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Role of Cucumovirus Capsid Protein in Long-Distance Movement within the Infected Plant

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Direct evidence is presented for a host-specific role of the cucumovirus capsid protein in long-distance movement within infected plants. Cucumber (*Cucumis sativus* L.) is a systemic host for cucumber mosaic cucumovirus (CMV). Tomato aspermy cucumovirus, strain 1 (1-TAV), multiplied to the levels of CMV (i.e., replicated, moved from cell to cell, and formed infectious particles) in the inoculated leaves of cucumbers but was completely unable to spread systemically. The defective long-distance systemic movement of 1-TAV was complemented by CMV in mixed infections. Coinfection of cucumbers with 1-TAV RNA with various combinations of transcripts from full-length cDNA clones of CMV genomic RNA 1, RNA2, and RNA3 showed that CMV RNA3 alone complemented 1-TAV long-distance movement. We obtained mutants containing mutations in the two open reading frames in CMV RNA3 encoding the 3a protein and the capsid protein (CP), both of which are necessary for cell-to-cell movement of CMV. Complementation experiments with mutant CMV RNA3 showed that only 3a protein mutants, i.e., those with an intact CP, complemented the long-distance movement of 1-TAV in cucumbers. Since CMV and TAV have common systemic host plants, the results presented here are strong evidence for an active, host-specific function of the CPs of these two cucumoviruses for long-distance spread in the phloem. The results also suggest that the plasmodesmata in the vascular system and/or at the boundary between the mesophyll and the vascular system, involved in long-distance movement through the phloem, and those in the mesophyll, involved in cell-to-cell movement, differ functionally.

Plant viruses enter host cells through wound sites induced either mechanically or by vector organisms. Spread of infection from the initially infected cells occurs by two processes. First, viruses spread from cell to cell in the epidermis and mesophyll; this is short-distance or cell-to-cell movement. A second process, long-distance movement, allows the viruses to enter the vascular system and spread systemically. The study of the mechanisms of virus movement in infected plants has received much attention during the last few years as a crucial point in plant virus interactions, and different aspects have been reviewed recently (2, 12, 16, 28). Further, the movement of viruses in infected plants provides appropriate experimental systems with which to analyze the more general problem of intercellular communications in plants (8, 9).

Substantial progress has been achieved in the understanding of cell-to-cell movement. It is generally accepted that cell-to-cell movement is a function of both viral and host genomes and that it involves virus-encoded "movement proteins" (MPs) and host factors. Although the nature of the host factors remain obscure, MPs have been identified for a number of plant viruses. It has also been shown that mechanisms by which MPs facilitate cell-to-cell movement may differ for different viruses. In some cases, such as tobacco mosaic tobamovirus, the MP interacts with plasmodesmata, thereby increasing their permeability and facilitating the passage of the virus in a nonvirion form (3, 56). In addition, it may be that the RNA-binding property of the tobacco mosaic virus MP plays a role in cell-to-cell movement of tobacco mosaic virus RNA (11). In other cases, such as cowpea mosaic comovirus, the MP induces the formation of tubular structures, extending from the plasmodesmata of infected cells, which are considered to facilitate cell-to-cell spread of cowpea mosaic virus in the form of virions (52, 53, 55).

Less is known about the mechanism(s) of long-distance movement via the phloem. It is not clear whether viral MPs are directly involved in the process of long-distance movement. In some cases, proteins other than MP have been shown to be related to long-distance movement. Such is the case for replication-related proteins in barley stripe mosaic hordeivirus (37, 54), brome mosaic bromovirus (51), and tobacco mosaic virus (33), although it is unclear whether this is due to anything other than replication effects per se. Also, for a number of both rod-shaped and spherical viruses, including the cucumoviruses (6, 50), the capsid protein (CP) is required for systemic infection. However, the role of CP in the long-distance transport of these viruses is unclear.

The cucumoviruses are spherical viruses with a tripartite, single-stranded, positive-sense RNA genome. RNA1 and RNA2 encode proteins (1a and 2a, respectively) involved in replication. RNA3 encodes the 3a protein and the CP, which is expressed from subgenomic RNA4 (for a review see 36). Using transcripts generated from mutated full-length cDNA clones of cucumber mosaic virus (CMV) RNAs, Suzuki et al. (50) and Boccard and Baulcombe (6) showed that both the 3a protein and CP were dispensable for replication in protoplasts but were essential for cell-to-cell movement and infection of whole plants. It was suggested that the 3a protein potentiates the cell-to-cell movement of the virus as the MP. The role of the CMV CP in establishing the systemic infection remained obscure.

