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Characterization of Cucumber Mosaic Virus

IV. Movement Protein and Coat Protein Are Both Essential for Cell-to-Cell Movement of Cucumber Mosaic Virus

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cDNA clones of cucumber mosaic virus (CMV) RNA 3 were modified to express the jellyfish green fluorescent protein (GFP) in place of the 3a movement protein (MP) or coat protein (CP), as fusions to the N (GFP–3a) or C (3a–GFP) terminus of the MP or from a separate open reading frame as part of tricistronic RNAs 3. CMV RNA transcripts containing the individual modified RNAs 3 were unable to infect either *Nicotiana tabacum* or *Nicotiana benthamiana* systemically. Infection, as measured by confocal microscopy of GFP fluorescence, generally was limited to one to three epidermal cells at each inoculation site. Limited cell-to-cell movement, but not systemic movement, could be detected by complementation involving expression of MP and CP from two different RNA 3 constructs, each also expressing GFP. Infection involving RNA 3 expressing the GFP–3a fusion showed bright granules of variable size distributed predominantly and nonuniformly throughout the cytoplasm and, to a lesser extent, associated with the cell wall in single fluorescent cells, while infections expressing the 3a–GFP fusion showed bright, punctate fluorescence associated only with the cell wall. Infected cells expressing either 3a–GFP or free GFP showed a halo of less bright, fluorescent, neighboring cells, indicating limited movement of GFP. The initially infected cells also allowed movement of 10-kDa fluorescent dextran to the neighboring halo cells, while infection did not spread, suggesting different requirements for movement of either MP or dextran versus RNA.

INTRODUCTION

The movement of plant viruses from cell to cell and systemically into more distal parts of the plant has come under considerable scrutiny (reviewed by Atabekov and Taliansky, 1990; Maule, 1991; McLean *et al.,* 1993; Lucas and Gilbertson, 1994; Carrington *et al.,* 1996; Séron and Haenni, 1996), since the initial observation that virus movement was an active process requiring viral gene products (Nishiguchi *et al.,* 1978). In the case of cucumber mosaic cucumovirus (CMV), all five gene products have been implicated in virus movement (Suzuki *et al.,* 1991; Gal-On *et al.,* 1994; Ding *et al.,* 1995a; Hellwald and Palukaitis, 1995; Kaplan *et al.,* 1995; Taliansky and García-Arenal, 1995). While some of those gene products specifically affect movement in certain hosts (Gal-On *et al.,* 1994; Ding *et al.,* 1995a; Taliansky and García-Arenal, 1995), the 3a protein and coat protein (CP) are needed for movement in all hosts tested (Suzuki *et al.,* 1991). The CMV 3a protein has been designated as a movement protein (MP), since it binds to single-stranded RNA (Li and Palukaitis, 1996), traffics itself and viral RNA from cell to cell in microinjection studies (Ding *et al.,* 1995b), complements the movement of 3a gene-defective mutants of CMV in *trans* in transgenic plants expressing the 3a gene (Kaplan *et al.,* 1995), associates with cell walls in both infected plants and transgenic plants expressing the CMV 3a gene (Vaquero *et al.,* 1994, 1996; Cooper and Dodds, 1995), and has sequence and functional similarity to the MP of tobacco mosaic tobamovirus (TMV) (Melcher, 1990; Kaplan *et al.,* 1995). The CMV CP also is necessary for virus movement (Suzuki *et al.,* 1991). However, since CMV particles have not been found inside plasmodesmata (Ding *et al.,* 1995b) and CMV does not form intercellular tubules that promote the movement of virus particles (Ding *et al.,* 1995b), as has been observed with caulimoviruses, comoviruses, geminiviruses, and nepoviruses (van Lent *et al.,* 1990; Kim and Lee, 1992; Perbal *et al.,* 1993; Wieczorek and Sanfaçon, 1993), it appears that CMV does not move as virions from cell to cell. Thus, the role of CP may be one of stabilizing the viral RNAs (Suzuki *et al.,* 1991; Boccard and Baulcombe, 1993). In contrast, the CP of TMV is not required for cell-to-cell movement, although CP does stabilize TMV RNA and allow it to accumulate (Siegel *et al.,* 1962). Hence, the precise role of CP in cell-to-cell movement of CMV has not been elucidated.

It is also unknown as to whether any cell-to-cell movement occurs in the absence of MP or CP. In the case of the bromoviruses brome mosaic virus (BMV) and cowpea

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chlorotic mottle virus (CCMV), the MP is required for even limited movement to epidermal cells adjacent to the initially inoculated cell (Mise and Ahlquist, 1995; Schmitz and Rao, 1996; Rao, 1997). For BMV, the CP is also required for even such limited movement (Schmitz and Rao, 1996). However, for CCMV, CP is not required for virus movement to epidermal cells (Rao, 1997), and CP, but not virion formation, is required for long-distance movement (Schneider *et al.,* 1997). To determine whether MP and CP are essential for limited cell-to-cell movement of CMV, we have replaced the genes encoding those proteins with the gene encoding the green fluorescent protein (GFP) from the jellyfish *Aqueorea victoria* (Chalfie *et al.,* 1994) and examined such constructs for their ability to promote CMV movement in epidermal cells of inoculated plants by fluorescence microscopic visualization of the expressed GFP. We have also examined the subcellular distribution of fusion proteins formed between the CMV MP and the GFP, and demonstrated a correlation between cell wall localization of the MP and its ability to increase the plasmodesmatal size exclusion limit for fluorescent dextran.

MATERIALS AND METHODS

Preparation of cDNA constructs expressing GFP

cDNA constructs expressing GFP, either as fusion proteins with the CMV MP or as free GFP (i.e., GFP not fused to any other other protein), were prepared by ligation of various DNA fragments, generated by the polymerase chain reaction (PCR), to previously digested plasmids containing sequences of CMV (Fny or LS strain) or tomato aspermy cucumovirus TAV RNA 3 and transformation into *Escherichia coli* DH5α cells using standard procedures (Sambrook *et al.,* 1989).

pF:GFP/CP. A DNA fragment (1) containing the GFP gene was amplified by the PCR from plasmid pS65T-C1 (Clontech) using primers containing *Bsp*HI and *Spe*I sites, upstream and downstream, respectively, of sequences identical to termini of the GFP gene. Fragment 1 was digested with *Bsp*HI and *Spe*I. A second DNA fragment (2) corresponding to the 5′ noncoding region (NCR) of Fny-CMV RNA 3 also was obtained by the PCR from a full-length cDNA clone of Fny-CMV RNA 3, pFny309-His (Li and Palukaitis, 1996), using the reverse sequencing primer from the plasmid vector (pUC18), as well as a primer complementary to Fny-CMV RNA 3 sequences 110–134 and containing a *Bsp*HI site. Fragment 2 was digested with *Bsp*HI and *Kpn*I. Fragments 1 and 2 were combined in a three-piece ligation with fragment 3 (pFny309-His, digested previously with *Kpn*I and *Spe*I) to create pF:GFP/CP (Fig. 1), which has the 3a gene replaced with the gene encoding the GFP.

pF:3a-GFP/CP. A DNA fragment (4) containing the 3a gene and the 5′ NCR of Fny-CMV RNA 3 was amplified by the PCR from pFny309-His using the reverse sequencing primer from pUC18 and a primer complementary to Fny-CMV RNA 3 sequences 944–955 and containing a *Bsp*HI site. Fragment 4 was digested with *Bam*HI and *Bsp*HI. Another DNA fragment (5) was obtained from pF:GFP/CP by digestion with *Bsp*HI and *Spe*I. Fragments 4 and 5 were combined in a three-piece ligation with fragment 6 (pF:GFP/CP, previously digested with *Bam*HI and *Spe*I), creating pF:3a-GFP/CP (Fig. 1), which has the gene encoding the GFP fused to the 3a gene sequences encoding the C terminus of the MP.

