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Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection

(bromoviruses/plant virus movement/virus evolution)

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ABSTRACT **RNAs 1 and 2 of the tripartite cowpea chlorotic mottle virus (CCMV) genome are sufficient for RNA replication in protoplasts, whereas systemic infection of cowpea plants additionally requires RNA3, which encodes the 3a noncapsid protein and coat protein. By using biologically active CCMV cDNA clones, we find that deletions in either RNA3 gene block systemic infection. Thus, though some plant RNA viruses are able to spread systemically without encapsidation, both the coat and 3a genes are required for systemic infection of cowpeas by CCMV. When plants were coinoculated with CCMV RNAs 1 and 2 and both the 3a and coat deletion mutants of RNA3, 30–60% rapidly developed systemic infection. Progeny RNA recovered from systemically infected leaves in such infections contained neither of the starting deletion mutants but rather a single full-length RNA3 component with both genes intact. Nucleotide substitutions introduced into the coat protein deletion mutant as an artificial marker were recovered in the full-length progeny RNA, confirming its recombinant nature. Intermolecular RNA recombination *in planta* can, therefore, rescue a complete infectious genome from coinoculated mutants independently disabled for systemic spread. These results have implications for the repair of defective genomes produced by frequent natural replication errors, the possible emergence of newly adapted RNA viruses upon coinfection of new hosts, and further studies of RNA virus recombination.**

Cowpea chlorotic mottle virus (CCMV) is a tripartite, (+)-strand RNA virus that infects legumes and several other dicotyledonous plants (1). The genomic RNAs of this bromovirus are designated RNAs 1–3 by decreasing size and are packaged separately in icosahedral capsids. Monocistronic RNAs 1 and 2 encode the 1a and 2a proteins, which are involved in RNA replication. Dicistronic RNA3 serves directly as the mRNA for the 32-kDa 3a noncapsid protein, whereas the 20-kDa coat protein is translated from a subgenomic mRNA designated RNA4. Full-length biologically active cDNA clones have been constructed for all three CCMV genomic RNAs (2) and the entire genome has been sequenced (ref. 3; J. Bujarski, personal communication).

As with other bromoviruses (4, 5), only RNAs 1 and 2 are required for CCMV RNA replication in protoplasts, whereas systemic infection of cowpea plants requires inoculation with all three genomic RNAs (ref. 6; unpublished results). Therefore, one or both of the proteins encoded by RNA3 must be involved in the movement of the viral genome to achieve normal systemic infection. The role of 3a as the only nonstructural protein without apparent influence on RNA replication, its position in the genome, its size, and the clear correspondence between all remaining gene products of tobacco mosaic virus (TMV) and bromoviruses circumstantially relate the 3a protein to the TMV 30-kDa protein, whose involvement in movement of infection has been established (3, 7–10). The 3a and 30-kDa proteins share little sequence similarity, however (3, 7). The ability to achieve long-range systemic spread in the absence of coat protein appears to vary among viruses, hosts, or both. Systemic infection of spinach by beet necrotic yellow vein virus requires encapsidation but tobacco rattle virus RNA1 undergoes slow systemic spread in plants in the absence of its cognate RNA2, which encodes the viral coat protein (11, 12). Systemic infection has also been reported for TMV isolates encoding encapsidation-defective coat protein (13) and some but not all TMV mutants with engineered deletions in the coat gene move systemically, though with impaired efficiency (14, 15). Specific amino acid substitutions in TMV coat protein influence host responses in some virus-host combinations (16, 17).

Brome mosaic virus (BMV), which is closely related to CCMV (3, 18), was the first plant RNA virus for which active recombination was demonstrated: Bujarski and Kaesberg (19) showed that a viable but poorly accumulating BMV RNA3 mutant with a small deletion in the 3′ noncoding region gave rise to progeny RNA3 in which the deletion was restored by recombination with the conserved 3′ sequences of wild-type (wt) RNA1 or RNA2. However, recombinants were only detected several days after the starting virus population was well established in systemic infection. Moreover, Dreher *et al.* (20) failed to see similar recombinational repair of a number of other viable but deleterious mutations in the 3′ end of BMV RNA3. As noted by Dreher *et al.* (20), these results suggested that bromovirus RNA recombination might be a rare event, perhaps effectively visible only within large starting populations and with corresponding limitations on its potential contribution to rapid virus evolution.

