. 3A).

Experiments using the TMV-based construct, TMV.GFP-3/4 (Fig. 1C), showed practically the same localization of GFP fusion with the ORF3 protein as the PVX vector. GFP was detected in cytoplasmic granules, in nucleoli and in inclusion bodies, which might be the amorphous "viroplasms" induced by TMV (e.g., Esau and Cronshaw, 1967; Matthews, 1981) (Fig. 3B and 3C).

GFP fusion with GRV ORF4 protein localizes to plasmodesmata

The fusion of the GRV ORF4 protein with GFP produced in *N. benthamiana* and *N. tabacum* plants infected with PVX.4-GFP was found in cell walls of epidermal and mesophyll cells (Fig. 3D). The sites of green fluorescence were located in close proximity to plasmodesmata detected by immunofluorescent staining of callose, an integral part of plasmodesmata (Northcote *et al.,* 1989; Lucas *et al.,* 1993), using fluorescent anti-callose antibodies (Fig. 3E). The same localization of the GFP-tagged ORF4 protein was observed when *N. benthamiana* plants were inoculated with TMV.4-GFP (Fig. 3F). These results suggested the targeting of the GRV ORF4 protein to plasmodesmata.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Cell-to-cell spread of movement-deficient PVX mutants is rescued by ORF4 of GRV

The cell-to-cell movement of PVX requires all three proteins encoded by the TGB and also the coat protein (Chapman *et al.,* 1992a; Angell *et al.,* 1996; S. Santa Cruz, unpublished results). To examine functional interchangeability between these PVX proteins and the ORF4 protein of GRV, either the PVX TGB or the CP gene or both were replaced by GRV ORF4 (Fig. 1D). The GFP gene was inserted into genomes of these chimeric viruses as a reporter to monitor the course of infection. The GFP was expressed either as a fusion with PVX CP or as a free protein in the case of constructs lacking the CP gene. The ability of recombinant PVX coding for free GFP or GFP-tagged CP to move from cell-to-cell and systemically has been demonstrated previously (Baulcombe *et al.,* 1995; Santa Cruz *et al.,* 1996).

In control experiments, *N. benthamiana* plants were inoculated with PVX.ΔTGB.GFP or PVX.GFP.ΔCP (Fig. 1D) (S. Santa Cruz, unpublished results; Baulcombe *et al.,* 1995), in which the TGB or CP gene, respectively, were deleted. Illumination with long-wavelength UV light and confocal laser scanning microscopy showed that, as expected (Chapman *et al.,* 1992a; Angell *et al.,* 1996; S. Santa Cruz, unpublished results), deletion of either the TGB or the CP gene in the PVX genome led to inability of the virus to move from cell to cell. Infection was restricted to single inoculated epidermal cells as shown in Figs. 3G and 3H, respectively.

To study possible rescue of virus movement of these recombinant PVX derivatives by GRV ORF4 protein, the following constructs were generated, based on the vector plasmid pTXS.P3C2, and all carrying GRV ORF4 under transcriptional control of a duplicated subgenomic promoter of the CP gene: (i) pTXS.ΔTGB.4.GFP–CP lacking the TGB and containing a fusion between the GFP and the PVX CP, (ii) pTXS.4.GFP.ΔCP lacking the CP gene and coding for free GFP, and (iii) pTXS.ΔTGB.4.GFP.ΔCP with deleted TGB and CP gene and coding for free GFP (Fig. 1D). The replication of *in vitro*-generated transcripts from these plasmids was assayed in *N. benthamiana* protoplasts. Figure 4 shows that all constructs were replicated. However, in agreement with a previous report

