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A hybrid plant RNA virus made by transferring the noncapsid movement protein from a rod-shaped to an icosahedral virus is competent for systemic infection

(bromoviruses/30-kDa movement protein/plant virus movement/capsid protein/virus evolution)

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ABSTRACT **For many plant RNA viruses, multiple viral gene products, including noncapsid movement proteins and capsid proteins, contribute to the spread of infection within plants. The extent to which these factors interact to support infection spread is not known, but, for movement protein mutants of certain viruses, the inability of coinoculated "helper" viruses to complement defective movement has suggested a possible requirement for coadaptation between noncapsid movement proteins and other virus factors. To test directly for required coadaptation, the 3a movement protein gene of cowpea chlorotic mottle virus, an icosahedral bromovirus, was replaced with the nonhomologous 30-kDa movement protein gene of sunn-hemp mosaic virus, a rod-shaped, cowpea-adapted tobamovirus. The resulting hybrid virus is competent for systemic infection of cowpea, with systemic infection dependent upon expression of the 30-kDa gene. In view of the dramatic differences between cowpea chlorotic mottle virus and sunn-hemp mosaic virus in genetic organization and particle morphology, the ability of the hybrid to systemically infect cowpea implies that the tobamovirus 30-kDa movement protein functions independently of sequence-specific interactions with other viral components or sequences. Similarly, the required contribution of bromovirus capsid protein to infection movement appears to be independent of specific interaction with the natural 3a movement protein. In addition to other implications concerning movement protein and coat protein function, the results are consistent with the possibility that two or more distinguishable transfer processes may be involved in crossing different tissue barriers to achieve full systemic spread of infection.**

The spread of virus infection in plants is an active process, dependent on multiple viral genes (1, 2). One class of movement functions is provided by plant virus movement proteins, which are noncapsid proteins that are dispensable for nucleic acid replication but required for cell-to-cell spread. Among such movement proteins are the 30-kDa protein of tobacco mosaic virus (TMV), a tobamovirus (3, 4), and the 3a protein of cowpea chlorotic mottle virus (CCMV), a bromovirus (5).

TMV 30-kDa protein is thought to act at plasmodesmata to permit transport of viral material to adjacent cells. The 30-kDa protein is localized at these intercellular channels (6, 7) and increases their size exclusion limit (8). The 30-kDa protein also cooperatively binds single-stranded nucleic acids *in vitro*, and the elongated RNA–protein complex predicted to result from this binding may facilitate the transport of viral RNA through plasmodesmata (9). No sequence specificity was found for 30-kDa protein binding to single-stranded RNA or single-stranded DNA *in vitro* (9), but sequence specificity in RNA binding by some proteins is only evident under particular assay conditions (10, 11).

Movement proteins are not the only virus factors contributing to infection spread. For both the rod-shaped tobamoviruses and icosahedral bromoviruses, encapsidation-competent coat protein is required for long-range, systemic spread of infection (5, 12–14). Moreover, in the absence of coat protein, initial cell-to-cell spread of bromovirus infection is delayed and reduced (ref. 5; K. Mise and P.A., unpublished results). Other viral genes, including those involved in RNA replication, may also directly or indirectly influence the systemic spread of infection (15–17).

The extent to which infection spread depends on interactions between viral gene products is not known. In some cases, helper virus coinfection assists the spread of a second, distinct virus in an otherwise nonpermissive host (1, 18), but such coinfection experiments do not reveal which or how many helper virus functions are involved in complementation. A recent transgenic tobacco study concluded that TMV 30-kDa protein was necessary but insufficient to reproduce TMV complementation of the transport deficiency of red clover mottle virus in tobacco, suggesting combined action by the 30-kDa protein and other TMV product(s) (19). In some other cases, putative helper viruses fail to complement the spread of a defective or misadapted virus (20), suggesting incompatibility between helper virus movement functions and the dependent virus. For example, we have been unable to complement even the local movement defect(s) of a CCMV 3a deletion mutant in cowpea plants by coinoculating sunn-hemp mosaic virus (SHMV), also known as the cowpea strain of TMV (this paper and M. Janda and P.A., unpublished results). Since CCMV and SHMV are each well adapted to cowpea, this lack of complementation must either reflect a lack of required coadaptation between SHMV function(s) and CCMV or spatial or temporal limitations in expression of potentially complementing SHMV functions.