pF:GFP-3a/CP. A DNA fragment (7) containing the 3a gene was amplified by the PCR from pFny309-His using a primer specific to RNA 3 sequences 120–131 and containing a *Sac*I site at the 5′ end and a second primer complementary to the 3′ end of the 3a gene and containing an *Spe*I site at the 5′ end. [The latter primer also incorporated sequences encoding six histidine residues fused to the C terminus of the 3a protein (Li and Palukaitis, 1996).] Fragment 7 was digested with *Sac*I and *Spe*I. PCR fragment 8 was obtained by amplification of the GFP gene from pS65T-C1 using primers containing sites for *Bsp*HI (5′ of sequences corresponding to nucleotides 1–14 of the GFP gene) and *Sac*I (5′ of sequences complementary to nucleotides 693–704 of the GFP gene). Fragment 8 was digested with *Nco*I and *Sac*I. Fragments 7 and 8 were combined in a three-piece ligation with fragment 9 (pF:GFP/CP, previously digested with *Nco*I and *Spe*I), to create pF:GFP–3a/CP (Fig. 1), which has the gene encoding the GFP fused to the 3a gene sequences encoding the N terminus of the MP.

pL:3a/GFP. A DNA fragment (10) containing the 3′ NCR of LS-CMV RNA 3 was amplified by the PCR from the full-length cDNA clone of LS-CMV RNA 3, pLS-CMV 3 (Zhang *et al.,* 1994), using one primer corresponding to nucleotides 1880–1892 of LS-CMV RNA 3 and containing an *Spe*I site at the 5′ end and the forward sequencing primer of pUC18. After digestion with *Spe*I and *Pst*I, fragment 10 was ligated into fragment 11 (pF:GFP/CP, digested with *Spe*I and *Pst*I) to create pFL:GFP/CP. After digestion of pFL:GFP/CP with *Bsp*HI and *Pst*I, fragment 12, containing LS-CMV RNA 3 and GFP sequences, was obtained. Fragment 13, containing LS-CMV RNA 3 sequences from the intergenic region (IGR) to the 3′ end, was obtained by PCR, using the reverse sequencing primer of pUC18 as well as a primer corresponding to the 3′ end of the IGR of LS-CMV RNA (nucleotides 1205–1217) and containing a 5′ *Bsp*HI site. Fragment 13 was digested with *Nhe*I and *Bsp*HI, and fragments 12 and 13 were combined in a three-piece ligation with fragment 14 (pLS-CMV 3, previously digested with *Nhe*I and *Pst*I) to create pL:3a/GFP (Fig. 1), which has the gene encoding the GFP replacing the CP gene.

pLF:3a/GFP/CP. The plasmid pL:3a/GFP was digested with *Spe*I and *Pst*I, and the larger fragment was ligated to the smaller fragment obtained by digestion of pF:GFP/CP with the same two restriction enzymes, to generate

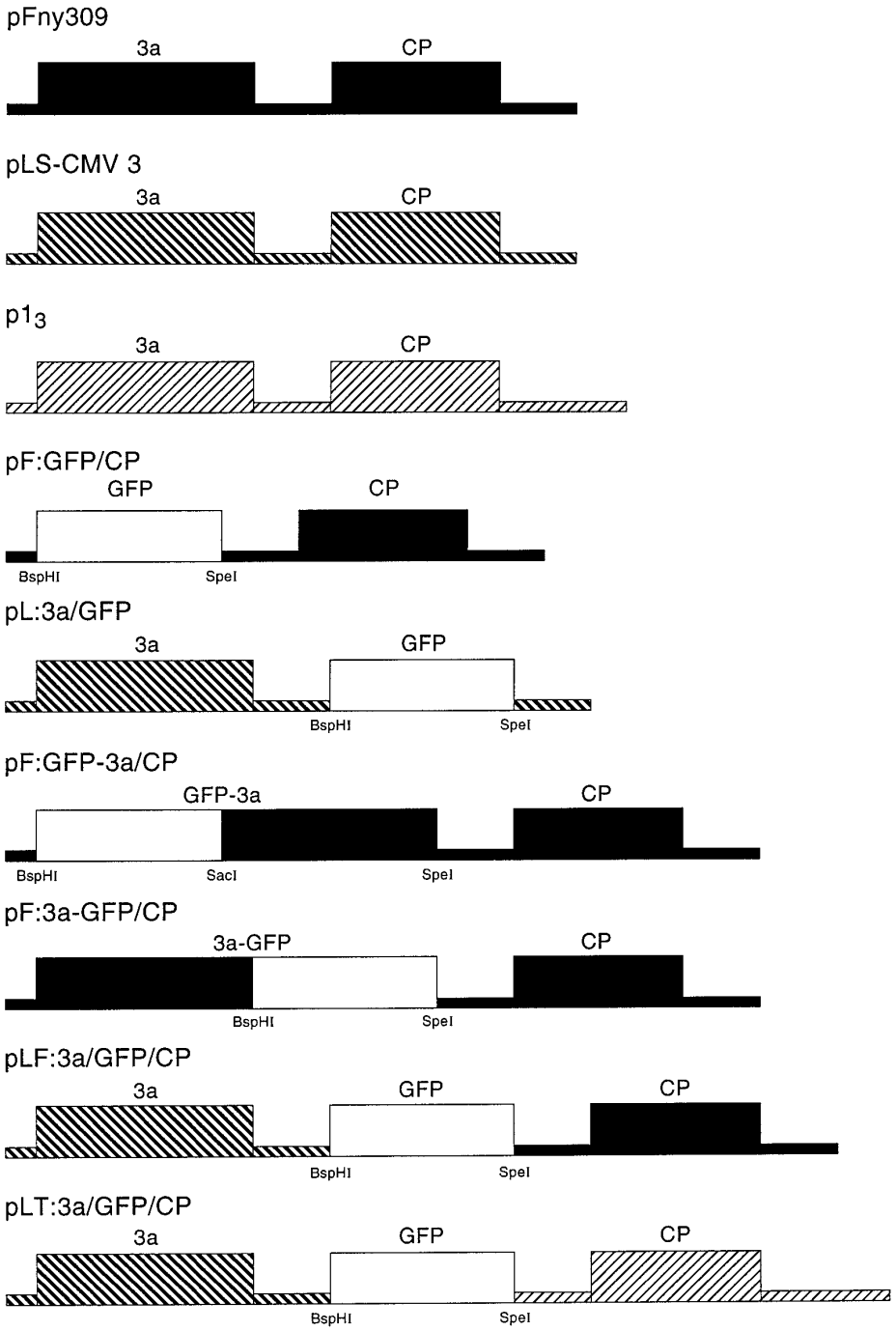

FIG. 1. Schematic representation of cDNA constructs of cucumoviral RNAs 3 used for expression of GFP. cDNA constructs used for synthesis of Fny-CMV RNA 3 (pFny309), LS-CMV RNA 3 (pLS-CMV 3), and 1-TAV RNA 3 ($p1_3$) were modified for expression of GFP, as free GFP in place of either the Fny-CMV 3a protein (pF:GFP/CP) or the LS-CMV CP (pL:3a/CP); or expressed as an additional open reading frame from an LS-CMV RNA 3 IGR in either pLF:3a/GFP/CP or pLT:3a/GFP/CP; or expressed as either an N-terminal (pF:GFP–3a/CP) or a C-terminal (pF:3a–GFP/CP) fusion with the Fny-CMV 3a protein. Restriction endonuclease cleavage sites introduced into the RNA 3 sequences by PCR to facilitate the generation of the various GFP-expressing constructs are indicated.

pLF:3a/GFP/CP (Fig. 1), a tricistronic RNA 3 containing (in the 5′ to 3′ orientation) the 5′ NCR, 3a gene, and IGR from LS-CMV RNA 3, the GFP gene, and the IGR, CP gene, and 3′ NCR of Fny-CMV RNA 3.

pLT:3a/GFP/CP. A DNA fragment (15) containing the IGR, CP gene, and 3′ NCR of TAV RNA 3 was amplified by the PCR from the full-length cDNA clone of 1-TAV RNA 3 [$p1_3$ (Moreno *et al.,* 1997)], using primers corresponding to the 5′ end of the IGR of TAV RNA 3 (nucleotides 967–982) and the 3′ end of TAV RNA 3 (nucleotides 2407–2418), containing sites at the 5′ ends for the restriction enzymes *Spe*I and *Pst*I, respectively. Fragment 15 was

digested with *Spe*I and *Pst*I and ligated into pLF:3a/GFP/CP, digested with the same enzymes, to create pLT:3a/GFP/CP (Fig. 1), a tricistronic RNA 3 containing the 5′ NCR and IGR of LS-CMV RNA 3, the GFP gene, and the IGR, CP, and 3′ NCR of TAV RNA 3.

pSLJ4D4:GFP. The gene encoding the GFP was introduced into the transient-expression plasmid pSLJ4D4 (Jones *et al.,* 1992) by digesting pL:3a/GFP with *Bsp* HI and *Spe*I and ligating the fragment containing the GFP gene into pSLJ4D4 previously digested with *Nco*I and *Xba*I. The resulting plasmid, pSLJ4D4:GFP, contained the GFP gene between the cauliflower mosaic virus 35S RNA promoter and the octopine synthase 3′ polyadenylation signal sequences (Jones *et al.,* 1992).