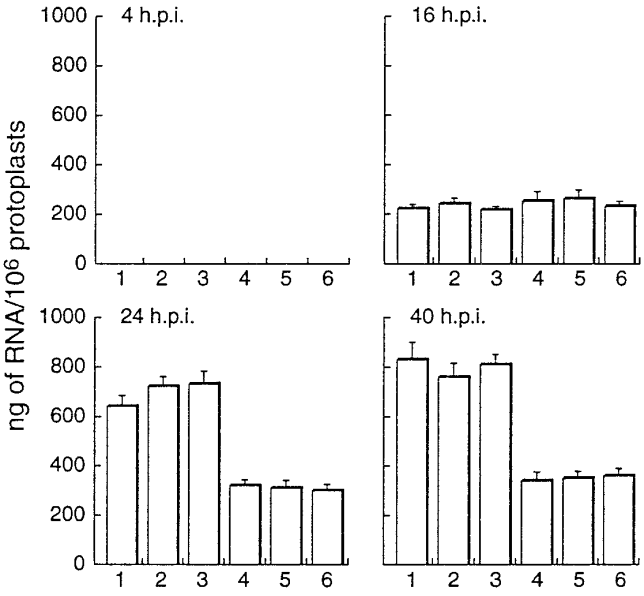

FIG. 4. Time course of PVX genomic RNA accumulation in protoplasts of *N. benthamiana* inoculated with PVX.GFP–CP (1), PVX.ΔTGB.GFP (2), PVX.ΔTGB.4.GFP–CP (3), PVX.GFP.ΔCP (4), PVX.4.GFP.ΔCP (5), and PVX.ΔTGB.4.GFP.ΔCP (6). Accumulation of RNA was determined by quantitation of dot blots of extracts made 4, 16, 24, and 40 h post inoculation (h.p.i.) Data are means and standard deviation from two independent experiments each with three replicates.

(Chapman *et al.,* 1992a), CP deletions (PVX.GFP.ΔCP, PVX.4.GFP.ΔCP, PVX.ΔTGB.4.GFP.ΔCP) resulted in some reduction of PVX accumulation compared with that of transcripts containing CP (PVX.GFP–CP, PVX.ΔTGB.GFP, PVX.ΔTGB.4.GFP–CP).

Confocal laser scanning microscopy of leaves inoculated with PVX.ΔTGB.4.GFP–CP showed that the chimeric PVX lacking TGB but expressing GRV ORF4 protein was able to spread from initially inoculated cells to neighboring ones (Fig. 3I). Until 2–3 d.p.i., the growth of infection foci in the epidermis occurred at a rate similar to that observed with PVX.GFP–CP (Table 1). After that time, the spread of PVX.ΔTGB.4.GFP–CP slowed down compared to that of PVX.GFP–CP (Table 1). Only a small proportion of mesophyll cells underlying epidermal sites of PVX.ΔTGB.4.GFP–CP infection contained virus (Table 1). Thus, this virus was able to move from cell to cell in

FIG. 3. The development of green fluorescence in cells and tissues infected with GFP-expressing derivatives of PVX and TMV containing GRV ORFs. (A–F) Confocal images of *Nicotiana tabacum* (A, D, E) and *N. benthamiana* (B, C, F) epidermal cells infected with PVX and TMV derivatives expressing GFP-tagged ORF3 and ORF4 proteins of GRV. (A, B) Intracellular localization of the GFP-ORF3 expressed from PVX (A) and TMV (B) protein in an inclusion body (IB) in the cytoplasm and also in the nucleolus (N). Unlabeled arrows indicate membranous vesicles surrounded by GFP–ORF3 protein. Membranes, stained with rhodamine B hexyl ester, appear red. Bars, 10 μm. (C) Nucleolus targeted by GFP–ORF3 protein expressed from TMV. Bar, 2 μm. (D) Localization in plasmodesmata of the ORF4–GFP fusion protein expressed from PVX. Bar, 50 μm. (E) Colocalization of the ORF4–GFP fusion protein expressed from PVX with plasmodesmata labeled with fluorescent antibodies to callose (presented in red and indicated by arrows). Bar, 25 μm. (F) Localization in plasmodesmata of the ORF4–GFP fusion protein expressed from TMV. Bar, 10 μm. (G–K) Confocal images of *N. benthamiana* leaves infected with PVX derivatives containing GFP and GRV ORF4. The recombinant viruses were expressing the GFP gene (G, H, J, K) or GFP–CP fusion gene (I) as reporters. (G) Single epidermal cell infected with PVX.ΔTGB.GFP, 3 d.p.i. Bar, 100 μm. (H) Single epidermal cell infected with PVX.GFP.ΔCP, 3 d.p.i. Bar, 100 μm. (I) A multiple-cell infection site of PVX.ΔTGB.4.GFP-CP, 3 d.p.i. Bar, 250 μm. (J) A multiple-cell infection site of PVX.4.GFP.ΔCP, 3 d.p.i. Bar, 250 μm. (K) A multiple-cell infection site of PVX.ΔTGB.4.GFP.ΔCP, 3 d.p.i. Bar, 250 μm.