To test directly whether the function(s) of the tobamovirus 30-kDa movement protein requires specific adaptations to other viral gene products or sequences, we replaced the 3a open reading frame (ORF) of CCMV with the SHMV 30-kDa movement gene and assayed the ability of the resulting hybrid virus to systemically infect cowpea. Despite the lack of significant sequence similarity between the 3a and 30-kDa proteins (21) and the clear differences between SHMV and CCMV in particle morphology and genomic organization (22, 23), this hybrid virus can systemically infect cowpea, implying that the movement functions of the 30-kDa gene are independent of specific features of the viral coat protein, RNA sequence, or other virus components.

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Abbreviations: CCMV, cowpea chlorotic mottle virus; ORF, open reading frame; SHMV, sunn-hemp mosaic virus; TMV, tobacco mosaic virus; wt, wild-type.

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MATERIALS AND METHODS

CCMV and Hybrid cDNA Clones. Viral cDNA clones, their transcripts, and progeny RNAs will be referred to by the brief descriptive names listed below, with laboratory plasmid designations following in parentheses. C1 (pCC1TP1), C2 (pCC2TP2), and C3 (pCC3TP4) are wild-type (wt) cDNA clones of CCMV RNA1, RNA2, and RNA3, respectively (24). C3(3a-Δ) (pCC3RA3), pCC3TP8, and pCC3TP10 have been described (5, 25, 26).

cDNA to the SHMV 30-kDa ORF was synthesized (27) with the primer d(GCGAGATCTAG*CTAGGAGTCGGAATC*). The italicized bases are complementary to the last 15 nucleotides of the 30-kDa ORF (28); the underlined bases contain a *Bgl* II restriction site and are complementary to the first eight bases following the CCMV 3a ORF. The cDNA was amplified by the polymerase chain reaction (29) using the first strand cDNA primer and d(CGCGGATCCCG*ATGTCTGAGGTGTCT*). The italicized bases are identical to the first 15 nucleotides of the 30-kDa ORF; the underlined bases contain a *Bam*HI restriction site and are identical to the eight bases preceding the 3a ORF in pCC3TP8. C3(30K) (Fig. 1; pCC30KWD11) was constructed by replacing the *Bam*HI/*Bgl* II 3a ORF cassette of pCC3TP8 with the amplified SHMV 30-kDa ORF, also digested with *Bam*HI and *Bgl* II. C3(30K-fs) (Fig. 1; pCC30KWD16) is a C3(30K) derivative bearing a four-base (CCCC) insertion between bases 131 and 132 of the 30-kDa ORF, whereas C3(3a-fs) (Fig. 1; pCC3KS1) is a C3 derivative with a two-base (CC) insertion in the 3a coding region between bases 395 and 396 (30).

Plant Inoculation and RNA Isolation and Analysis. Transcripts were prepared, and barley protoplasts (31) or cowpea plants (24) were inoculated as described. For SHMV coinoculations, 1–2 μg of SHMV RNA was used for each primary leaf.

Total RNA was prepared from protoplasts (32) or from cowpea leaves (5, 33) as described. For dot blots, 5–10 cm^2 of leaf tissue was ground in a microcentrifuge tube and centrifuged for 1 min at 12,000 × *g*. The supernatant was transferred to a new tube, and sodium dodecyl sulfate was added to a final concentration of 1%. After 5 min at 65°C, the extract was centrifuged again, and 5 μl was dotted directly onto a nylon membrane. Probes to detect (+)- and (−)-strand CCMV RNAs have been described (5, 26). A T7 RNA polymerase transcript from p30KWD14, constructed by cloning the *Bam*HI/*Bgl* II 30-kDa cassette of pCC30KWD11 into the *Bam*HI site of the vector pGEM-1 (Promega), was used to detect (−)-strand 30-kDa sequences. A T7 RNA polymerase transcript from pCC3WD18, constructed by cloning a *Sal* I–*Sau*3AI fragment (coat gene bases 4–468) of pCC3TP10 into the *Sal* I–*Bam*HI sites of pGEM-1, was used to detect (+)-strand CCMV coat gene sequences.