We show here that deletions in either the 3a noncapsid gene or the coat gene block systemic infection of cowpea by CCMV. However, when plants were coinoculated with 3a and coat gene deletion mutants, wild-type RNA3 was rapidly regenerated and gave rise to systemic infection. Thus, intermolecular RNA recombination *in planta* not only is able to modify infection-competent genomes after their systemic amplification but also can rescue infectious virus from coinoculated virus genomes lacking proper adaptation to the host plant. The biological and evolutionary significance of

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Abbreviations: CCMV, cowpea chlorotic mottle virus; BMV, brome mosaic virus; TMV, tobacco mosaic virus; wt, wild type.

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these results for genetic exchanges within and between viruses is discussed in connection with emerging concepts of recombination as an important force in RNA virus divergence.

MATERIALS AND METHODS

CCMV cDNA Clones and Plant Inoculation. Wt cDNA clones were pCC1TP1, pCC2TP2, and pCC3TP4 (2). pCC3TP4 deletion mutants pCC3RA3 and pCC3RA5 were as described (6). pCC3RA6 was made from pCC3TP8, which contains a *Bam*HI marker (6), by the same method used for pCC3RA5 construction. Transcript preparation and cowpea or barley inoculation were as described (2, 21).

RNA Isolation and Analysis. Virion RNA was phenol/chloroform [1:1 (vol/vol)] extracted and ethanol precipitated from virion samples prepared from infected plants by the method of Verduin (22). Total RNA was extracted by homogenizing 1 g of leaf tissue with a Brinkman Polytron in 2 ml of 100 mM glycine, pH 9.5/100 mM NaCl/10 mM EDTA/1% SDS/bentonite (1 mg/ml). Homogenate was extracted twice with phenol/chloroform and RNA was recovered by ethanol precipitation. Total and viral RNA were separated by electrophoresis on 0.8% agarose gels and Northern blots were made and analyzed using standard procedures (23). The CCMV RNA probe was a T3 RNA polymerase transcript from pCC3RA518, which contains the *Dra* I–*Xba* I cDNA fragment from pCC3TP4 (bases 1994–2173) cloned into *Sma* I–*Xba* I sites of pT7T318U (Pharmacia). This 179-nucleotide fragment includes the common 3′ terminal nucleotides shared by all four CCMV RNAs (24).

Sequence Analysis. Full-length RNA3 cDNA was synthesized with the 3′ terminal primer d(CAGTCTAGATGGTCTCCTTAGAGAT) (2, 25). This cDNA was amplified by 35 cycles of polymerase chain reaction (26) with the 3′ cDNA primer and a 5′ primer corresponding to bases 155–171 of (+)-strand cDNA. Melting, annealing, and polymerizing times and temperatures of 60 sec at 94°C, 45 sec at 50°C, and 150 sec at 74°C, respectively, were used under conditions specified for *Thermus aquaticus* polymerase (United States Biochemical), in a Coy temperature cycler (Coy Laboratory Products, Ann Arbor, MI). Amplified DNA was purified on 2% NuSieve agarose gels (FMC) and sequenced with Sequenase (United States Biochemical) by using the manufacturer's specifications. The 5′ noncoding sequences (see Fig. 4) were determined with a primer complementary to bases 281–297. The RA3–RA5 overlap region was sequenced with primers that hybridized to bases 1462–1478 and bases 1078–1094 of the (+)- and (−)-strand cDNAs, respectively. Dideoxynucleotide sequencing of RNA templates was as described (24).

RESULTS

Requirement for CCMV 3a and Coat Genes in Systemic Infection. We have shown (6) that deletions in the 3a and coat genes do not block CCMV RNA3 amplification in protoplasts coinfected with CCMV RNAs 1 and 2. The possible involvement of these genes in systemic spread of CCMV infection in whole plants was assessed with selected deletion mutants of a full-length biologically active RNA3 cDNA clone (Fig. 1). The deletion in pCC3RA3 removes the 3′ half of the 3a gene. *In vitro* translation of pCC3RA3 *in vitro*-synthesized transcripts produced a protein with the electrophoretic mobility predicted for this truncated 3a derivative. The deletion in pCC3RA5 removes 75% of the coat protein gene, fusing the first 9 codons in-frame to the last 38 codons of the coat gene. These deleted RNA3 clones, their *in vitro* transcripts, and their progeny RNAs will be referred to as RA3 and RA5, and the wt cDNA clones of CCMV genomic RNAs 1, 2, and 3 and their transcripts and progeny will be referred to as C1, C2, and C3, respectively. Although RA3 and RA5 are replicated in protoplasts coinfected with C1 and C2, the RA5 deletion and other modifications blocking expression of a functional coat protein reduce accumulation of all three CCMV RNAs, apparently due to reduced RNA stability in the absence of encapsidation (Fig. 2) (6). Direct encapsidation assays (2) confirmed that all four viral RNA species seen in RA3-inoculated protoplasts were packaged in PEG-precipitable virions (data not shown).