In the present work, we analyzed viral factors involved in the long-distance movement of two different cucumoviruses, CMV and tomato aspermy cucumovirus (TAV). The results indicate that in addition to its functions in cell-to-cell movement and

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TABLE 1. Accumulation of viral products in cucumbers inoculated with 1-TAV and Fny-CMV[a]

Inoculum	Leaf	Amt of CP (μg/g of leaf)[b]	Amt of genomic RNA (μg/g of leaf)[c]		Infectivity of virus particles[d]	% Infected cells[e]
			RNA1	RNA2		
1-TAV	Inoculated cotyledons[f]	56 ± 5	4.5 ± 0.4	8.0 ± 0.2	41 ± 7	68 ± 6
	Upper leaves[f]	0	0	0	0	0
Fny-CMV	Inoculated cotyledons	64 ± 6	5.0 ± 0.2	6.0 ± 0.2	37 ± 4	65 ± 8
	Upper leaves	86 ± 8	4.8 ± 0.2	7.5 ± 0.3	62 ± 8	78 ± 5
1-TAV	Inoculated true leaves	44 ± 5	2.5 ± 0.2	5.0 ± 0.2	35 ± 5	51 ± 4
	Upper leaves	0	0	0	0	0
1-TAV+Fny-CMV	Inoculated cotyledons[g]	85 ± 6	2.7 ± 0.3	5.0 ± 0.2	NT[h]	59 ± 5
		84 ± 4	3.5 ± 0.2	4.8 ± 0.3	NT	68 ± 4
	Upper leaves[g]	64 ± 3	3.5 ± 0.2	4.5 ± 0.3	NT	54 ± 8
		95 ± 5	7.5 ± 0.2	11.5 ± 0.4	NT	74 ± 2

[a] Data are mean ± standard error of three independent experiments with three replicates in each.
[b] Determined by ELISA.
[c] Determined by dot-blot hybridization.
[d] Determined by inoculation of purified virus preparations on *Chenopodium quinoa* and presented as the average number of lesions per half leaf. Half leaves were inoculated with an amount of purified virions equivalent to 0.1 g of infected tissue.
[e] Determined by immunofluorescent staining of protoplasts isolated from inoculated cotyledons or upper leaves.
[f] Accumulation of viral products in inoculated and noninoculated (upper) leaves measured 7 and 12 days p.i.; tests for determination of viral products in second upper leaves of 1-TAV-infected cucumbers repeated 4 weeks p.i. did not reveal any infection.
[g] The top line of each pair is for TAV; the bottom line is for CMV.
[h] NT, not tested.

virus assembly, the CMV CP possesses host-specific determinants for long-distance movement.

MATERIALS AND METHODS

Viruses and plants. CMV Fny and M (in subgroup I) and TrK7 and LS (in subgroup II) and TAV 1, V, C, and P have been described previously (30, 34, 36, 45, 46). Virus isolates were propagated in *Nicotiana tabacum* L. cv. Xanthi-nc plants. The viruses were purified as described previously (22, 29). RNA was extracted from purified virions with phenol-sodium dodecyl sulfate.

Tobacco and cucumber (*Cucumis sativus* L. cv. Ashley) plants were grown either in a greenhouse or in a growth chamber at 25°C with a 14-h light/10-h dark cycle.

Recombinant plasmids, generation of mutants for Kin-CMV RNA3, and in vitro transcription. Recombinant plasmids with cDNA clones representing the full-length RNA1 (pK1), RNA2 (pK2), and RNA3 (pK3) of Kin-CMV (5) were the gift of David Baulcombe, The Sainsbury Laboratory, Norwich, United Kingdom. Plasmids were multiplied in *Escherichia coli* DH5α, and DNA was purified by standard procedures (47).

Four mutant forms of pK3 (see Fig. 3) were constructed. pΔHH was generated by the replacement of a *Hpa*I (nucleotide [nt] 125)-*Hpa*I (nt 923) fragment by an inverted *Hpa*I (nt 125)-*Sac*I (nt 492) fragment after removal of the *Sac*I 3′ protruding end with T4 DNA polymerase. pC1 has a frameshift mutation at nt 560 generated by cleavage at a *Cla*I site (nt 560), filling the recessed 3′ termini with the Klenow fragment of DNA polymerase I, and religating. pR was generated by cleavage at an *Rsr*II site (nt 1289), filling the recessed 3′ termini, and religating. The result was an in-frame insertion of 3 nt which inserted a Ser residue between Arg-23 and Ser-24. pΔRT was generated by deletion of the *Rsr*II (nt 1290)-*Tht*111I (nt 1875) after filling the recessed 3′ termini and religation. Restriction sites and nucleotide numbers are those of Boccard and Baulcombe (6). The identity of the mutants was confirmed by restriction analyses and DNA sequencing (as in reference 32).

Plasmids pK1, pK2, and pK3 and the mutants were linearized with *Bgl*II and used as templates for in vitro transcription in the presence of m^7GpppG (New England Biolabs) as described by Boccard and Baulcombe (5).

Plant inoculation. Inoculation of plants was performed on expanded cotyledons of cucumbers 10 days after sowing or on true leaves 20 days after sowing. The suspension of total viral RNA of CMV and TAV used as inoculum (10 μl per plant) was 200 μg/ml in 0.1 M Na_2HPO_4. For inoculation with RNA transcripts, 2 to 5 μg of each transcript was used per plant. At different times postinoculation, between 1 and 4 weeks, virus multiplication was analyzed by enzyme-linked immunosorbent assay (ELISA), infectivity assay, dot-blot hybridization, Northern (RNA) blot analysis, and immunofluorescent screening of isolated protoplasts. Results were obtained from two or three independent experiments with three replicate plants per treatment.