RESULTS

Coinoculation of SHMV and CCMV 3a Truncations. CCMV is an icosahedral virus, with a tripartite RNA genome (34). Monocistronic RNA1 and RNA2 (hereafter C1 and C2) encode factors necessary and together sufficient for RNA replication in protoplasts. For systemic infection, dicistronic RNA3 (C3) is also required, which encodes the 3a and coat proteins (Fig. 1).

When primary cowpea leaves are inoculated with wt CCMV, viral RNAs accumulate to high levels and rapidly spread to uninoculated trifoliate leaves (Fig. 2). C3 mutant C3(3a-Δ) lacks the 3′ half of the 3a gene (Fig. 1). As shown previously (5) and validated here (Fig. 2), this 3a deletion reduces the level of CCMV RNA detected in inoculated leaves by at least 100-fold and prevents the appearance of CCMV RNAs in uninoculated leaves. To independently

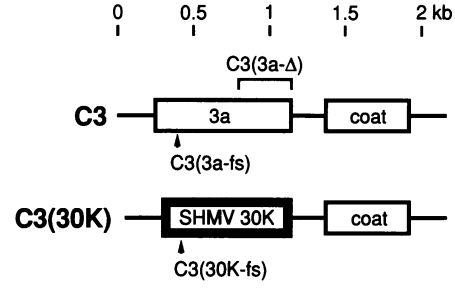

FIG. 1. Structure of wt CCMV RNA3 (C3), hybrid RNA C3(30K), and their deletion and frameshift derivatives. C3 is shown with boxes representing the 3a and coat protein ORFs and horizontal lines representing noncoding sequences. The 30-kDa ORF of SHMV is illustrated with a darker box in RNA3 hybrid C3(30K). Arrows denote the site of translational frameshift insertions in derivatives C3(3a-fs) and C3(30K-fs). A bracket above C3 indicates the sequence deleted in C3(3a-Δ). kb, Kilobases.

corroborate the requirement for 3a protein in systemic infection, a translational frameshift mutation was introduced into the CCMV 3a gene to create mutant C3(3a-fs) (Fig. 1). The insertion of two cytidines after nucleotide 395 of C3 leads to the introduction of a proline residue in the 3a protein at amino acid 54 and shifts the reading frame so that a premature stop codon is encountered 11 codons downstream. C3(3a-fs) is replicated in inoculations with C1 and C2 as well as wt C3 in barley protoplasts (Fig. 3), but, like C3(3a-Δ), this frameshift mutation blocked accumulation of CCMV RNAs in inoculated and uninoculated leaves (Fig. 2). Fluorescent antibody and other localization studies show that these effects result from blockage of infection transport from initially infected cells (K. Mise and P.A., unpublished results).

We tried to complement the movement defect of the CCMV 3a truncations by coinoculating these mutants with SHMV, a strain of TMV adapted to infect cowpeas (22).