TABLE 1

Analysis of Cell-to-Cell Movement of PVX–GRV Hybrid Viruses Expressing GFP Gene in *N. benthamiana*[a]

Inoculum	Average number of epidermal cells per infection focus[b]				% of infected cells in mesophyll (7 dpi)[c]	
	2 d.p.i.	3 d.p.i.	4 d.p.i.	6 d.p.i.	Palisade mesophyll	Spongy mesophyll
PVX.ΔTGB.4.GFP–CP	5.53 ± 1.43	7.22 ± 1.92	9.95 ± 3.44	10.05 ± 4.01	11.1 ± 1.0	0
PVX.4.GFP.ΔCP	6.89 ± 1.94	16.33 ± 4.92	18.9 ± 5.49	42.88 ± 13.42	93.9 ± 1.0	86.9 ± 2.2
PVX.ΔTGB.4.GFP.ΔCP	5.55 ± 2.27	7.5 ± 1.96	11.5 ± 4.11	14.47 ± 3.32	13.4 ± 2.9	3.87 ± 0.5
PVX.GFP–CP	6.58 ± 2.27	20.05 ± 5.06	24.33 ± 6	42.67 ± 8.78	97.8 ± 1.0	92.5 ± 1.4

[a] Infection was examined by observation of the fluorescence in inoculated leaves.

[b] Average for 9 to 21 foci on four plants examined for each virus ± standard deviation.

[c] Percentage of infected cells was expressed as percent of fluorescent cells to total number of cells in a section; average for six sections of three foci examined ± standard deviation. The total number of either palisade or spongy mesophyll cells in foci sections ranged from 30 to 70.

both epidermal and mesophyll tissues but at a level reduced in comparison with that of "normal" PVX. Systemic spread of PVX.ΔTGB.4.GFP–CP to uninoculated leaves was not detected even after 3 weeks p.i. These effects on movement could not be explained by reduced rates of replication of the hybrid virus, because the accumulation of viral RNA in protoplasts was similar for the hybrid virus (PVX.ΔTGB.4.GFP–CP) and for "normal" PVX expressing the GFP gene (PVX.GFP–CP) (Fig. 4).

PVX.ΔTGB.4.GFP.ΔCP, in which both the TGB and the CP genes were deleted and replaced by GRV ORF4, spread within the inoculated leaf at a rate similar to that of PVX.ΔTGB.4.GFP–CP, which lacked only the TGB. Like PVX.ΔTGB.4.GFP–CP, PVX.ΔTGB.4.GFP.ΔCP spread efficiently at early stages (up to 3 d.p.i.) in epidermal cells but further movement in epidermis, as well as in mesophyll, was significantly reduced (Fig. 3K, Table 1) and this virus did not spread to noninoculated leaves. Thus, there was no evidence that the limitation on cell-to-cell movement and lack of systemic spread were due to interference between the PVX CP and GRV ORF4 MP.