Plasmid $p1_3\Delta$BssHI, containing a 6-nt deletion in the 3a gene, was produced from the full-length cDNA clone of 1-TAV RNA 3 [$p1_3$ (Moreno *et al.,* 1997)] and also was provided by Dr. C. Oncino, Cornell University, Ithaca, New York.

Inoculation of plants and protoplasts

Viral RNAs were transcribed from full-length cDNA clones pFny109, pFny209, and pFny309, representing Fny-CMV RNAs 1, 2, and 3, respectively, as well as from a movement-deficient cDNA clone of 1-TAV RNA ($p1_3\Delta$BssHI, containing a deletion in the TAV MP) and from the various GFP-expressing RNA 3 constructs in Fig. 1. The plasmids were linearized and the RNA transcripts were synthesized using T7 RNA polymerase as described previously (Zhang *et al.,* 1994). Transcript mixtures were concentrated by ethanol precipitation prior to use in mechanical inoculations on aluminum oxide-dusted leaves of either *Nicotiana tabacum* cv. Samsun NN or *Nicotiana benthamiana.* Mesophyll protoplasts were prepared from 6- to 8-week old *N. tabacum* cv. Samsum NN plants, and 10^6 protoplasts were electroporated with RNA transcripts, as described by Gal-On *et al.* (1994). Samples were collected for analysis of the viral RNAs after 48 hr of incubation.

Biolistic infection and transfection

The "hand gun" biolistic delivery system of Gal-On *et al.* (1997) was used for infection of plants with a mixture of RNA transcripts of pFny109, pFny209, pL:3a/GFP, and $p1_3\Delta$BssHI, as well as for transfection of plants with the transient-expression plasmid pSL4D4:GFP. RNA transcripts or undigested plasmids were adsorbed to tungsten beads (Bio-Rad) as described by Gal-On *et al.* (1995).

Detection of GFP fluorescence

Inoculated leaves were viewed under an epifluorescence microscope (Nikon) equipped with an FITC/TRITC (rhodamine) multiband filter set (Chroma, Catalog No. 51004). Fluorescence was also detected using an MRC 1000 confocal laser scanning microscope (Bio-Rad) with excitation at 488 nm and emission at 522 nm. Whole plants and leaves were also viewed using a Black Ray long-wave ultraviolet lamp (Model B 100Ap, UV Products). Photographs on the FITC/TRITC epifluorescence microscope were taken with Kodak Ektachrome Elite 400 film using a microscope-coupled SLR camera and an HFX automatic expometer (Nikon) at variable exposure times, depending on the intensity of the fluorescence. Photographs of whole leaves were taken using Kodachrome 200 Professional film. Images from the confocal microscope were processed using Adobe Photoshop software (Adobe Systems).

In vitro translation and analysis of translation products

RNA 3, transcribed from pFny309 or various plasmids containing a GFP gene integrated into a CMV RNA 3 cDNA clone (Fig. 1), was translated in the rabbit reticulocyte cell-free system (Promega), using [^{35}S]methionine (Amersham). The labeled proteins were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography (Sambrook *et al.,* 1989).

Analysis of nucleic acids from plants and protoplasts

Viral RNAs were extracted from either protoplasts (10^6) or plant leaves (1-cm disks), by grinding them in 200 μl of 50 m*M* Tris–HCl, pH 8.0, 10 m*M* EDTA, 2% SDS. Samples were extracted with phenol, and RNA was precipitated with ethanol as described previously (Palukaitis *et al.,* 1985). RNA samples were recovered by centrifugation, fractionated by electrophoresis in 1.5% agarose gels under denaturing conditions (6% formaldehyde), blotted to nitrocellulose membranes, hybridized to [^{32}P]RNA probes complementary to either the 3′ NCR of each Fny-CMV RNA (Gal-On *et al.,* 1994) or the GFP gene, washed, and autoradiographed, all as described by Sambrook *et al.* (1989).

To characterize the sites of recombination, viral RNAs extracted from an infected plant were used as templates for reverse transcription–PCRs, using primers corresponding to the 5′ end of the LS-CMV RNA 3 IGR and the 3′ end of the Fny-CMV RNA 3 IGR. The PCR products were subjected to automated DNA sequencing on an ABI PRISM system (Perkin–Elmer).

Microinjection of epidermal cells

Texas Red 10-kDa dextran (Molecular Probes) was filtered through Microcon 30 and 10 membrane-exclusion filters, to eliminate contaminating low- or high-molecular-weight molecules. The filtered, 10-kDa fluorescent dextran was used for microinjection of *N. tabacum* cv. Samsun NN epidermal cells (showing GFP fluorescence), 2 to 4 days after inoculation of the plants with viral transcript RNAs, as described by Oparka *et al.* (1990, 1996a).

TABLE 1

Size of Cucumoviral RNAs 3 and Subgenomic RNAs Derived from RNA 3[a]

RNA source[b]	RNA 3	RNA 4[c]	RNA 4*[d]
Fny-CMV[e]	2216	1031	
LS-CMV	2197	1028	
1-TAV	2389	1239	
pF:GFP/CP	2097	1031	
pF:3a–GFP/CP	2934	1031	
pF:GFP–3a/CP	2948	1031	
pL:3a/GFP	2257	1088	
pLF:3a/GFP/CP	3197	1031	2028
pLT:3a/GFP/CP	3393	1239	2224

[a] Size of specified RNA given in number of nucleotides.

[b] Strain of CMV, TAV, or of construct encoding modified RNAs 3 from Fig. 1.

[c] RNA 4 is the subgenomic RNA encoding the coat protein mRNA3 derived from RNA 3.

[d] RNA 4* is the additional subgenomic RNA derived from RNA 3 of two constructs expressing tricistronic RNAs 3.

[e] Fny-CMV RNAs 1 and 2, used in all inoculations with modified RNA 3 transcripts, contain 3357 and 3050 nucleotides, respectively.

RESULTS

Description and characterization of RNA 3 variants expressing GFP

To determine the genetic requirements for cell-to-cell movement and localization of the 3a MP, six constructs were generated that could express GFP when RNA 3 was amplified by Fny-CMV RNAs 1 and 2 during viral infection (Fig. 1). Two of the constructs contained a replacement of either the MP gene of Fny-CMV RNA 3 (pF:GFP/CP) or the CP gene of LS-CMV RNA 3 (pL:3a/GFP) with the gene encoding the GFP. RNA transcripts of these constructs should express GFP *in vivo* (not fused to any other open reading frame, i.e., as "free GFP") in place of either the 3a or CP, respectively. The expected sizes of the corresponding RNAs 3 and RNA 4 (generated from RNA 3 as a subgenomic RNA for expression of the CP gene) were similar to those of the wild-type (wt) viral RNAs (Table 1). Two of the constructs encoded GFP fused to either the N (pF:GFP–3a/CP) or C (pF:3a–GFP/CP) terminus of the MP. (Fig. 1). While the RNAs 4 were the same size as for RNA 4 produced from wt RNA 3, the RNAs 3 were considerably longer (Table 1) and near CMV RNA 2 (3050 nt) in size. constructs pLF:3a/GFP/CP and pLT:3a/GFP/CP contained an additional open reading frame encoding the GFP that should be expressed from a duplicated subgenomic RNA promoter, yielding tricistronic RNAs 3. These two constructs should produce RNAs 3 either similar in size to CMV RNA 1 (3357 nt) or between CMV RNAs 1 and 2 in size (Table 1). However, besides producing an RNA 4, replication of these tricistronic constructs should also yield an additional subgenomic RNA (RNA 4*, Table 1), near RNA 3 in size. In an attempt to avoid recombination occurring between duplicated IGR sequences, these two constructs contained a 5′ proximal IGR from LS-CMV RNA 3 and a 3′ proximal IGR from either Fny-CMV RNA 3 or 1-TAV RNA 3. The IGR of Fny-CMV RNA 3 contains 75 and 62% sequence identity to the LS-CMV RNA 3 IGR and 1-TAV RNA 3 IGR, respectively.