Preparation and transfection of protoplasts. Protoplasts were isolated from fully expanded cotyledons or mature leaves of cucumber plants as described for *N. tabacum* (39). Cellulase Onozuka R10 and Macerocyme R10 were used at concentrations of 1.5 and 0.3%, respectively. Protoplasts isolated from cotyledons or true leaves of inoculated plants were used for immunofluorescence analyses. Only protoplasts from cotyledons were used for inoculation experiments. Transfections were carried out with 3 to 5 μg of each RNA transcript (tK1, tK2, or tK3) or with 5 μg of virion RNA per 10^6 protoplasts. RNA in 25 μl of ice-cold water was added to 10^6 pelleted protoplasts. After incubation for 2 min on ice, 1 ml of 40% (wt/vol) polyethylene glycol (molecular weight 6,000) in 9% mannitol was added (40). 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 to 24°C under continuous illumination. Virus multiplication was analyzed at different times (0, 4, 16, 24, and 42 h) postinoculation. All transfections were performed three times.

Analysis of virus progeny. For ELISA analyses of CP accumulation, samples of 10^6 protoplasts or 0.2 g of leaf tissues were homogenized in 1 ml of phosphate-buffered saline (pH 7.2). The indirect ELISA procedure (13) was used to detect

TABLE 2. Accumulation of CP in cucumber plants coinoculated with 1-TAV RNA and with RNA transcripts generated from full-length clones of Kin-CMV RNAs

Inoculum	Amt of CP (μg/g of leaf)[a] in:			
	Inoculated leaves[b]		Upper leaves[b]	
	1-TAV CP	CMV CP	1-TAV CP	CMV CP
tK1 + tK2 + tK3	NT[c]	69 ± 6	NT	75 ± 4
tK1 + tK2	NT	0	NT	0
tK3	NT	0	NT	0
1-TAV	59 ± 7	NT	0	NT
1-TAV + tK1 + tK2 + tK3	47 ± 5	62 ± 4	54 ± 6	75 ± 6
1-TAV + tK1 + tK2	48 ± 6	0	0	0
1-TAV + tK3	51 ± 6	4.2 ± 0.6	49 ± 4	5.7 ± 0.4

[a] Determined by ELISA. Data are mean ± standard error of three independent experiments with three replicates each.
[b] Accumulation of CP in inoculated and upper leaves was determined 9 and 16 days p.i., respectively. Tests repeated 24 days p.i. did not reveal any infection for either of the treatments showing no infection 16 days p.i.
[c] NT, not tested.

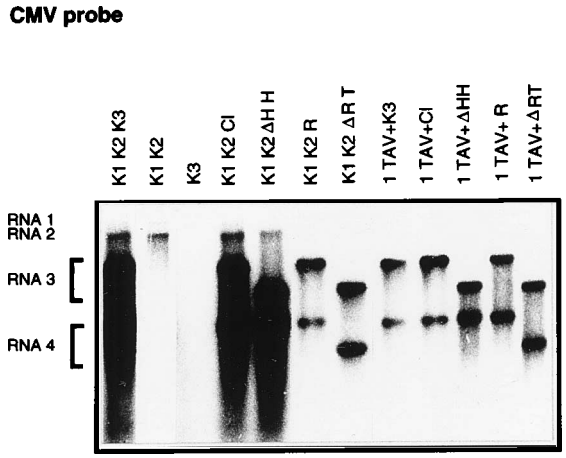

FIG. 1. Accumulation of viral RNA in cucumber protoplasts 24 h after inoculation with transcripts from clones of Kin-CMV RNAs and with 1-TAV RNA. Northern blot analyses were of total nucleic acids extracted from protoplasts after electrophoresis in 1.2% agarose–TBE gels. Inocula are indicated above each line. 1-TAV, 1-TAV virion RNA; K1, K2, K3, Kin-CMV RNA transcripts from full-length cDNA clones pK1, pK2, and pK3, respectively; Cl, ΔHH, R, ΔRT, transcripts from mutant Kin-CMV RNA3 clones pCl, pΔHH, pR, and pΔRT, respectively. The mobilities of RNA1, RNA2, and RNA3 and of subgenomic RNA4 are indicated.

both CMV and TAV with polyclonal antisera, the gift of R. I. B. Francki, Adelaide, Australia, and of M. T. Serra, Madrid, Spain. Concentrations of CP were estimated by comparing the readings of dilution series of the samples with those of purified CMV or TAV particles.

The infectivity of virus preparations was assessed by measuring the mean number of necrotic local lesions induced in six to eight randomized half leaves of *Chenopodium quinoa*, a common necrotic host for both CMV and TAV.

The percentage of infected cells in inoculated and systemically infected leaves was determined by immunofluorescence staining of protoplasts with anti-CMV (or anti-TAV) serum as described by Van Lent et al. (52).

Total RNA was extracted from leaf tissue (0.4 g) or from protoplasts (10^6) by the method of Palukaitis et al. (35). Dot-blot hybridization for quantification of individual RNA1 and RNA2 of CMV and TAV was performed on nitrocellulose sheets by the method of Sambrook et al. (47). Blots were hybridized to cRNA probes transcribed from cDNA clones representing nt 1535 to 2337 of V-TAV RNA1 (4), nt 82 to 877 of V-TAV RNA2 (31), nt 851 to 1694 of CMV RNA1 (43), and nt 1250 to 2139 of CMV RNA2 (42). The concentrations of RNA were determined by comparing dilution series of the samples with concentration standards of individual viral RNA1 and RNA2.