The chimeric virus PVX.4.GFP.ΔCP, which lacked the CP gene (Fig. 1D), was able to move from cell to cell (Fig. 3J). Moreover, in spite of reduced rates of replication in protoplasts (Fig. 4), the rate of cell-to-cell movement of PVX.4.GFP.ΔCP did not slow down after 3 d.p.i., as was the case for PVX.ΔTGB.4.GFP–CP, but continued at a level similar to that of PVX.GFP–CP (Table 1). The chimeric virus accumulated and spread effectively into palisade and spongy mesophyll cells underlying infected areas of epidermis (Table 1). Similar growth of foci was observed in the case of normal PVX expressing a GFP–CP fusion as a reporter (Table 1). However, in spite of its effective accumulation and cell-to-cell movement in inoculated leaves, systemic spread of PVX.4.GFP.ΔCP *via* vascular tissues was not observed.

In contrast, GRV ORF3 could functionally replace neither the PVX TGB (in the recombinant PVX.ΔTGB.3.GFP–CP lacking the TGB and containing a fusion between the GFP and the PVX CP) nor the PVX CP gene (in the recombinant PVX.3.GFP.ΔCP lacking the CP gene and coding for free GFP) in virus movement. Neither of these chimeric viruses moved beyond the initially infected epidermal cells (data not shown).

DISCUSSION

In this study of the localization and functional analysis of the proteins encoded by two GRV genes, ORF3 and ORF4, we describe the use of two different plant virus vectors, PVX and TMV (Chapman *et al.,* 1992b; Oparka *et al.,* 1996b). These viruses have different cytopathic effects on infected cells and therefore any effects they may have on the proper localization of the GRV proteins should not be the same.

The ORF4 28-kDa protein fused with GFP expressed from either PVX or TMV was localized in or near plasmodesmata. In contrast, free GFP, expressed from PVX or TMV, was localized in the cytoplasm and nucleoplasm (see Baulcombe *et al.,* 1995; data not shown). Thus, the affinity of the fusion protein for plasmodesmata must be a property of the 28-kDa protein rather than the GFP moiety of the fusion. Localization to plasmodesmata is a common attribute of several plant virus MPs (Tomenius *et al.,* 1987; Atkins *et al.,* 1991; Ding *et al.,* 1995; Oparka *et al.,* 1995, 1997). Therefore, these results further confirm the idea, based on the amino acid sequence similarity with a number of plant virus MPs, particularly the CMV MP (Taliansky *et al.,* 1996), that the ORF4 28-kDa protein of GRV is a MP.

A database search with the sequence of the GRV ORF3 27-kDa protein revealed no significant similarity with any other viral or nonviral proteins, except the corresponding proteins encoded by another umbravirus and by pea enation mosaic virus (Taliansky *et al.,* 1996). In this study, we found that the 27-kDa protein was localized in large inclusion bodies, possibly induced by the virus vectors (PVX and TMV) and with which the process

of vector virus replication may be associated (Hills *et al.,* 1987). It is important to note that besides the affinity to these plant virus vector-induced structures, the ORF3 27-kDa was also found in the cytoplasm, forming granules associated with membranes, and was also targeted to nucleoli. The nucleolus is a prominent subnuclear domain, which is the site of transcription of rDNA, processing of the pre-rRNAs and biogenesis of preribosomal particles. It is interesting that the localization of GFP-tagged 27-kDa protein within nucleoli corresponds in position to the so-called granular component, in which later stages of ribosome biogenesis occurs (Beven *et al.,* 1996). Unfortunately, these observations alone do not provide a key to understanding the possible functions of the 27-kDa protein, and further studies are required.