The presence of the GFP gene in CMV RNA 3 for expression of GFP, either as a free protein (Fig. 2A, lane 4) or as a fusion product with the 3a protein (Fig. 2A, lane 5), did not have a negative effect on viral RNA replication or on the expression of the subgenomic RNA 4 in tobacco protoplasts. The accumulation of the variant RNAs 3 in protoplasts suggests that these RNAs were being encapsidated, despite the larger size versus the wt RNA 3. Proteins of the expected size were translated *in vitro* from RNA 3 transcripts expressing either free GFP in place of the 3a MP (Fig. 2B, lane 3) or a GFP–3a fusion (Fig. 2B, lane 4). Thus, these variant RNAs 3 appeared to be able to replicate *in vivo* as well as express the expected proteins.

MP and CP are both required for cell-to-cell movement

Inoculation of tobacco or *N. benthamiana* plants by RNA transcripts representing Fny-CMV RNAs 1 plus 2 and RNA 3 of the six GFP-encoding variants (Fig. 1) resulted in expression of GFP in the initially infected epider-

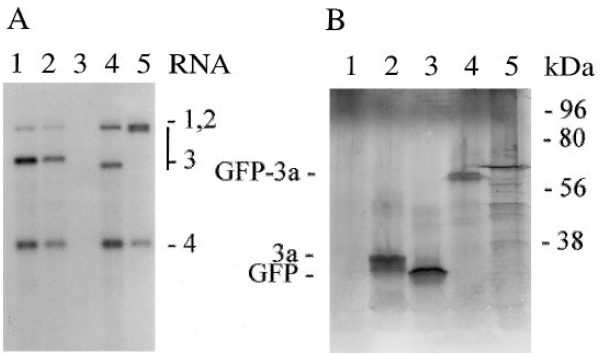

FIG. 2. Replication and gene expression of RNA transcripts expressing free GFP and a GFP-3a fusion protein. (A) Autoradiogram of a Northern blot of RNAs extracted from tobacco protoplasts inoculated with CMV RNAs 1 plus 2 and transcripts of pFny309 (lane 1), pFny309-His (lane 2), pF:GFP/CP (lane 4), pF:GFP–3a/CP (lane 5), or mock inoculated (lane 3). CMV RNAs were detected using a ^{32}P-labeled RNA probe complementary to the 3′ NTR of each CMV RNA. The expected positions of the CMV RNAs (1–4; Table 1) are indicated on the right side of the autoradiogram. (B) Autoradiogram of an SDS–polyacrylamide gel containing the *in vitro* translation products of RNAs transcribed from pFny309 (lane 2), pF:GFP/CP (lane 3), pF:GFP–3a/CP (lane 4), the luciferase mRNA control provided with the *in vitro* translation system (lane 5), or no added RNA (lane 1). The molecular marker proteins are indicated on the right side of the autoradiogram. RNAs derived from pFny309, pF:GFP/CP, and pF:GFP-3a/CP would be expected to yield proteins of molecular weight 30 kDa (3a protein), 27 kDa (GFP), and 57 kDa (GFP-3a fusion), respectively.

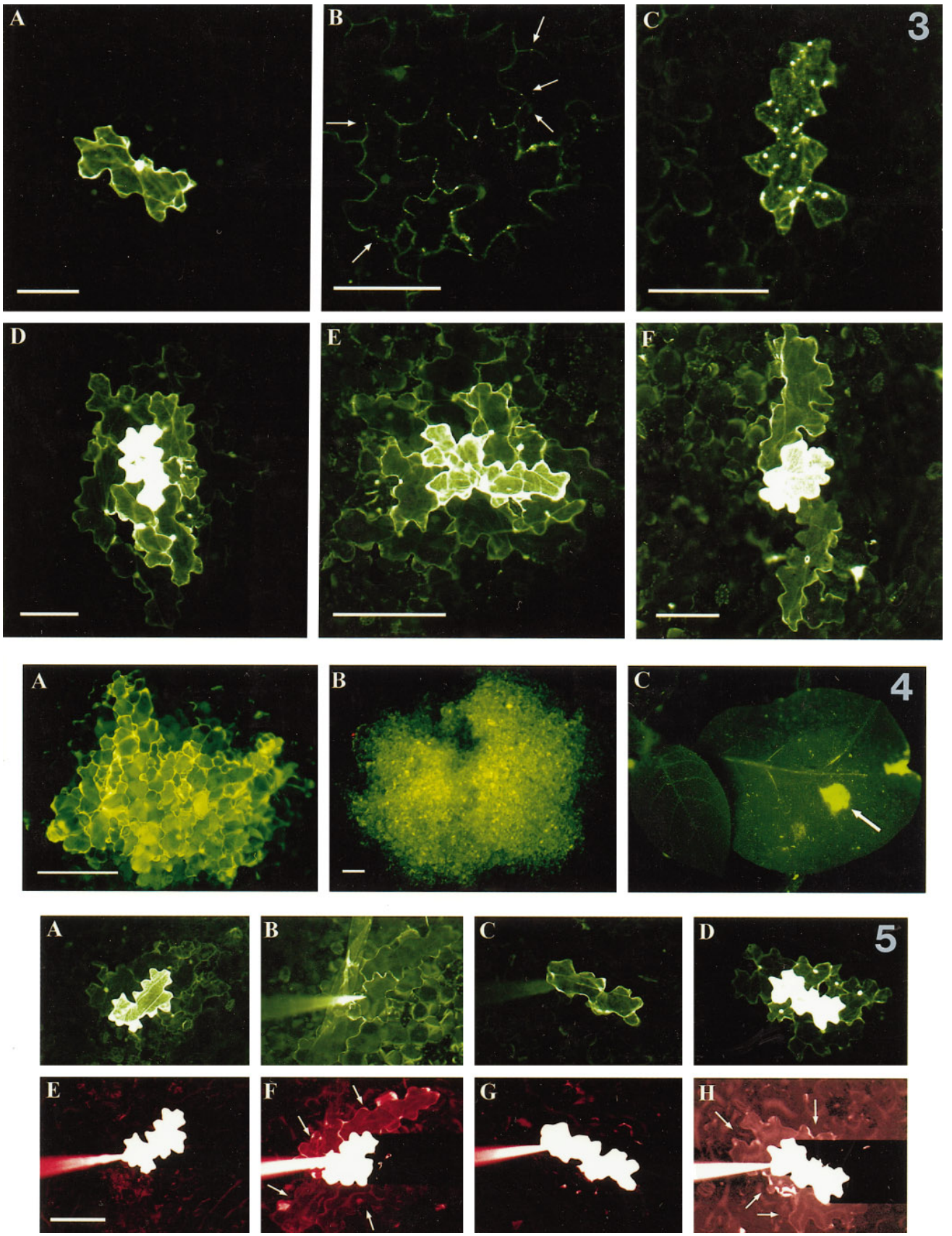

FIG. 3. Fluorescence by GFP expressed in CMV-infected, tobacco-leaf epidermal cells. Tobacco leaves were inoculated with RNA transcribed from pFny109 plus pFny209 (yielding Fny-CMV RNAs 1 plus 2) and pL:3a/GFP (A and D), pF:3a−GFP/CP (B), pF:GFP−3a/CP (C), or pF:GFP/CP (E), or were transfected with transient-expression plasmid pSLJ4D4:GFP using biolistic delivery (F). The infected cells in B, D, E, and F showed a halo