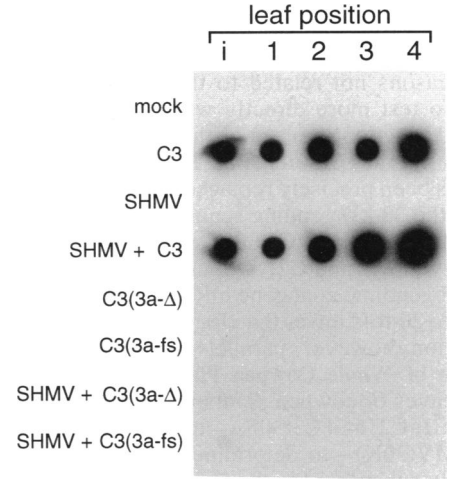

FIG. 2. Representative assays for CCMV-specific RNA sequences in inoculated primary and uninoculated trifoliate leaves of cowpea plants inoculated with transcripts C1 and C2 plus the C3 derivatives shown at left, together with SHMV RNA where indicated. Inoculated primary leaves were sampled 14 days post inoculation (dpi) and uninoculated first, second, third, and fourth trifoliate leaves above the inoculated leaf were sampled 15, 20, 28, and 29 dpi, respectively. Equal volumes of sample extracts (see text) were dotted onto a nylon membrane, which was hybridized to a ^{32}P-labeled probe complementary to the conserved 3′ terminal region of all CCMV RNAs and autoradiographed. "Mock" denotes samples from a mock-inoculated plant. Leaf positions are indicated at the top: i, inoculated leaf; 1, 2, 3, 4, first, second, third, and fourth trifoliate leaves.

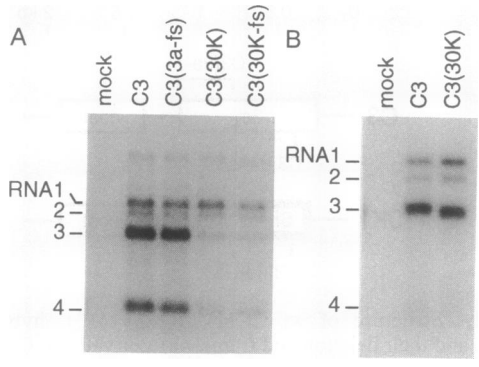

FIG. 3. Accumulation of C3, C3(30K), and their frameshift derivatives in protoplasts. Northern blot analysis of total RNAs isolated from barley protoplasts 20 hr after transfection with transcripts C1 plus C2 and the C3 derivative indicated above each lane. Mock, mock inoculated only. Positions of CCMV RNAs are indicated to the left. (*A*) (+)-Strand RNA accumulation, assessed with the same probe as in Fig. 2. Total RNA was electrophoresed under native conditions through a 1% agarose gel. (*B*) (−)-Strand RNA accumulation, assessed with a ^{32}P-labeled transcript complementary to the 5′ end of CCMV (−)-strand RNAs. Total RNA was glyoxylated prior to electrophoresis through a 1% agarose gel.

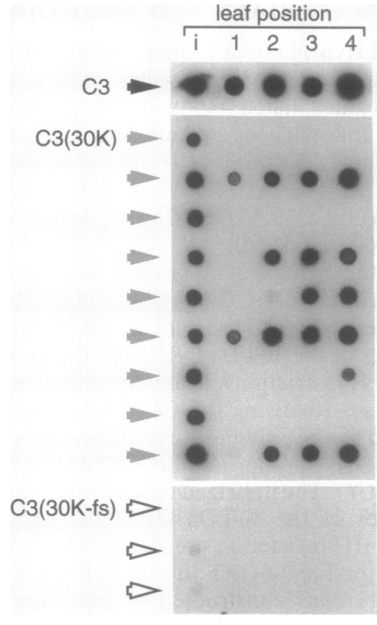

FIG. 4. Representative assays for CCMV-specific RNA sequences in inoculated primary and uninoculated trifoliate leaves of cowpea plants inoculated with transcripts C1 and C2 plus either C3, C3(30K), or C3(30K-fs). The dark arrow denotes samples from a plant inoculated with C3; shaded arrows indicate samples from nine plants inoculated with C3(30K); open arrows indicate samples from three plants inoculated with C3(30K-fs). Sampling technique, sample size, hybridization, autoradiograph exposure time, and figure labeling were all similar to Fig. 2.