Cell-to-cell movement of PVX requires all three proteins encoded by the TGB and also the CP. The CP of PVX neither gates plasmodesmata nor is localized in plasmodesmata in uninfected transgenic plants, but it is necessary for transport of viral RNA to, and possibly through, plasmodesmata (Oparka *et al.,* 1996a). The TGB proteins are proposed to have a role in plasmodesmatal modification (Angell *et al.,* 1996). Recently, it has been shown that a defective 25-kDa protein encoded by the PVX TGB can be complemented by the MPs of tobamo- or dianthoviruses (Morozov *et al.,* 1997). Our work with PVX.ΔTGB.4.GFP–CP and PVX.ΔTGB.4.GFP.ΔCP demonstrated that the complete TGB of PVX, or even both the TGB and the CP genes of PVX, could be substituted by ORF4 of GRV, at least for the early stages of cell-to-cell movement in the epidermis. These results are the first evidence that a single MP-coding gene is able to replace functionally the complete transport complex of PVX, consisting of the MP genes (TGB) and the CP gene. However, in contrast to the initial phases of cell-to-cell movement, further spread of the resulting hybrid viruses in epidermal and mesophyll tissues was significantly reduced. As mentioned above, the movement of native PVX requires the CP, which is necessary for the transport of PVX, possibly in virion form (Oparka *et al.,* 1996a). On the other hand, GRV does not code for a CP and from the work presented here it seems clear that the GRV ORF4 MP facilitates transport of GRV in a nonvirion form. However, because PVX-derived hybrid viruses with and without CP genes both displayed the same limited cell-to-cell movement, the reason for this limitation probably does not lie in the interference of the PVX CP in the GRV ORF4 MP-mediated movement process.

Limited movement in epidermis has also been described by Mise and Ahlquist (1995) in cowpea leaves inoculated with a derivative of cowpea chlorotic mottle bromovirus in which the MP gene was replaced by that of a bromovirus not adapted to cowpea (brome mosaic virus). Because of the apparent absence of any preexisting anatomical boundary at the limit of infection spread, Mise and Ahlquist (1995) suggested that the barrier to further spread might have resulted from changes that occurred in the host tissue during early phases of infection. Reduced transport of the TGB-lacking PVX hybrids, supported by GRV ORF4 28-kDa protein, into and within mesophyll tissue might also indicate that the intercellular connections may be different between and within different plant tissues (epidermis and mesophyll). These observations suggest that different mechanisms may operate to facilitate efficient cell-to-cell movement of a plant virus in different tissues and/or at different stages of infection. Thus, the GRV ORF4 28-kDa MP by itself seems to be able to initiate cell-to-cell movement of the PVX genome in epidermis, but may require additional virus-coded factor(s) to trigger the mechanism involved in further movement in epidermis and mesophyll. Interestingly, the GRV 28-kDa MP could efficiently transport a PVX genome lacking only the CP gene (but still retaining the TGB) in epidermis and mesophyll. This result suggests that the GRV ORF4 28-kDa protein can function in concert with one or more TGB-encoded proteins to facilitate movement of the hybrid virus in epidermis and in mesophyll tissues. It has been suggested that in PVX infections CP is involved in trafficking of virus RNA through plasmodesmata that have been modified by one or more of the TGB proteins (Oparka *et al.,* 1996a; Angell *et al.,* 1996), and it seems likely that GRV ORF4 protein can replace PVX CP in the case of hybrid PVX.4.GFP.ΔCP virus. In the case of native GRV infection also, the ORF4 28-kDa protein might not be the only GRV-encoded protein involved in cell-to-cell movement, and another protein, for example, the ORF3 27-kDa protein, may also be required. A direct test of this suggestion using a PVX vector for expression of both GRV ORF3 and ORF4 would be difficult because of the high probability of recombination between practically identical nucleotide sequences of these overlapping ORFs. More attractive is the idea of using transgenic plants expressing one of these ORFs for inoculation with PVX hybrids containing the other.

However, in spite of the efficient cell-to-cell movement of the PVX.4.GFP.ΔCP in epidermis and in mesophyll, systemic spread of this hybrid virus did not occur. The absence of long-distance movement of PVX.ΔTGB.4.GFP-CP and PVX.ΔTGB.4.GFP.ΔCP might be explained by inefficient movement in the mesophyll, but in the case of PVX.4.GFP.ΔCP the absence of long-distance movement suggests that the PVX CP is essential for long-distance movement (transport in phloem or "cell-to-cell" movement into sieve elements). We cannot, with the available data, distinguish between the possibilities of either a direct or an indirect effect of the CP on long-distance movement. If the effect is indirect, it may be because the CP-lacking hybrid viruses accumulated reduced levels of viral RNA in the mesophyll cells, as suggested by the protoplast experiments. As a result of this effect, there may be insufficient viral RNA to reach