TABLE 2

Quantitation of GFP-Expressing Epidermal Cells Infected by CMV RNAs 1 + 2 and Various RNA 3 Variants

RNA 3 source[c]	Halo cells[d]	Number of infected sites with number of infected cells[a]										
		Tobacco[b]					3a-Transgenic tobacco[b]					
		1	2	3	5–10	>10	1	2	3	5–10	10–20	>20
pF:GFP/CP	−	18	1	0	0	0	44	0	0	0	0	0
	+	41	2	0	0	0	38	4	2	1	0	0
pL:3a/GFP	−	13	0	0	0	0	7	0	0	0	0	0
	+	53	3	2	0	0	15	1	0	0	0	0
pLF:3a/GFP/CP	−	26	0	0	0	0	2	1	0	7	1	3
	+	15	2	1	0	0	2	0	1	0	0	0
pLT:3a/GFP/CP	−	37	4	1	1	0	27	1	5	5	4	6
	+	17	2	1	0	0	15	1	0	0	0	0

[a] Each site of infection was detected by confocal microscopy of green fluorescence from epidermal cells infected by Fny-CMV RNAs 1 + 2 and a particular RNA 3 variant derived from a free GFP-expressing construct at 10–12 days p.i.

[b] Nontransformed *N. tabacum* cv. Samsun NN or the same cultivar transformed and expressing the CMV 3a gene.

[c] See Fig. 1.

[d] Cells at the site of infection were or were not associated with a halo of less intense fluorescent cells, varying from 1 to 20. The number of cells in the halo is not considered in the data, but only the numbers of bright fluorescent cells and whether or not those contained a halo. When more than 5 bright fluorescent cells were present at an infection site, the presence or absence of a halo was not recorded.

mal cells (Fig. 3 and data not shown). (All subsequent inoculations include RNA 1 plus 2 transcripts, although only the nature of the variable RNA 3 transcripts is mentioned.) The infections were confined to the initially inoculated cells (Figs. 3A–F, Table 2). The number of multiple infection sites (Table 2) did not increase with time, indicating no further spread of infection. Thus, inoculations involving variant RNA 3 transcripts, in which either the MP (pF:GFP/CP, Fig. 3E) or the CP (pL:3a/GFP, Fig. 3A) had been replaced with GFP, showed no movement. However, if inoculations involved a mixture of RNA 3 transcripts derived from pF:GFP/CP and pL:3a/GFP, then cell-to-cell movement was observed (Fig. 4A), and a cluster of infected cells was formed. Therefore, both CP and MP are required for even limited cell-to-cell movement of CMV. Curiously, although *trans*-complementation of cell-to-cell movement occurred using two RNA 3 transcripts (Fig. 4A), one producing MP and the other producing CP, little-or-no *trans*-complementation occurred if the inoculum containing RNA 3 transcripts of pF:GFP/CP was applied to transgenic tobacco plants expressing the Fny-CMV 3a protein (Table 2). Previously, plants of this transgenic line had been shown to complement 3a gene-defective mutants of CMV (Kaplan *et al.,* 1995).

Fluorescence due to free GFP was found distributed throughout the cytoplasm, but also concentrated in the nucleus (Figs. 3A, 3D, 3E), as was described by Baulcombe *et al.* (1995). Although we could not quantify the *in vivo* fluorescence intensities, it was apparent that infection involving RNA 3 derived from pL:3a/GFP, with GFP expressed from the subgenomic RNA 4 (the CP mRNA), produced more intense fluorescence than infection involving RNA 3 derived from pF:GFP/CP, with GFP expressed directly from RNA 3.

Limited cell-to-cell movement of tricistronic RNAs 3

Two tricistronic RNAs 3 were generated (Fig. 1), each containing a viral MP and a CP, as well as a separate open reading frame for GFP expression. Inocula con-

of faint, fluorescent epidermal cells around or adjacent to the inoculated cells. Arrows in B indicate localized fluorescence in the cell walls of halo cells. Cells were viewed 5–8 days p.i. The bars represent 100 μm.

FIG. 4. Complementation of cucumovirus cell-to-cell movement. Tobacco leaves were inoculated with RNA transcripts of pFny109 plus pFny209 and either pL:3a/GFP and pF:GFP/CP (A), or pL:3a/GFP and $p1_3\Delta$BssHI (B and C). The spread of virus from the site of inoculation in tobacco leaf epidermal cells was to either hundreds of cells (A) or thousands of cells (B), as viewed by epifluorescence microscopy, 10–14 days p.i. The bars represent 200 μm. At 20 days p.i., the lesion in B was viewed under long-wave UV illumination (C). The diameter of the UV fluorescent lesion (arrow) is about 1 cm.

FIG. 5. Movement of 10-kDa fluorescent dextran microinjected into single epidermal cells infected with CMV and expressing GFP from RNA 3. The RNAs 3 were derived from pF:GFP/CP (A and E), pF:3a-GFP/CP (B and F), pF:GFP–3a/CP (C and G), and pL:3a/GFP (D and H). The green fluorescent cells (A–D) were monitored for the movement of red fluorescent dextran within 10 min of microinjection (E–H). The tapered, white object is the needle used for microinjection. The black zone to the right of the white cell in E–H is caused by saturation of the detector after scanning through the injected cell at the high gain needed to view the movement of red fluorescent dextran to adjacent cells. Arrows in F and H indicate cells to which the red fluorescent dextran has diffused. The bar represents 100 μm.

taining RNA 3 from both constructs, pLF:3a/GFP/CP and pLT:3a/GFP/CP, showed predominantly single-cell infections in tobacco (Table 2), although the intensity of the fluorescent signals was much weaker than for inocula in which the free GFP was expressed from RNA 3 derived from other constructs (data not shown). It is known that the level of expression is lower for extra subgenomic RNAs distal to the viral 3′ end than for the 3′ proximal RNA 4 (Boccard and Baulcombe, 1993).

In CMV 3a transgenic plants, some limited complementation of cell-to-cell movement occurred when the inocula contained RNA 3 derived from both pLF:3a/GFP/CP and pLT:3a/GFP/CP (Table 2). Although infection involving RNA 3 derived from pLT:3a/GFP/CP showed limited cell-to-cell movement, there were no indications of systemic infection. By contrast, infection involving RNA 3 transcripts derived from pLF:3a/GFP/CP always resulted in systemic symptoms similar to infection by Fny-CMV, and the viral RNAs contained RNAs 3 and 4 of the wt size, with no additional subgenomic RNAs (data not shown). In addition, there was no fluorescence either in any systemic leaves or beyond the initial infection site in the inoculated leaves, indicating that the GFP gene had been lost by recombination. Sequence analysis of RNA 3 obtained from the systemically infected leaves confirmed that it was a recombinant, with the 3a gene derived from LS-CMV RNA 3 and the CP gene derived from Fny-CMV RNA 3 (data not presented).

Localization of CMV MP to cell walls

Expression of the fusions between the CMV MP and GFP in both orientations led to single-cell infection, but no subsequent movement in either *N. tabacum* or *N. benthamiana* (Figs. 3B, 3C, and data not shown). However, the intensity of the fluorescence and distribution of GFP were quite different for the two different fusion proteins. The expressed 3a–GFP fusion showed fluorescence almost completely confined to discrete, bright points associated with the cell wall (Fig. 3B), and was visible only at a magnification of 20× or higher, while the expressed GFP–3a fusion showed fluorescence readily visible at a magnification of 10× and was distributed as numerous granular spots of variable size, scattered unevenly throughout the cytoplasm, as well as associated with the cell wall (Fig. 3C). Once again, infections involving either RNA 3 variant expressing a fusion protein could not be complemented for movement in the 3a transgenic plants (data not shown).