However, after coinoculating SHMV with C1 and C2 and either C3(3a-Δ) or C3(3a-fs), the level of CCMV RNA detected in inoculated leaf extracts after long autoradiographic exposures consistently remained within the range of minor fluctuations seen in the absence of SHMV, and no CCMV RNA was detected in uninoculated leaves (Fig. 2 and similar experiments). The presence of SHMV RNA in all leaves of plants inoculated with SHMV was verified by probing parallel blots with a probe complementary to the SHMV 30-kDa ORF (data not shown). Coinoculation of SHMV with C1, C2, and C3 did not discernibly lower the levels of CCMV or SHMV RNAs in primary or trifoliate leaves (Fig. 2 and data not shown).

Replacement of CCMV 3a with SHMV 30-kDa Coding Sequence. The timing, level, or location of 30-kDa expression in SHMV coinfections might have prevented complementation for reasons not related to the function of the 30-kDa protein. To test more directly whether the SHMV 30-kDa gene could function in place of the 3a gene, we constructed the RNA3 hybrid C3(30K) (Fig. 1), in which the 3a ORF of CCMV has been precisely replaced (see *Materials and Methods*) with the 30-kDa coding sequence of SHMV. As shown in Fig. 3*A*, the hybrid C3(30K) is replicated in protoplast infections with C1 and C2, although both RNA3 (+)-strands and the subgenomic coat gene mRNA, RNA4, accumulate to levels 10- to 20-fold lower than for wt C3. C3(30K) (−)-strand accumulation, however, parallels that of wt C3 (Fig. 3*B*).

Infection of Whole Cowpea Plants by the Hybrid Virus. Primary leaves of cowpea plants were inoculated with transcripts of C1, C2, and C3(30K)—together defining the hybrid virus CCMV(30K)—to determine if the 30-kDa gene could functionally substitute for the 3a gene in systemic infection. As cowpeas inoculated with CCMV(30K) showed no symptoms, we tested for infection by probing extracts from inoculated and uninoculated leaves with a transcript complementary to the 3′ end of all CCMV RNAs (5). Despite the poor accumulation of C3(30K) (+)-strands in protoplasts, CCMV(30K) spread to uninoculated trifoliate leaves in many of the cowpea plants inoculated with this viral hybrid (Fig. 4). Unlike wt CCMV infections, systemic infections by CCMV(30K) often failed to infect the first and sometimes other early-developing trifoliate leaves above the inoculated primary leaves (Fig. 4), suggesting delayed spread from the inoculated leaf. Once infection was detected in a trifoliate leaf, however, infection was also detected in all trifoliate leaves that subsequently developed (Fig. 4). Though only one or two early trifoliate leaves were tested for most plants, systemic spread was documented in over one-third of more than 75 cowpeas inoculated to date.

Dependence of Systemic Infection on SHMV 30-kDa Gene Expression. To evaluate the relative contributions of the SHMV 30-kDa coding sequence and the 30-kDa protein in the ability of CCMV(30K) to systemically infect cowpea, a frameshifting insertion was engineered into the 30-kDa ORF of C3(30K) to generate C3(30K-fs) (Fig. 1). Four cytidine residues inserted after nucleotide 370 in C3(30K-fs) introduce a proline at amino acid 45, followed by a frameshift resulting in premature termination of the 30-kDa gene 11 codons downstream. C3(30K-fs) accumulated as well as C3(30K) in protoplast infections (Fig. 3). However, this frameshift greatly reduced the CCMV RNA signal detected in inoculated leaves, and C3(30K-fs) failed to direct systemic infection in any of 28 plants inoculated to date (Fig. 4).