the threshold level necessary for long-range movement. It is also possible that distinct mechanisms are involved in cell-to-cell and long-distance movement and PVX CP, in addition to its role in cell-to-cell movement, is directly required for long-distance movement. Neither GRV ORF4 nor GRV ORF3 (data not shown) could functionally replace the PVX CP gene to facilitate long-distance movement of the hybrid viruses. It cannot be excluded that in native GRV long-distance movement is mediated by both ORF3 and ORF4 products and perhaps a specific interaction between these genes.

MATERIALS AND METHODS

Plasmids, generation of chimeric cDNA constructs, and mutants

GRV cDNA clone grmp2 (Taliansky *et al.,* 1996) was used as a template for PCR amplification of ORF3, ORF4, and the ORF3–ORF4 block of GRV. Plasmid pTXS.GFP (Baulcombe *et al.,* 1995) containing GFP cDNA was used for amplification of the GFP gene sequences. Amplified fragments were cloned into the PVX-based vector pTXS.P3C2 (Baulcombe *et al.,* 1995; Fig. 1B) and into the TMV-based vector p30B, kindly provided by Dr. W. O. Dawson (unpublished; Fig. 1C). The main constructs generated in this work are illustrated in Figs. 1B, 1C, and 1D. Standard DNA manipulation techniques (Sambrook *et al.,* 1989) were used for their generation.

pTXS.GFP-3/4 (Fig. 1B). The fragment containing the GFP gene fused to ORF3 in the ORF3/ORF4 block of GRV was produced by overlap extension PCR (Higuchi *et al.,* 1988) using flanking oligonucleotides 5′-CATGATCGATATGAGTAAAGGAGAAG-3′ with a *Cla*I site preceding 16 nucleotides (nt) identical to those of the 5′ end of the GFP gene as the forward primer, 5′-CATGCTGCAGTTACGTCGCTTTGC-3′ with a *Pst*I site preceding 14 nt complementary to those of the 3′ end of GRV ORF4 as the reverse primer, and two complementary mutagenic oligonucleotides to produce the GFP-ORF3 fusion, one of which was 5′-GGATGAACTATACAAAATGGACACCC-3′ with the 5′ part identical to 16 3′ terminal nucleotides of the GFP gene and the 3′ part identical to the first 13 nt of ORF3. The amplified fragment was cloned between the *Cla*I and *Nsi*I sites of pTXS.P3C2 to give pTXS.GFP-3/4.

pTXS.3/4-GFP (Fig. 1B). The fragment containing the GFP gene fused to ORF4 in the ORF3/ORF4 block of GRV was produced by overlap extension PCR using flanking oligonucleotides 5′-CATGATCGATATGGACACCACCC-3′ with a *Cla*I site preceding 14 nt identical to those at the 5′ end of GRV ORF3 as a forward primer, 5′-CATGCTGCAGTTATTTGTATAGTTCAT-3′ with a *Pst*I site preceding 17 nt complementary to those at the 3′ end of the GFP gene as a reverse primer, and a pair of complementary mutagenic oligonucleotides to produce the ORF4-GFP fusion, one of which was 5′-CCGCAAAGCGACGATGAGTAAAGGAGAAG-3′ with the 5′ part identical to the 13 3′ terminal nucleotides of the GRV ORF4 gene and the 3′ part identical to the first 16 nt of the GFP gene. The amplified product was cloned between the *Cla*I and *Nsi*I sites of pTXS.P3C2 to give pTXS.3/4-GFP.

pTXS.4-GFP (Fig. 1B). The fragment containing the ORF4–GFP gene fusion was obtained in the same way as the ORF3/ORF4–GFP fusion except that the flanking oligonucleotide 5′-GCTAGTCGACCATGGCTTCGCAAGTGGC-3′ with 17 nt identical to those at the 5′ end of GRV ORF4 was used as the forward primer. The amplified product was cloned between *Eco*RV and *Nsi*I sites of pTXS.P3C2 to give pTXS.4-GFP.