Trafficking of MP and movement of fluorescent dextran

Many of the epidermal cells expressing free GFP showed a faint halo of fluorescence in some of the neighboring cells (Figs. 3D, 3E; Tables 2, 3). The number of fluorescent cells in the halo varied from 1 to more than 20 cells. However, the number of cells in the halo per site of infection did not increase with time into multicellular fluorescent lesions, as occurred when complementation of movement occurred (Fig. 4A). In addition, these sites of infection continued to show one to three intensely fluorescent cells associated with the same number of lower-intensity fluorescent halo cells. Surprisingly, these halos were present even in the case of infection involving RNA 3 derived from pF:GFP/CP (Fig. 3E), which lacks the viral MP. Moreover, halos also were observed at infection sites involving RNA 3 derived from pF:3a–GFP/CP (Fig. 3B), in which the GFP was expressed as a C-terminal fusion with the MP. However, in this case, as in the initially infected cells, the fluorescent signal for 3a–GFP also was confined to the cell wall of the halo cells (Fig. 3B). By contrast, the fluorescent signal produced at infection sites involving RNA 3 expressing GFP–3a never showed a halo (Fig. 3C), even in 3a trangenic tobacco leaves (data not presented). These data indicate that the 3a–GFP and free GFP were moving through plasmodesmata, but the GFP–3a fusion protein was not.

TABLE 3

Cell-to-Cell Movement of Fluorescent Dextran Microinjected into Tobacco Epidermal Cells Infected with CMV RNAs 1 + 2 and Various RNA 3 Variants

RNA 3 source[a]	Number of cells microinjected[b]	Number of cells showing movement[c]
None[d]	4	0
pF:GFP/CP	4	0
pF:3a–GFP/CP	4	4[e]
pF:GFP–3a/CP	5	0
pL:3a/GFP	5	3[e]

[a] See Fig. 1.

[b] Cells infected by various RNA 3 variants expressing GFP were microinjected with Texas Red, fluorescent 10-kDa dextran.

[c] Movement is defined as diffusion of red fluorescent dye to neighboring cells within 10 min of microinjection.

[d] Noninfected cells microinjected with 10-kDa fluorescent dextran.

[e] The microinjected cells with a halo of less intense green fluorescent cells to which the red fluorescent dye moved.

The cell-to-cell movement of free GFP in the absence of CMV MP (Fig. 3E) was quite unexpected. To determine whether this movement was a property of GFP itself or was due to movement assisted by other CMV-encoded proteins, free GFP was expressed transiently from a plasmid transfected into epidermal cells by biolistic delivery. Five days after transfection, numerous, fluorescent single cells could be seen, most of which (25 of 32) showed the presence of halos (Fig.3F), with variable numbers of halo cells around the initially inoculated cells. Thus, free GFP can traffic to a limited extent, even in the absence of viral-encoded proteins, at least in epidermal cells of tobacco (Table 2) and *N. benthamiana* (data not shown).

To verify whether fusion MPs expressed in singly in-

fected cells could increase the permeability of plasmodesmata, 10-kDa (Texas Red) fluorescent dextran was microinjected into cells intensely expressing GFP, i.e., the initially infected cells. In cells expressing free GFP and CMV CP, but no CMV MP, there was no movement of the microinjected fluorescent dextran (Fig. 5A vs Fig. 5E, Table 3). In cells expressing free GFP and the CMV MP, but not CP, movement of the microinjected fluorescent dextran was observed (Fig. 5D vs Fig. 5H, Table 3), but only to cells of the surrounding halo. Cells expressing 3a–GFP also showed movement of microinjected fluorescent dextran (Fig. 5B vs Fig. 5F, Table 3), while cells expressing GFP–3a did not (Fig. 5C vs Fig. 5G, Table 3). Thus, the ability to increase plasmodesmatal permeability of epidermal cells and allow passage of 10-kDa dextran required the presence of CMV MP, whereas GFP fused to the N terminus of the CMV MP disrupted this function.

CMV RNAs that express GFP do not move long distance

Although *trans*-complementation occurred when RNA 3 transcripts of pF:GFP/CP and pL:3a/GFP were included in the inoculum, virus movement was limited to clusters of hundreds of cells (Fig. 4A). In only 1 of 20 inoculations involving this combination of RNAs 3 was a systemic infection obtained, although in that case no fluorescence was observed, and the RNA 3 present was shown to be a recombinant (data not presented).

Trans-complementation was also done using RNA 3 derived from pL:3a/GFP and $p1_3\Delta$BssHI (a cDNA clone expressing a movement-deficient TAV RNA 3 containing a 6-nt deletion in the 3a gene). In this case, the cluster of infected cells was much larger, consisting of thousands of cells (Fig. 4B), and was even visible to the naked eye under UV illumination (Fig. 4C). Nevertheless, there was no apparent vascular movement of this virus. Extracts made from these lesions were not infectious when inoculated into other plants; i.e., single-cell infection could not be detected by fluorescence microscopy.

Analysis of the viral RNA in the fluorescent lesions showed that little accumulating CMV RNAs 3 or 4 was present during complementation involving RNAs 3 from pL:3a/GFP and $p1_3\Delta$BssHI (Figs. 6A and 6B, lane 1). While the levels of accumulating CMV RNAs 1–4 were higher in fluorescent lesions from complementation involving RNAs 3 from pL:3a/GFP and pF:GFP/CP (Fig. 6A, lane 2 vs lane 1), they were much lower than was detected in a similar area of tissue taken from tobacco leaves infected with wt Fny-CMV (Fig. 6A, lane 4 vs lane 2). Furthermore, the unusually high ratio of RNA 4 to RNA 3 in tissue infected by RNAs 3 derived from pL:3a/GFP and pF:GFP/CP (Fig. 6A, lane 2) stands in contrast to the low ratio of RNA 4 to RNA 3 observed when the same membrane was hybridized with a GFP gene-specific probe (Fig. 6B, lane 2), suggesting differential encapsidation of the various RNAs 3 and 4.

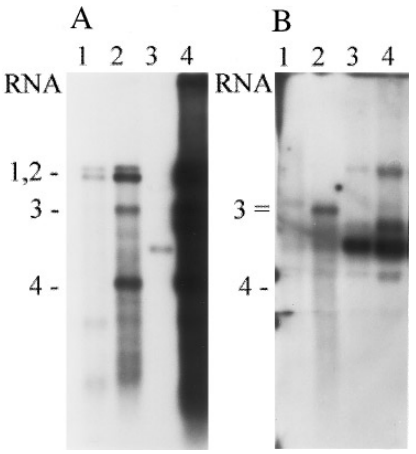

FIG. 6. Autoradiogram of Northern blot analysis of total RNAs extracted from fluorescent lesions on tobacco. The fluorescent lesions, containing multiple fluorescent cells, were from infections containing RNA 3 derived from pL:3a/GFP plus $p1_3\Delta$BssHI (lane 1), pL:3a/GFP plus pF:GFP/CP (lane 2), or wt pFny309 (lane 4). RNAs from an equal area of noninoculated tobacco leaf are present in lane 3. The blot was first incubated with ^{32}P-labeled RNA complementary to the 3′ NTR of the Fny-CMV RNAs [but also capable of detecting the LS-CMV RNAs (A)], washed to remove the probe, and then incubated with a probe specific to the GFP gene (B). Residual hybridization to some plant rRNAs (lanes 3 and 4) by the first probe is present and amplified by the longer exposure in B. The positions of the CMV RNAs in A and of TAV and CMV RNA 3 and CMV RNA 4 in B are indicated.