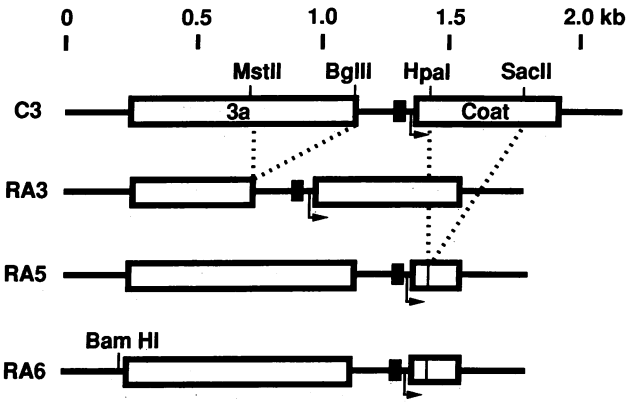

FIG. 1. Structure of RNA3 deletion mutants. wt CCMV RNA3, C3, is illustrated with open boxes representing coding regions and a solid box denoting the intercistronic oligo(A). An arrow denotes the initiation site of subgenomic RNA4. Restriction endonuclease sites bounding the deleted regions are marked, and dashed lines indicate the sites fused to create RA3 and RA5. RA6 differs from RA5 by only two nucleotide substitutions that create a *Bam*HI site within the 5′ noncoding region (see Fig. 4). kb, Kilobase(s).

To determine the effect of these deletions on systemic infection, primary leaves of 7-day-old cowpeas were inoculated with *in vitro* transcripts of C1 and C2 plus C3, RA3, or RA5. Although 100% of the plants treated with C3 inoculum developed systemic symptoms within 10–14 days, plants treated with either RA3 or RA5 inoculum remained asymptomatic throughout observation periods of up to 4 weeks. Northern blot analysis with a CCMV probe specific for the conserved 3′ termini easily detected CCMV RNA in PEG-precipitated virion fractions from all uninoculated secondary leaves sampled from C3-inoculated plants, at levels at least 100-fold over detection limits. No CCMV RNA signals were detected in equivalent fractions from either inoculated or uninoculated leaves of plants inoculated with RA3, in which the capsid protein gene is intact and expressed. For plants

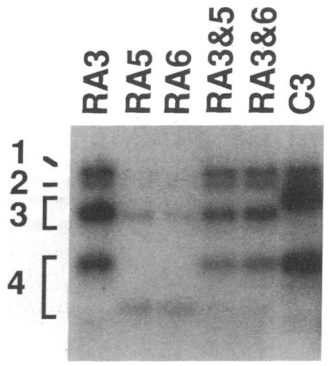

FIG. 2. Northern blot of agarose-gel-electrophoresed RNAs from barley protoplasts inoculated with C1, C2, and the indicated RNA3 components. Each lane contains the RNA from 2×10^4 inoculated cells. ^{32}P-labeled RNA complementary to the last 179 bases of all CCMV RNAs was used as a probe. RNAs are identified at the left with brackets indicating the size range of wt and deleted RNAs 3 and 4.

inoculated with the RA5 coat gene deletion mutant, total RNA samples were electrophoresed and probed for CCMV RNAs. Viral RNAs were weakly detectable in RA5-inoculated primary leaves, increasing between 1 and 4 days after inoculation and thereafter declining (data not shown). CCMV signals in total RNA samples from C3-inoculated primary leaves continued to increase for at least 7 days after inoculation, reaching levels hundreds of times greater than those in RA5-inoculated leaves. No viral signals were detected in total RNA samples from secondary leaves of RA5-inoculated plants during periodic sampling over a 20-day period.