pTMV.GFP-3/4 (Fig. 1C). The fragment containing the GFP gene fused to ORF3 in the ORF3/ORF4 block of GRV was amplified using oligonucleotides 5′-CATGATCGATATGAGTAAAGGAGAAG-3′ with a *Cla*I site preceding 16 nt identical to those of 5′ end of the GFP gene as a forward primer, 5′-CATGCTCGAGTTACGTCGCTTTGC-3′ with a *Xho*I site preceding 14 nt complementary to those of the 3′ end of GRV ORF4 as a reverse primer and pTXS.GFP-3/4 as template. The amplified fragment was cloned between the *Pme*I and *Xho*I sites of p30B to give pTMV.GFP-3/4.

pTMV.4-GFP (Fig. 1C). The fragment containing the ORF4–GFP gene fusion was amplified using oligonucleotides 5′-GTACGTTTAAACATGTCTTCGCAAGTGGC-3′ with a *Pme*I site preceding the 17 nt identical to those of the 5′ end of GRV ORF4 as a forward primer, 5′-CTAGCTCGAGTTATTTGTATAGTTCATCC-3′ with a *Xho*I site preceding 19 nt complementary to those of 3′ end of the GFP gene as a reverse primer, and pTXS.4-GFP as template. The amplified fragment was cloned between the *Pme*I and the *Xho*I sites of p30B to give pTMV.4-GFP.

pTXS.ΔTGB.4.GFP-CP (Fig. 1D). The fragment containing GRV ORF4 was amplified by PCR using oligonucleotides 5′-GCTAGTCGACCATGGCTTCGCAAGTGGC-3′ with 17 nt identical to those at the 5′ end of GRV ORF4 as a forward primer and 5′-CATGCTGCAGTTACGTCGCTTTGC-3′ with a *Pst*I site preceding 14 nt complementary to those of the 3′ end of the GRV ORF4 as a reverse primer. The PCR product was cloned into pTXS.P3C2 using the restriction sites *Eco*RV and *Nsi*I to give pTXS.4. Then, the *Apa*I–*Eco*NI fragment of pTXS.4 (positions 4945 to 5500 of the pTXS.P3C2 sequence) containing the TGB (C-terminal half of the gene for the 25-kDa protein and genes encoding the 12- and 8-kDa proteins) was excised to give pTXS.ΔTGB.4. To replace the CP gene of pTXS.ΔTGB.4 with the GFP–CP fusion gene the appropriate fragment of the plasmid pTXS.GFP-CP (Santa Cruz *et al.,* 1996) was amplified using oligonucleotides 5′-CGCGCCCGGGCCGTTGAACGGTTAAG-3′ with a *Sma*I restriction site preceding sequences identical to nucleotides 5614 to 5629 of pTXS.P3C2 as the forward primer and 5′-AACAGCTATGACCATG-3′, complementary to nucleotides 6607 to 6622 of pTXS.GFP-CP, located downstream of the *Spe*I site at the 3′ end of the PVX sequence,

as the reverse primer. The resulting fragment, digested with *Sma*I and *Spe*I restriction endonucleases, was used to replace the *Sma*I–*Spe*I fragment of pTXS.ΔTGB.4 to give pTXS.ΔTGB.4.GFP–CP.

pTXS.4.GFP.ΔCP (Fig. 1D). To replace the CP gene of pTXS.4 with the GFP gene, a fragment of plasmid pTXS.GFP.ΔCP (Fig. 1D) (Baulcombe *et al.,* 1995) was amplified with the same oligonucleotides as were used for amplification of the GFP–CP gene-containing fragment mentioned above. The resulting fragment, digested with *Sma*I and *Spe*I restriction endonucleases, was used to replace the *Sma*I–*Spe*I fragment of pTXS.4 to give pTXS.4.GFP.ΔCP.

pTXS.ΔTGB.4.GFP.ΔCP (Fig. 1D). The *Sma*I/*Spe*I-treated PCR fragment amplified from pTXS.GFP.ΔCP, containing the GFP gene, was used to replace the *Sma*I–*Spe*I fragment of pTXS.ΔTGB.4 to give pTXS.ΔTGB.4.GFP.ΔCP.