DISCUSSION

Localization of MP to cell walls and intercellular trafficking

The localization of the 3a–GFP to cell walls was not unexpected, and also had been observed in mesophyll cells and leaf trichome cells when CMV 3a–GFP was expressed from a potato virus X (PVX) vector (Oparka *et al.,* 1996b). Several other studies involving subcellular fractionation also have indicated a cell wall association of CMV MP (Vaquero *et al.,* 1994, 1996; Cooper and Dodds, 1995). By contrast, our own subcellular fractionation studies did not indicate such an association, except in older leaves (Kaplan *et al.,* 1995; Gal-On *et al.,* 1996). These differences in localization are not due to some effect of the GFP fused to the C terminus of the 3a MP, since both the 3a protein (Vaquero *et al.,* 1996), produced during infection by Fny-CMV, and the 3a–GFP fusion expressed from the PVX vector (L. Blackman, P. Boevink, and K. J. Oparka, unpublished observations) have been localized to plasmodesmata by immunoelectron microscopy. These observations are consistent with the ability of the 3a–GFP fusion to increase the permeability of plasmodesmata and promote its own movement as well as that of 10-kDa dextran. However, the inability of 3a–GFP to traffic viral RNA from cell to cell suggests that

there are differences in the mechanism of transport for viral RNA versus other molecules. This was also the case for inhibition of movement by transgenic plants expressing replicase gene sequences, where neither TMV MP trafficking nor TMV MP-potentiated movement of 9.4-kDa fluorescent dextran was inhibited, but TMV MP-potentiated movement of TMV RNA was inhibited, even in transgenic plants expressing a defective CMV replicase gene and showing no resistance to TMV (Nguyen *et al.,* 1996).

Requirement for MP and CP for cell-to-cell movement

The previous genetic data indicating that CMV MP and CP are both required for movement in the inoculated leaf (Suzuki *et al.,* 1991) have been confirmed and extended to demonstrate that both genes (and presumably their gene products) are required for movement of virus out of the initially infected cells. Since MP alone was shown to be capable of translocating viral RNA between mesophyll cells (Ding *et al.,* 1995b), it is not at all understood what the role of CP is in this process, although the data here show that there is such a requirement in epidermal cells. Whether these differences in requirements for intercellular movement are based on cell types or differences in the techniques employed (microinjection vs infection, which may relate to the effects of other gene products present during infection) remains to be determined.

Could halo cells reflect movement of viral RNA from the inoculated cell to adjacent cells followed by replication and GFP accumulation? The absence of an increase in either the number of cells in a halo or the number of infection sites containing multicellular fluorescent lesions with time, by inocula lacking either MP or CP, argues against movement of viral RNA and subsequent infection of adjacent cells. Moreover, the presence of transiently expressed free GFP in the halo cells shows that a low level of GFP movement occurs independent of virus movement (Fig. 3F). In addition, the presence of 3a–GFP in the cell walls of halo cells (Fig. 3B) in lesser amounts, which again does not increase in fluorescence intensity with time, is also indicative of an absence of virus movement and accumulation in cells adjacent to the inoculated cell, even though 3a–GFP could still promote the movement of 10-kDa fluorescent dextran (Fig. 5F). Finally, the inability of GFP–3a to traffic itself to form halo cells (Fig. 3C) is consistent with its inability to increase the size-exclusion limit for 10-kDa fluorescent dextran (Fig. 5G), indicating that GFP–3a is incapable of promoting movement. Together these observations are inconsistent with poor rather than no movement of viral RNAs occurring in the absence of either MP or CP.

Protection of the viral RNAs against degradation has been suggested as the role for the CP in virus movement (Suzuki *et al.,* 1991; Boccard and Baulcombe, 1993). However, while the level of accumulated viral RNA was much lower in protoplasts infected with CP deletion mutants than for wt CMV RNA (Suzuki *et al.,* 1991; Boccard and Baulcombe, 1993), and no movement was observed in those studies or these (Fig. 3) in the absence of CP, the level of accumulated viral RNA was also much lower here in fluorescent lesions showing complementation of cell-to-cell movement than was observed for wt CMV (Fig. 6). Moreover, the more intense GFP fluorescence observed at infection sites involving RNA 3 from pL:3a/GFP than RNA 3 from pF:GFP/CP suggests that sufficient RNAs 3 and 4 were synthesized during virus replication even in the absence of CP, making it unlikely that the CP merely serves a role in protecting viral RNA against degradation and enabling sufficient viral RNA to be produced for subsequent movement. In addition, sequences in the CP of CMV and TAV have been implicated in host-specific restrictions to virus movement in zucchini squash (Shintaku and Palukaitis, 1990) and cucumber (Taliansky and García-Arenal, 1995), respectively, also indicating an active role for CP in virus movement.

The ability of GFP to move from the initially infected cell to adjacent cells to form halos, even in the absence of CMV MP, appear to be an intrinsic property of free GFP and does not require other CMV-encoded proteins (Fig.3F). Whether this property is manifested in inoculated cells versus nonstressed cells is unknown. PVX constructs expressing free GFP and no CP (also needed for PVX movement) also showed a halo in the adjacent cells (Oparka *et al.,* 1996a), while stable PVX GFP–CP fusions did not (Santa Cruz *et al.,* 1996), similar to the situation with the GFP–3a fusions described here. While some of these fusion proteins may be too large for trafficking, size is not the only factor, since the 3a–GFP fusion protein was still able to traffic itself from cell to cell.

Inhibition of long-distance movement

Although several variant RNAs 3 were able to demonstrate *trans*-complementation of cell-to-cell movement, their inability to potentiate long-distance movement may relate to their lower accumulation levels (Fig. 6). The reason for the lower levels of accumulation of the latter viral RNAs in tissues is unknown, since individual variant RNAs 3 accumulated efficiently in protoplasts (Fig. 2 and data not shown). The failure to move long distance could be be due to an indirect effect of different efficiencies of packaging some of these RNAs. Whether this is due to some effect of the GFP-encoding sequences or differences in the sizes of the RNAs (Table 1), vis-à-vis what is optimal for encapsidation, is unknown. On the other hand, lower levels of accumulation may also result in less MP being synthesized, and there may be a requirement for both high MP levels and high viral RNA levels to promote rapid movement. A low accumulation of MP leading to a severely reduced rate of cell-to-cell and long-

distance movement of TAV has been demonstrated (Moreno *et al.,* 1997), and differences in levels of accumulation of MP have been shown to affect the movement into, and accumulation of, CMV in systemically infected tobacco leaves by two strains of CMV (Gal-On *et al.,* 1996). While reduced MP levels had only a slight effect on the rate of cell-to-cell movement of CCMV and no effect on the rate of systemic infection (De Jong *et al.,* 1997), the level of MP needed for these various aspects of the movement process may vary between these viruses or in different hosts. Thus, a reduction in the level of MP without a reduction in the level of accumulating viral RNA may lead to only a reduced efficiency of virus movement, while a reduction in both may lead to a block in systemic movement.

The slow growth of the fluorescent lesions during *trans*-complementation could also be explained by an alternative model. If the virus moves slower from cell-to-cell, then there may be sufficient time for the host to establish some form of inducible resistance to long-distance movement. Such a response has been suggested for the arrest of further cell-to-cell movement of CCMV expressing a BMV MP after 40–80 epidermal cells became infected (Mise and Ahlquist, 1995). A host response also may be occurring in cucurbits infected by CMV that contains the MP encoded by a variant of the Sny strain of CMV, since such chimeric viruses moved more slowly within the cotyledons and failed to infect several cucurbit species systemically (Kaplan *et al.,* 1997). The extent to which inhibition of virus movement occurs by either or both of these models may vary with different viral systems.

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REFERENCES

Atabekov, J. G., and Taliansky, M. E. (1990). Expression of plant virus-coded transport function by different viral genomes. *Adv. Virus Res.* 38, 201–248.

Baulcombe, D. C., Chapman, S., and Santa Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J.* 7, 1045–1053.

Boccard, F., and Baulcombe, D. (1993). Mutational analysis of *cis*-acting sequences and gene function in RNA 3 of cucumber mosaic virus. *Virology* 193, 563–578.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K., and Schaad, M. C. (1996). Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* 8, 1669–1681.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescence protein as a marker for gene expression. *Science* 263, 802–805.

Cooper, B., and Dodds, J. A. (1995). Differences in the subcellular localization of tobacco mosaic virus and cucumber mosaic virus movement proteins in infected and transgenic plants. *J. Gen. Virol.* 76, 3217–3221.

De Jong, W., Mise, K., Chu, A., and Ahlquist, P. (1997). Effects of coat protein mutations and reduced movement protein expression on infection spread of cowpea chlorotic mottle virus and its hybrid derivatives. *Virology* 232, 167–173.

Ding, S. W., Li, W-X., and Symons, R. H. (1995a). A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J.* 14, 5762–5772.