For Northern blot analyses, an amount of total extracted RNA corresponding to 0.08 g of leaf tissue or 0.2×10^6 protoplasts was electrophoresed in a 1.2% agarose gel in TBE. Electrophoresed nucleic acids were transferred to a Zeta-Probe (Bio-Rad) membrane, and Northern blots were performed as described by Sambrook et al. (47).

RESULTS

CMV complements the defective long-distance movement of 1-TAV in cucumber. To establish an experimental system to analyze determinants of long-distance movement of CMV and TAV, it was necessary to identify isolates defective for this process in differential hosts. It was known that some TAV strains infect only the inoculated cotyledons, and not the true leaves, of cucumber (*Cucumis sativus* L.), a systemic host for CMV (26). Of the TAV strains available, some, i.e., V-TAV, P-TAV, and C-TAV, did not infect cucumber at all, but a fourth strain, 1-TAV, infected inoculated cotyledons of cucumber. Table 1 shows that the accumulation of 1-TAV in cucumber cotyledons was similar to that of Fny-CMV, according to ELISA quantitation of CP accumulation and dot-blot hybridization quantitation of RNA1 and RNA2 accumulation. Virions were purified from the infected cotyledons, which showed that 1-TAV particles were formed in vivo, as was the case for CMV. Production of infectious particles in cucumber cotyledons was similar for both viruses (Table 1). Moreover, isolation and immunofluorescence screening of protoplasts showed that cell-to-cell spread in inoculated cucumber cotyledons was similarly active for both viruses (Table 1). However, the data in Table 1 show that only Fny-CMV, but not 1-TAV, accumulated in noninoculated upper true leaves. No 1-TAV particles, CP, or RNA were detected in noninoculated leaves 4 weeks postinoculation (p.i.). This was true regardless of which leaf, from the first above the cotyledons to the sixth (not shown), was analyzed. When true leaves from cucumber were directly inoculated with 1-TAV, they were susceptible to the virus (Table 1), but, again, they did not serve as a source of infection for other, upper true leaves. Thus, the failure of 1-TAV to systemically infect cucumbers was not due to inefficient viral accumulation in the inoculated leaves, or to resistance of true leaves versus cotyledons. Rather, it was due to the incapacity of 1-TAV for long-distance movement in this host plant.

TABLE 3. Accumulation of CMV CP in cucumber protoplasts inoculated with RNA transcripts generated from full-length clones of Kin-CMV RNAs

Inoculum	Amt of CP (ng/10^6 protoplasts)[a]	
	CMV[b]	TAV[b]
tK1 + tK2 + tK3	840 ± 20	NT[c]
tK1 + tK2	0	NT
tK3	0	NT
tK1 + tK2 + tCl	820 ± 35	NT
tK1 + tK2 + tΔHH	750 ± 20	NT
tK1 + tK2 + tR	105 ± 10	NT
tK1 + tK2 + tΔRT	0[d]	NT
1-TAV + tK3	150 ± 15	750 ± 25
1-TAV + tCl	120 ± 20	850 ± 40
1-TAV + tΔHH	120 ± 15	870 ± 35
1-TAV + tR	110 ± 10	920 ± 40
1-TAV + tΔRT	0[d]	780 ± 30

[a] Determined by ELISA. Data are mean ± standard error of three replicate transfections.
[b] Accumulation of CP in inoculated protoplasts was determined 24 h p.i.
[c] NT, not tested.
[d] CP gene deleted.

When 1-TAV was coinoculated onto cucumber cotyledons with Fny-CMV, 1-TAV accumulated to high levels in upper, noninoculated leaves (Table 1). Thus, Fny-CMV complemented the defective long-distance movement of 1-TAV in cucumbers. Quantitation of the accumulation of CP, of RNA1 and RNA2, and of the proportion of infected cells showed that once in the upper noninoculated leaves, 1-TAV replication and cell-to-cell movement were highly efficient. Also, 1-TAV formed infectious particles in these leaves (Table 1). Fny-CMV is a highly aggressive strain belonging to subgroup I of CMV strains (36). Other, milder strains of CMV, both in subgroup I (e.g., M-CMV) and in subgroup II (e.g., LS-CMV and TrK7-CMV), also complemented the long-distance movement of 1-TAV (data not shown).

Identification of the CMV genomic segment involved in the complementation of long-distance movement of 1-TAV in cucumber plants. Full-length cDNA clones of the three genomic RNAs of Kin-CMV (pK1, pK2 and pK3 [5]) obtained from David Baulcombe were used to identify the portion of the CMV genome involved in the complementation of 1-TAV long-distance movement. Capped transcripts corresponding to RNA1 (tK1), RNA2 (tK2), and RNA3 (tK3) were infectious in cucumber protoplasts (Tables 2 and 3; Fig. 1).