In vitro transcription and plant inoculation

PVX-based and TMV-based plasmids were linearized by digestion with the restriction enzymes *Spe*I and *Kpn*I, respectively, and *in vitro* transcripts were synthesized with T7 RNA polymerase using the mCAP RNA capping kit (Stratagene). Transcripts were inoculated directly to leaves of 3- to 4-week-old *Nicotiana benthamiana* and *N. tabacum* plants by rubbing corundum-dusted leaves with the transcription products derived from 0.2 μg plasmid template.

Detection of GFP in whole plants, leaves, or within cells

Plants were illuminated with long-wavelength UV light and photographed as described previously (Baulcombe *et al.,* 1995; Oparka *et al.,* 1995). GFP fluorescence in plant tissues was viewed with a Bio-Rad MRC 1000 confocal laser scanning microscope. The methods were as described previously (Baulcombe *et al.,* 1995; Oparka *et al.,* 1995). For some experiments, the leaf tissue was sectioned transversely into approximately 200-μm slices using a Vibraslice (Campden Instruments Ltd. UK) and examined in water under a coverslip. To determine the growth rate of the viral infection sites, the number of fluorescent epidermal cells was counted at intervals over a 6-day period for 9–21 infection foci in four plants for each virus. The number of fluorescent mesophyll cells underlying epidermal infection foci was counted for six transverse (0.2 mm thick) sections in three sites of infection for each virus.

Staining of membranes and immunolocalization of β-1,3-glucan (callose)

In some experiments, endoplasmic reticulum was stained with the fluorescent probe rhodamine B hexyl ester (excitation, 568 nm) at a concentration of 1 μg ml^{-1}, essentially as described by Grabski *et al.* (1993). Callose was localized in epidermal cells using a monoclonal rabbit primary antibody (Genosys Biotechnologies Ltd., Cambridge, UK) and an anti-rabbit secondary antibody conjugated to CY3 (Sigma Immunochemicals). Epidermal tissue, containing viral infection sites, was removed from underlying mesophyll tissue by paradermal sectioning with a Vibraslice. To aid the penetration of antibody, the undersurface of the epidermis was abraded lightly with corundum applied by a paintbrush and then incubated on solutions containing antibody (Oparka *et al.,* 1997).

Protoplast experiments

Protoplasts were isolated from fully expanded mature leaves of *N. benthamiana* as described by Power and Chapman (1985). For inoculation, 5 μg of RNA transcript in 25 μl of ice-cold water was added to the pellet of 1×10^6 protoplasts. After incubation for 2 min on ice, 1 ml of 40% (w/v) polyethylene glycol (MW 6000) in 9% mannitol was added (Power *et al.,* 1989). The suspension was gently mixed. After incubation for 30 min at room temperature, the protoplasts were washed and incubated in Murashige and Skoog medium containing 9% mannitol at 23–24°C under continuous illumination. RNA accumulation was analyzed at different times (0, 4, 16, 24, and 42 h) postinoculation. All transfection experiments were repeated three times.

Total RNA preparations from protoplasts were extracted by the method of Blok *et al.* (1994). For dot blot hybridization analysis, RNA samples were spotted onto Hybond N nylon membrane (Amersham) and immobilized by UV crosslinking in a Stratalinker 2400 (Stratagene). The hybridization probe was the *Bam*HI (nucleotide 543)–*Avr*II (nucleotide 3939) fragment of TXS.P3C2, which represents sequences of the PVX replicase gene, labeled with ^{32}P dATP using a random primers DNA labeling kit (Life Technologies). Quantitative analysis of dot blots was done by densitometry of the autoradiographic images, using a Bio Image Intelligent Quantifier Version 2.5.0. A dilution series of PVX RNA was used as concentration standard.

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