Maintenance of the 30-kDa Gene in Progeny RNA. To examine the nature of the progeny viral RNA in CCMV(30K)-infected plants, total RNA was isolated from uninoculated leaves of mock-, wt CCMV-, and CCMV(30K)-inoculated plants 4–7 weeks after inoculation and subjected to Northern blot analysis (Fig. 5). As in C3(30K) protoplast infections, the hybrid RNA3 was detected most readily in the form of (−)-strand RNA3. Hybridization with a probe complementary to the conserved 5′ end of all CCMV (−)-strand RNAs (26) showed that (−)-strand RNA1, RNA2, and RNA3 all accumulated in CCMV(30K) infections to levels similar to wt CCMV infections (Fig. 5*A*). Probing the same samples with an RNA probe specific for the (−)-strand of the SHMV 30-kDa gene confirmed the presence of this gene in (−)-strand RNA3 of the recombinant- but not wt virus-infected samples (Fig. 5*B*). Conversely, probing with a transcript specific for the (−)-strand of the CCMV 3a gene gave a clear hybridization signal for samples from wt CCMV-inoculated

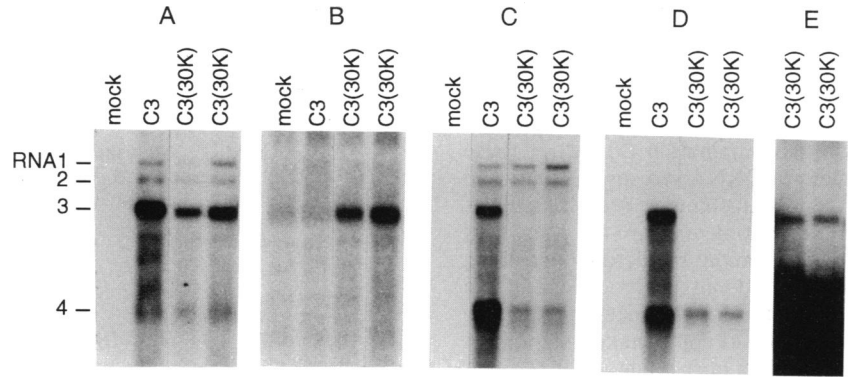

FIG. 5. Representative Northern blot analyses of viral RNAs from uninoculated leaves of cowpea plants inoculated with transcripts C1 plus C2 and either C3 or C3(30K), as indicated. Total RNA was extracted from the fourth or sixth trifoliate leaf, glyoxylated, electrophoresed through 1% agarose, and transferred to a nylon membrane. Five micrograms of total RNA was loaded in each lane. Lanes and viral RNA positions are labeled as in Fig. 3; the C3(30K) samples are from independent plants. (*A*) (−)-Strand RNAs, detected with the same probe used in Fig. 3*B*. The autoradiogram was exposed 11 days with an intensifying screen. (*B*) Detection of SHMV 30-kDa sequences with a ^{32}P-labeled probe complementary to the entire (−)-strand 30-kDa ORF. The autoradiogram was exposed 12 days with an intensifying screen. (*C*) (+)-Strand RNAs, detected with the same probe used in Fig. 2. The autoradiogram was exposed 10 min without an intensifying screen. (*D*) Detection of CCMV coat-specific (+)-strands with a ^{32}P-labeled probe complementary to bases 4–468 of the coat gene. The autoradiogram was exposed 30 min without an intensifying screen. (*E*) Longer exposure of C3(30K) lanes from *D*. The autoradiogram was exposed 20 hr with an intensifying screen.

plants but no signal from the CCMV(30K)-inoculated plants (data not shown).

Probing samples from CCMV(30K)-infected plants with a transcript complementary to the conserved 3′ end of CCMV (+)-strand RNAs revealed that, as in protoplasts, (+)-strand RNA1 and RNA2 accumulation was equivalent to wt infection, whereas RNA4 and particularly RNA3 accumulation was much lower than wt (Fig. 5*C*). This explains why, in Fig. 4, the intensities of hybridization signals from CCMV(30K)-infected leaves were generally lower than those from wt CCMV-infected leaves. Probing with a transcript specific for the (+)-strand of the CCMV coat gene eliminated the background caused by hybridization to RNA1 and RNA2 degradation products, demonstrating that at the time of sampling (+)-strand C3(30K) was present in CCMV(30K) uninoculated leaves at levels ranging from 15 to 100 times less than wt RNA3 (Fig. 5 *D* and *E*).