Ding, B., Li, Q., Nguyen, L., Palukaitis, P., and Lucas, W. J. (1995b). Cucumber mosaic virus 3a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants. *Virology* 207, 345–353.

Gal-On, A., Kaplan, I. B., Roossinck, M. J., and Palukaitis, P. (1994). The kinetics of infection of zucchini squash by cucumber mosaic virus indicate a function of RNA 1 in virus movement. *Virology* 205, 280–289.

Gal-On, A., Meiri, E., Huet, H., Hua, W. J., Raccah, B., and Gaba, V. (1995). Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic virus. *J. Gen. Virol.* 76, 3223–3227.

Gal-On, A., Kaplan, I. B., and Palukaitis, P. (1996). Characterization of cucumber mosaic virus. II. Identification of movement protein sequences that influence its accumulation and systemic infection in tobacco. *Virology* 226, 354–361.

Gal-On, A., Meiri, E., Elman, C., Gray, D. J. and Gaba, V. (1997). Simple hand-held devices for the efficient infection of plants with viral-encoding constructs by particle bombardment. *J. Virol. Methods* 64, 103–110.

Hellwald, K-H., and Palukaitis, P. (1995). Viral RNA as a potential target for two independent mechanisms of replicase-mediated resistance against cucumber mosaic virus. *Cell* 83, 937–946.

Jones, J. D. G., Shlumukov, L., Carland, F., English, J., Scofield, S. R., Bishop, G. J., and Harrison, K. (1992). Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. *Transgenic Res.* 1, 285–297.

Kaplan, I. B., Shintaku, M. H., Li, Q., Marsh, L. E., and Palukaitis, P. (1995). Complementation of virus movement in transgenic tobacco expressing the cucumber mosaic virus 3a gene. *Virology* 209, 188–199.

Kaplan, I. B., Gal-On, A., and Palukaitis, P. (1997). Characterization of cucumber mosaic virus. III. Localization of sequences in the movement protein controlling systemic infection in cucurbits. *Virology* 230, 343–349.

Kim, K-S., and Lee, K-W. (1992). Geminivirus-induced macrotubules and their suggested role in cell-to-cell movement. *Phytopathology* 82, 664–669.

Li, Q., and Palukaitis, P. (1996). Comparison of the nucleic acid- and NTP-binding properties of the movement protein of cucumber mosaic cucumovirus and tobacco mosaic tobamovirus. *Virology* 216, 71–79.

Lucas, W. J., and Gilbertson, R. L. (1994). Plasmodesmata in relation to viral movement within leave tissues. *Annu. Rev. Phytopathol.* 32, 387–411.

Maule, A. J. (1991). Virus movement in infected plants. *Crit. Rev. Plant. Sci.* 9, 457–474.

McLean, B. G., Waigmann, E., Citovsky, V., and Zambryski, P. (1993). Cell-to-cell movement of plant viruses. *Trends Microbiol.* 1, 105–109.

Melcher, U. (1990). Similarities between putative transport proteins of plant viruses. *J. Gen. Virol.* 71, 1009–1018.

Mise, K., and Ahlquist, P. (1995). Host-specificity restriction by bromovirus cell-to-cell movement protein occurs after initial cell-to-cell spread of infection in nonhost plants. *Virology* 206, 276–286.

Moreno, I. M., Bernal, J. J., García de Blas, B., Rodríguez-Cerezo, E., and García-Arenal, F. (1997). The expression level of the 3a movement protein determines differences in severity of symptoms be-

tween two strains of tomato aspermy cucumovirus. *Mol. Plant–Microbe Interact.* 10, 171–179.

Nguyen, L., Lucas, W. J., Ding, B., and Zaitlin, M. (1996). Viral RNA trafficking is inhibited in replicase-mediated resistant transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA* 93, 12643–12647.

Nishiguchi, M., Motoyoshi, F., and Oshima, N. (1978). Behaviour of a temperature sensitive strain of tobacco mosaic virus in tomato leaves and protoplasts. *J. Gen. Virol.* 39, 53–61.

Oparka, K. J., Murphy, R., Derrick, P. M., Prior, D. A. M., and Smith, J. A. C. (1990). Modification of the pressure probe technique permits controlled intracellular microinjection of fluorescent probes. *J. Cell Sci.* 99, 557–563.

Oparka, K. J., Roberts, A. G., Roberts, I. M., Prior, D. A. M., and Santa Cruz, S. (1996a). Viral coat protein is targeted to, but does not gate, plasmodesmata during cell-to-cell movement of potato virus X. *Plant J.* 10, 805–813.

Oparka, K. J., Boevink, P., and Santa Cruz, S. (1996b). Studying the movement of plant viruses using green fluorescent protein. *Trends Plant Sci.* 1, 412–418.

Palukaitis, P., Cotts, S., and Zaitlin, M. (1985). Detection and identification of viroids and viral nucleic acids by "dot-blot" hybridization. *Acta Horticult.* 164, 109–118.

Perbal, M-C., Thomas, C. L., and Maule, A. J. (1993). Cauliflower mosaic virus gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. *Virology* 195, 281–285.

Rao, A. L. N. (1997). Molecular studies on bromovirus capsid protein. III. Analysis of cell-to-cell movement competence of coat protein-defective variants of cowpea chlorotic mottle virus. *Virology* 232, 385–395.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Santa Cruz, S., Chapman, S., Roberts, A. G., Roberts, I. M., Prior, D. A. M., and Oparka, K. J. (1996). Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. *Proc. Natl. Acad. Sci. USA* 93, 6286–6290.

Schmitz, I., and Rao, A. L. N. (1996). Molecular studies on bromovirus capsid protein. I. Characterization of cell-to-cell movement-defective RNA 3 variants of brome mosaic virus. *Virology* 226, 281–293.

Schneider, W. L., Greene, A. E., and Allison, R. F. (1997). The carboxy-terminal two-thirds of the cowpea chlorotic mottle bromovirus capsid protein is incapable of virion formation yet supports systemic movement. *J. Virol.* 71, 4862–4865.

Séron, K., and Haenni, A-L. (1996). Vascular movement of plant viruses. *Mol. Plant–Microbe Interact.* 9, 435–442.

Shintaku, M., and Palukaitis, P. (1990). Genetic mapping of cucumber mosaic virus. *In* "Viral Genes and Plant Pathogenesis" (T. P. Pirone and J. G. Shaw, Eds.), pp. 156–164. Springer-Verlag, New York.

Siegel, A., Zaitlin, M., and Sehgal, O. P. (1962). The isolation of defective tobacco mosaic virus strains. *Proc. Natl. Acad. Sci. USA* 48, 1845–1851.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N., and Takanami, Y. (1991). Functional analysis of deletion mutants of cucumber mosaic virus RNA 3 using an *in vitro* transcription system. *Virology* 183, 106–113.

Taliansky, M. E., and García-Arenal, F. (1995). Role of cucumovirus capsid protein in long-distance movement within the infected plant. *J. Virol.* 69, 918–922.

Van Lent, J., Wellink, J., and Goldbach, R. (1990). Evidence for the involvement of the 58K and 48K proteins in the intercellular movement of cowpea mosaic virus. *J. Gen. Virol.* 71, 219–223.

Vaquero, C., Turner, A. P., Demangeat, G., Sanz, A., Serra, M. T., Roberts, K., and García-Luque, I. (1994). The 3a protein from cucumber mosaic virus increases the gating capacity of plasmodesmata in transgenic tobacco plants. *J. Gen. Virol.* 75, 3193–3197.

Vaquero, C., Sanz, A. I., Serra, M. T., and García-Luque, I. (1996). Accumulation kinetics of CMV RNA 3-encoded proteins and subcellular localization of the 3a protein in infected and transgenic tobacco plants. *Arch. Virol.* 141, 987–999.

Wieczorek, A., and Sanfaçon, H. (1993). Characterization and subcellular localization of tomato ringspot virus putative movement protein. *Virology* 194, 734–742.

Zhang, L., Hanada, K., and Palukaitis, P. (1994). Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. *J. Gen. Virol.* 75, 3185–3191.