Cotyledons of cucumber plants were inoculated with 1-TAV RNA alone or in combination with (i) tK1 plus tK2 plus tK3,

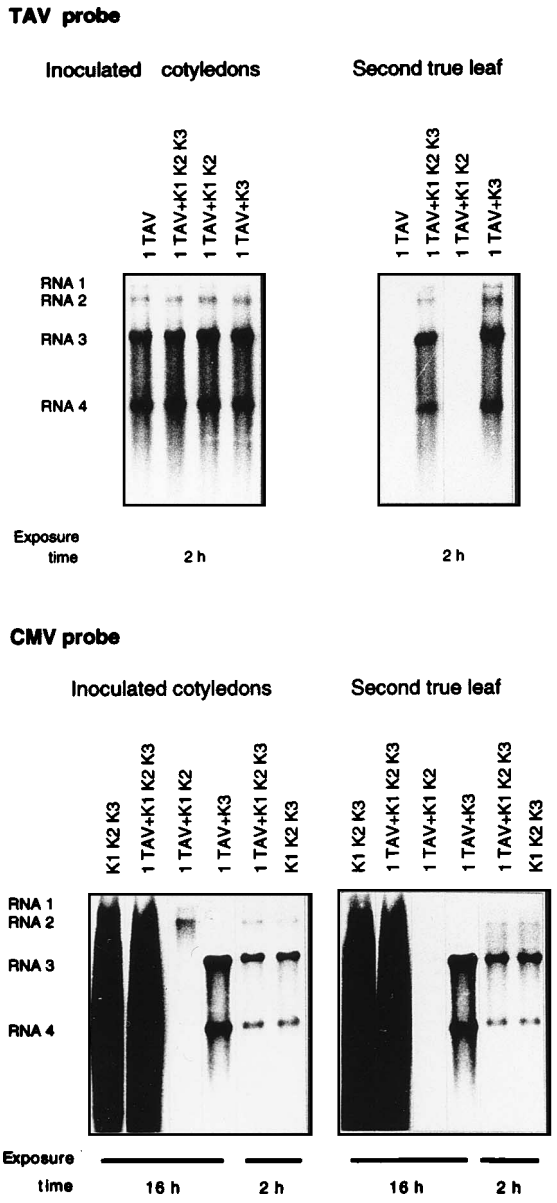

FIG. 2. Accumulation of viral RNA in cucumber plants inoculated with 1-TAV RNA and RNA transcripts from full-length clones of Kin-CMV RNAs. Northern blot analyses were of total nucleic acids extracted from leaves after electrophoresis in 1.2% agarose–TBE gels. Inocula are indicated above each lane. 1-TAV, 1-TAV virion RNA; K1, K2, K3, Kin-CMV RNA transcripts from full-length cDNA clones pK1, pK2, and pK3 respectively. Electrophoretic mobilities of RNA1 to RNA4 of TAV and CMV and exposure times for autoradiography are indicated.

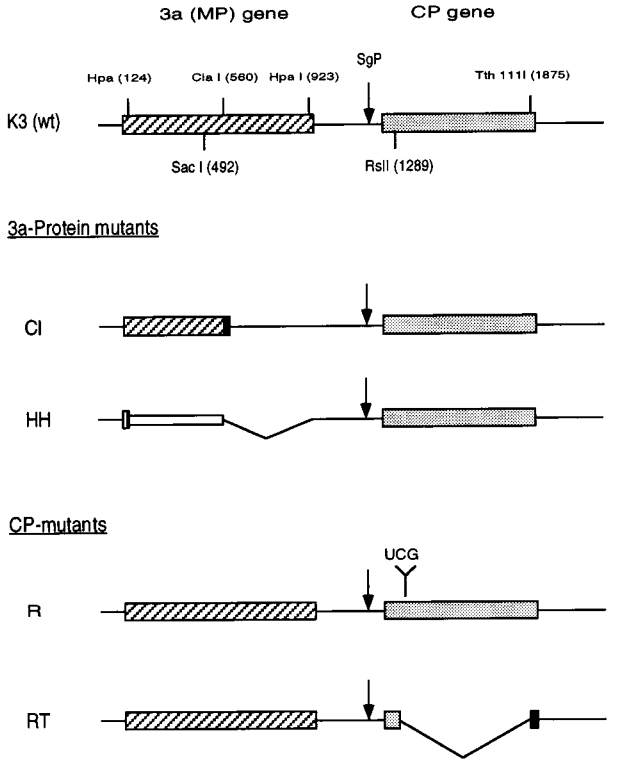

FIG. 3. Schematic map of RNA3 K3 (wild type [wt]) and 3a protein and CP mutants. Translated sequences in the frame of the 3a gene and of the CP gene are indicated by hatched and dotted boxes, respectively. Deleted sequences are indicated. The inverted *Hpa*I (nt 124)-*Sac*I (nt 492) fragment in ΔHH is indicated by an empty box. Insertion of UCG in-frame in the CP gene in the R mutant is shown. The position of the subgenomic promoter (SgP) for production of RNA4 is shown by an arrow. Restriction enzyme sites and positions are those of Boccard and Baulcombe (6).