DISCUSSION

We have shown that the SHMV 30-kDa ORF can substitute for the required 3a ORF of CCMV to support not only local movement but also long-range systemic infection of cowpea plants. Because the use of a hybrid virus allowed helper-independent complementation, and because of the dramatic differences between CCMV and SHMV in genetic organization, particle morphology, and other properties (22, 23), these findings strongly imply that the contribution(s) of the SHMV 30-kDa protein to infection spread does not require sequence-specific interactions with other virus-encoded factors. The results are consistent with models of 30-kDa function where the 30-kDa protein acts alone, in conjunction with host factors, indirectly with other viral proteins, or by interactions that are not sequence-specific. If 30-kDa protein facilitates cell-to-cell spread of infection through binding to viral RNA, the CCMV(30K) results presented here strongly suggest that this binding is not dependent on selective interaction with a virus-specific initiation site, consistent with prior *in vitro* studies (9). Since it seems unlikely that virus processes would nonselectively transport cellular as well as viral RNAs between cells, other factors might contribute specificity to such a pathway by mechanisms yet to be recognized.

Recently it was also shown that the 30-kDa gene could be transferred between two closely related strains of TMV (35) and could complement defects in the 29-kDa gene of tobacco rattle virus (36), with which it shares significant sequence homology (37). These studies complement our findings, although the close relation of the viruses used in these studies supports a more limited range of conclusions.

No statistically significant sequence similarity has been found between the movement proteins of bromoviruses and tobamoviruses in general (38, 39) or the CCMV 3a and SHMV 30-kDa proteins in particular (21). Despite this low level of sequence similarity, the results presented here show that the activities of the CCMV 3a and the SHMV 30-kDa proteins are functionally equivalent and may be biochemically related.

Implications for the Role of Coat Protein in Infection Spread. Encapsidation-competent coat protein is essential for the movement of bromovirus infection from inoculated to uninoculated leaves (5, 14). These results and similar results with TMV indicate that virions, or conceivably another form of coat protein–RNA complex, perform a crucial role in the long-range movement of both bromovirus and tobamovirus infection (13, 14). The ability of 30-kDa protein from rod-shaped SHMV to support systemic infection by an icosahedral virus shows that coat protein or virions need not be directly adapted to the 30-kDa protein for successful spread to occur.

The small size of plasmodesmatal exclusion limits (8) suggests that the movement of coat protein–RNA complexes between cells or between cells and vascular channels requires either specific coat protein–host interactions or the assistance of other viral factor(s) (1). The 30-kDa protein may indirectly assist the movement of such complexes through its partial relaxation of the plasmodesmatal exclusion limit (8). Although this relaxation is insufficient to allow the passage of intact virions, it may permit free coat protein, subvirion coat protein–RNA complexes, or a coat protein–RNA–30-kDa protein complex to pass through plasmodesmata. Coat protein may also mediate separate interactions with host factors to further facilitate movement through 30-kDa-modified plasmodesmata. Alternatively, or in addition, coat protein or virions may independently mediate infection movement across some barriers without 30-kDa movement protein assistance. One such transfer might occur in exiting the vascular system at sites distal from the primary infection, where noncapsid movement proteins may not be available. Thus, two or more distinct transfer processes may be involved in the full systemic spread of infection.

Virus Evolution and Engineering. To the best of our knowledge, CCMV and SHMV are the most divergent RNA viruses from which essential components have been combined to

make a viable hybrid. Other work in our laboratory has shown that the SHMV coat gene and origin of assembly can functionally replace the brome mosaic virus (BMV) coat gene, encapsidating BMV RNAs in rod-shaped virions *in vivo* (40), and that defective viral RNAs can obtain required genes from coinfecting RNAs by recombination (5). All of these findings support the concept that RNA viruses have and can continue to evolve in a modular fashion by recruiting genes from their hosts or other coinfecting viruses (41). Viral host range frequently appears to be limited by factors controlling cell-to-cell spread (1, 42). Based on the precedent of the CCMV(30K) exchange, the natural or engineered exchange of movement genes between viruses could make a significant contribution toward altering virus host range.

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