(ii) tK1 plus tK2, or (iii) tK3. ELISA quantitation of CP accumulation is presented in Table 2, and Northern blot analyses of RNA accumulation, using 3′-end probes that specifically recognize RNA1 to RNA4 of CMV or TAV, are presented in Fig. 1. The data showed that the transcript of Kin-CMV RNA3 (tK3), but not the transcripts of RNA1 plus RNA2 (tK1 plus tK2), complemented the systemic movement of 1-TAV as efficiently as did the mixture of transcripts of all three Kin-CMV RNAs (tK1 plus tK2 plus tK3). It should be noted that Kin-CMV RNA3 and RNA4 accumulated in both inoculated and systemically infected leaves when Kin-CMV was coinoculated with 1-TAV but accumulated at lower levels than when coinoculated with Kin-CMV RNA1 plus RNA2. Levels were also lower than those of 1-TAV RNA3 plus RNA4 (Table 2; Fig. 2). Experiments with cucumber protoplasts (Fig. 1) showed that replication of Kin-CMV RNA3 by 1-TAV was less efficient than by Kin-CMV. Nevertheless, the reduced amount of CMV-RNA3 observed was sufficient to spread systemically and to complement the systemic movement of 1-TAV. Thus, determinants for the complementation of systemic spread in cucumbers of 1-TAV by CMV are on RNA3. Data also showed that CMV RNA1 plus RNA2 were able to replicate, and probably to spread from cell to cell, in inoculated leaves in the presence of 1-TAV (Fig. 2).

Generation and characterization of Kin-CMV mutants with mutations in the 3a and CP open reading frames. To identify the CMV gene(s) involved in complementation of 1-TAV long-range movement in cucumbers, 3a and CP mutants were generated with plasmid pK3. Two different 3a mutants were generated (Fig. 3). Mutant ΔHH was constructed by replacing a *Hpa*I (nt 125)-*Hpa*I (nt 923) fragment with an inverted *Hpa*I (nt 125)-*Sac*I (nt 492) fragment; thus, most of the 3a protein, except 10 amino acids at the N terminus, was lost. Mutant CI contained a frameshift mutation generated at the *Cla*I (nt 560) site such that the C-terminal half of the 3a protein was lost. Two CP mutants were also generated. Mutant R had an in-frame insertion of 3 nt at the *Rsr*II (nt 1289) site, leading to the addition of a Ser residue between Arg-23 and Ser-24 in the CP. Mutant ΔRT was constructed by deleting an *Rsr*II (nt 1290)-

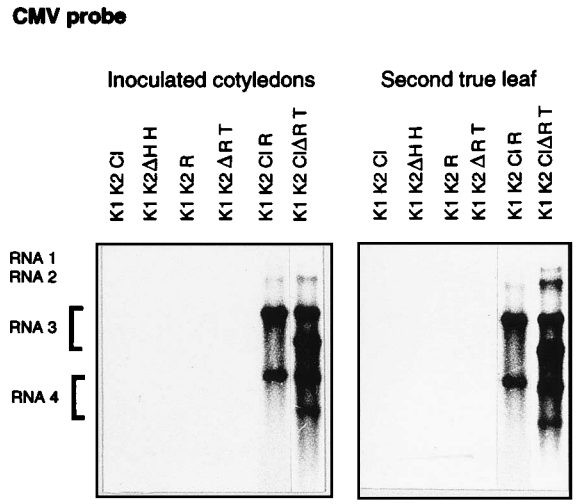

FIG. 4. Accumulation of mutant Kin-CMV RNA3 in cucumber plants. Northern blot analyses were of total nucleic acids extracted from leaves after electrophoresis in 1.2%–agarose TBE gels. Inocula are indicated above the lanes. Abbreviations are as in Fig. 1.

TABLE 4. Accumulation of CP in cucumber plants coinoculated with 1-TAV RNA and with RNA transcripts generated from mutated clones of Kin-CMV RNA3

Inoculum	Amt of CP (μg/g leaf)[a] in:			
	Inoculated leaves[b]		Upper leaves[b]	
	1-TAV	CMV	1-TAV	CMV
tK1 + tK2 + tK3	NT[c]	69 ± 6	NT	75 ± 4
tK1 + tK2 + tCl	NT	0	NT	0
tK1 + tK2 + tΔHH	NT	0	NT	0
tK1 + tK2 + tR	NT	0	NT	0
tK1 + tK2 + tΔRT	NT	0	NT	0
1-TAV	59	NT	0	NT
1-TAV + tCl	64 ± 6	8.4 ± 1.1	48 ± 4	3.4 ± 0.2
1-TAV + tΔHH	57 ± 8	6.1 ± 0.4	52 ± 4	2.5 ± 0.1
1-TAV + tR	72 ± 7	5.2 ± 0.7	0	0
1-TAV + tΔRT	74 ± 6	0[d]	0	NT
tK1 + tK2 + tCl + tR	NT	24 ± 2	NT	21 ± 2
tK1 + tK2 + tCl + tΔRT	NT	35 ± 4	NT	18 ± 2

[a] Determined by ELISA. Data are mean ± standard error of two or three independent experiments with three replicates in each.
[b] Accumulation of CP in inoculated and upper leaves was determined 9 and 16 days p.i., respectively.
[c] NT, not tested.
[d] CP gene deleted.

*Tth*111I (nt 1875) fragment, so that the most of the CP open reading frame was missing.

The replication of in vitro-generated transcripts from these four mutants (tΔHH, tCl, tR, and tΔRT) in cucumber protoplasts was assayed by coinoculation with tK1 plus tK2. Figure 1 shows that all mutant RNA3 were replicated and produced subgenomic RNAs (RNA4) of the expected size. In agreement with previous reports (6, 50), both CP mutations resulted in a reduction of CMV RNA accumulation compared with that of RNA3 transcripts containing a wild-type CP (tK3, tCl, and tΔHH). Table 3 shows ELISA analyses confirming the infectivity in protoplasts of tCl and tΔHH. Data also showed that the R-mutated CP retained antigenic affinity to CMV antibodies. Figure 1 and Table 3 also show that all four mutant CMV RNA3, i.e., Cl, ΔHH, R, and ΔRT, replicated in cucumber protoplasts when coinoculated with 1-TAV RNA. Accumulation levels were similar to those of nonmutated K3 and lower than when coinoculated with tK1 plus tK2, although 1-TAV accumulated to levels as high as those of CMV (Table 3).

The data in Table 4 and Fig. 4 show that none of the mutants were detected in inoculated or upper leaves of cucumber when coinoculated with tK1 plus tK2. This confirmed the conclusions of Suzuki et al. (50) and Boccard and Baulcombe (6) on the requirement of both 3a and CP for cell-to-cell spread of CMV.

Identification of the CMV gene involved in the complementation of long-distance movement of 1-TAV. Transcripts from the four mutant Kin-CMV RNA3 (tCl, tΔHH, tR, and tΔRT) were coinoculated into cucumber cotyledons with 1-TAV RNA. Table 4 and Fig. 5 show that all four mutant Kin-CMV RNA3 and their subgenomic RNAs accumulated in the inoculated cotyledons at levels similar to each other but lower than those of 1-TAV RNA3 and RNA4. Accumulation levels of these mutant RNAs were similar to those of wild-type Kin-CMV RNA3 and RNA4 when coinoculated with 1-TAV (compare Fig. 2 and 5). Thus, 1-TAV, in addition to complementing the replication of these mutant CMV RNA3, probably complemented their defective cell-to-cell spread. Table 4 and Fig. 5 also show that both 3a mutants, Cl and ΔHH, were able to complement the long-distance movement of 1-TAV in cucumber plants. In contrast, the CP mutants, R or ΔRT, did not complement the long-distance movement of 1-TAV (Table 4; Fig. 5). This was not due to nonfunctionality of the CP mutant RNAs, since both of them, R and ΔRT, efficiently complemented the 3a mutant Cl in both inoculated and systemically infected leaves of cucumber when coinoculated with tK1 plus tK2 (Fig. 4; Table 4). Note that mutant Cl, in both the tK1 plus tK2 plus tCl plus tR and the tK1 plus tK2 plus tCl plus tΔR inocula, provided a functional CP. Thus, Cl *trans*-complemented the CP-deficient mutants R and ΔRT, so that CMV CP is detected in the upper, noninoculated leaves. Thus, the failure of Kin-CMV RNA3 R and ΔRT to complement the long-distance movement of 1-TAV indicated that the CP was required for this function.

DISCUSSION

Long-distance movement of virus infection in host plants is a complex process including several steps: (i) entrance of the virus from the mesophyll cells of the primary inoculated leaf into the vascular system, (ii) transport through the vascular system, and (iii) exit from the vascular system into the mesophyll cells of the upper leaves. It has been known for several decades that long-distance movement occurs via the phloem and parallels the distribution of photosynthates from sources to sinks (2, 28). There is also long-standing evidence suggesting that long-distance movement of viruses is not a passive phenomenon but requires factors from the virus and the host. For example, Holmes (27) described many host plant species in which TMV was able to replicate and spread from cell to cell, but remained confined to initially inoculated leaves. Restriction to primarily inoculated leaves without necrotic resistance has also been described for CMV in pepper (20), cowpea chlorotic mottle bromovirus in soybean (21), barley stripe mosaic virus in *Nicotiana benthamiana* (37), and TAV in cucumber (26). These reports suggest that at least some step in long-distance movement is an active process requiring the interaction of virus and host components, which differentiates long-distance movement from cell-to-cell movement. This is further supported by reports on the requirement of a functional CP for the systemic movement of a large number of viruses, both spherical and rod shaped, including viruses that

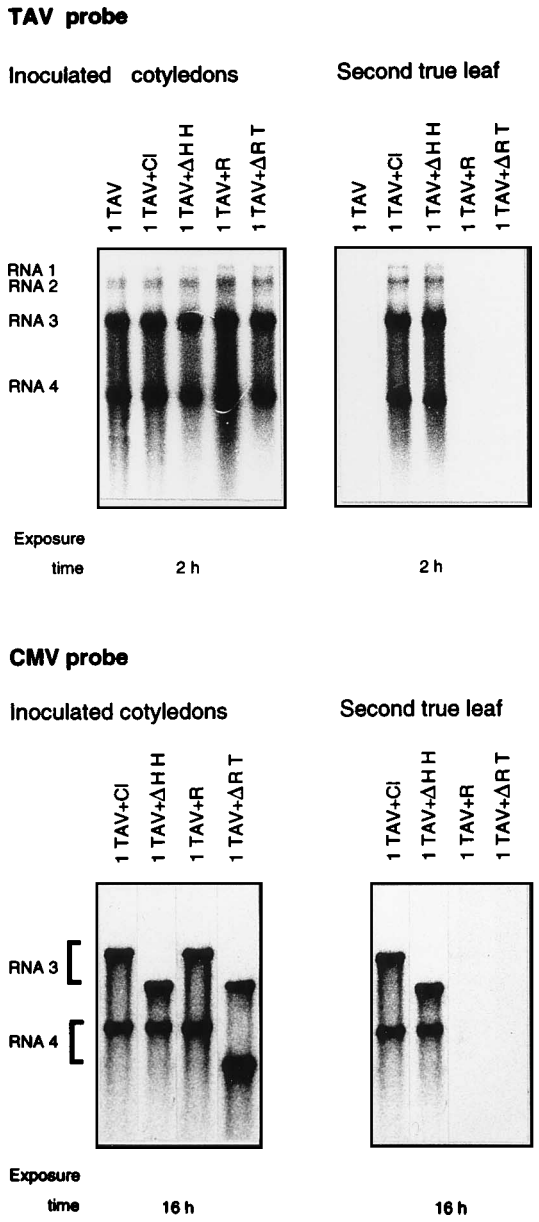

FIG. 5. Accumulation of viral RNA in cucumber plants inoculated with 1-TAV RNA and Kin-CMV RNA, wild type and mutants. Northern blot analyses were of total nucleic acids extracted from leaves after electrophoresis in 1.2% agarose–TBE gels. Inocula are indicated above the lanes. Abbreviations are as in Fig. 1.

do not require a CP for cell-to-cell movement (1, 6, 7, 15, 19, 23, 24, 41, 49, 50, 58). It has not been established in these instances whether the coat protein has a role in addition to that of protecting the viral RNA from degradation, but for some cases there is evidence of a more active role (25). A number of plant viruses do not require CP for systemic movement (14, 38, 44, 48, 57). At any rate, MP do not appear to be sufficient for long-distance movement.

The complementation of the defective long-distance movement of 1-TAV in cucumber by CMV is strong evidence that some virus-encoded product(s) is involved in the long-distance movement of the cucumoviruses. It is shown here that CMV RNA3, encoding the 3a protein and the CP, is enough to complement the long-distance movement of 1-TAV. Analysis of different CMV RNA3 carrying mutations either in the 3a or in the CP open reading frames shows that the virus-encoded product involved in long-distance movement is the CP.

Our results also clearly show that in the cucumoviruses, functional MP are not sufficient for long-distance movement to occur. Both 1-TAV and CMV accumulated to similar levels and infected a similar proportion of mesophyll cells in inoculated cotyledons of cucumber, but only CMV was able to systemically infect this host plant. For TMV MP, a role in increasing the plamodesmata size exclusion limit (17, 56) has been shown. It has also been proposed that TMV MP chaperones TMV RNA traffic through the plasmodesmata (10, 11). It is not known if the 3a protein of the cucumoviruses also has these roles in vivo. An important difference between TMV and CMV is the need by CMV for both 3a protein and CP for cell-to-cell movement, as reported by Suzuki et al. (50) and Boccard and Baulcombe (6) and as shown in the present work. It has not been established whether CMV CP has a specific role in cell-to-cell movement, whether CMV moves from cell to cell as virus particles, or whether the CP simply stabilizes viral RNA by encapsidation. This last function is suggested by the depression of CMV RNA accumulation in infected protoplasts when a functional CP is not expressed (6, 50; see above). Since the colonization of the mesophyll of cucumber cotyledons is similarly efficient for 1-TAV and for CMV, but only CMV moves systemically, the role of CP in long-distance movement is likely to be different from its role in cell-to-cell movement. The role of CMV CP for long-distance movement goes beyond the role of stabilizing the viral RNA. 1-TAV is able to form stable, infectious particles in inoculated cucumber cotyledons but does not move systemically.

The data presented here are strong evidence for the CP having an active role in the long-distance movement of these two cucumoviruses. 1-TAV and CMV share different systemic hosts, e.g., tobacco and tomato (see, for instance, reference 30). Thus, our present data are also evidence that the CP of cucumoviruses has host-specific determinants for long-distance movement, as was suggested for TMV (25). This suggests that a specific interaction of CP and some host component(s) is required for this process to occur. The nature of this interaction remains a subject for speculation. Plant viruses colonize their host plants through the symplast (2), entering each new cell through plasmodesmata. The different viral factors enabling the infection of mesophyll cells (i.e., MP) and vascular cells (i.e., CP) may reflect differences between the plasmodesmata connecting mesophyll cells and those conecting mesophyll and bundle-sheath cells or vascular cells. Evidence of this was presented by Ding et al. (18), who showed that in transgenic tobacco plants expressing TMV MP, the MP was associated with secondary plasmodesmata of mesophyll and vascular cells but the SEL of only the former was increased. It was suggested that CP was a second viral factor required for penetrating the bundle-sheath/phloem parenchyma barrier. Our data support this suggestion in a different biological system.

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