Coinoculation of RA3 and RA5 in Whole Cowpea Plants. To investigate whether coinoculation of RA3 and RA5 might overcome their independent defects in systemic infection by complementation, recombination, or both, cowpeas were inoculated with a mixture of all four transcripts, C1, C2, RA3, and RA5. Of 20 plants so inoculated, 12 developed the normal mosaic symptoms of CCMV systemic infection. These symptoms appeared and spread through the affected plants without detectable delay compared to inoculations with wt transcripts C1, C2, and C3. Contemporaneous, parallel inoculation of 16 plants with either transcripts C1, C2, and RA3 or transcripts C1, C2, and RA5 produced no systemic infection, arguing against inoculum contamination as a possible source of infection. Mock-inoculation controls conducted in all experiments were also consistently negative by hybridization and symptomology. Standard CCMV extraction procedures produced normal yields of virion RNA from the noninoculated leaves of symptomatic RA3 plus RA5 coinoculated plants. Agarose gel electrophoresis of these preparations revealed only three genomic RNAs and one subgenomic RNA, which comigrated with the wt CCMV virion RNAs (Fig. 3). Although RNAs RA3 and RA5 are electrophoretically distinct from wt RNA3 in agarose gels (Fig. 2), there was no evidence of these starting deletion mutants. In some samples variations were noted in the relative levels of RNAs 3 and 4, as discussed further below.

Recovery of an Artificial Marker in Recombinant Progeny. To confirm the recombinant nature of the wt length RNA3 that appeared after RA3 plus RA5 coinoculation, a second coat protein deletion mutant, pCC3RA6, was constructed (Fig. 1). This mutant differs from pCC3RA5 by only two nucleotide substitutions, creating a new *Bam*HI site adjacent to the 5′ side of the 3a gene initiation codon. As shown by comparison with RA5 in Fig. 2, these substitutions have no detectable effect on the accumulation of RA6 RNA in protoplast infections. Of a total of 24 cowpea plants inoculated with the C1, C2, RA3, and RA6 transcript combination in several independent experiments, 7 became systemically infected and showed the appearance of a wt length RNA3 band. The region surrounding the 3a gene initiation codon was then examined by dideoxynucleotide sequencing of both progeny viral RNA and cDNA copies of this region amplified by the polymerase chain reaction. In each of three independent experiments, progeny RNA3 derived from RA3 plus RA6-inoculated plants was found to contain the RA6-specific nucleotide substitutions, and progeny RNA3 derived from RA3 plus RA5-inoculated plants contained the expected wt sequence (Fig. 4).

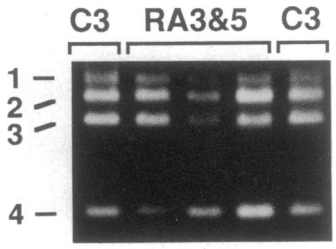

FIG. 3. Ethidium bromide-stained 0.8% agarose gel showing recombinant RNA3s in virion RNA extracts from systemically infected leaves of three plants inoculated with C1, C2, and deletion mutant RA3 and RA5 (lanes RA3&5). Lanes C3 contain virion RNA from plants receiving the wt inoculum C1, C2, and C3. RNAs 1–4 are identified at the left.

Northern blot analysis (with the same probe as in Fig. 2) was then used to see if recombinant molecules could be detected in virion extracts of primary leaves inoculated with transcripts C1, C2, RA3, and RA5. Four days after such inoculation, primary leaves from one of eight plants tested contained C1, C2, full-length recombinant RNA3, and RNA4 at levels approximately 100 times less than those seen in parallel leaves inoculated with wt C1, C2, and C3 transcripts. The parental RA3 and RA5 bands were not visible in the recombinant sample, and extracts from the other seven inoculated plants lacked any detectable viral RNA bands. However, even though both inoculated primary leaves were removed for this fourth-day sampling, one of these seven plants later became systemically infected, as did the plant showing recombination in the inoculated leaf. The population of cells infected by the parental RA3 and RA5 viruses thus appears to remain small even in the inoculated leaf, but nevertheless gives rise to the generation of recombinants and development of systemic infection.

Progeny Sequencing Reveals Homologous Recombination. To combine complete copies of the 3a and coat genes into a single RNA, RA3–RA5 or RA3–RA6 recombination must occur between the inner endpoints of the 3a and coat deletions—i.e., within the 0.2-kilobase intercistronic region or the first 27 nucleotides of the coat protein gene (Fig. 1). Comigration with wt RNA3 on agarose gels showed that the recombinants did not contain major deletions or duplications but did not rule out nonhomologous recombination within the largely noncoding target region for RA3–RA5 recombination. Altered levels of RNA3 and RNA4 in some recombinant samples (Fig. 3) also appeared consistent with deviations

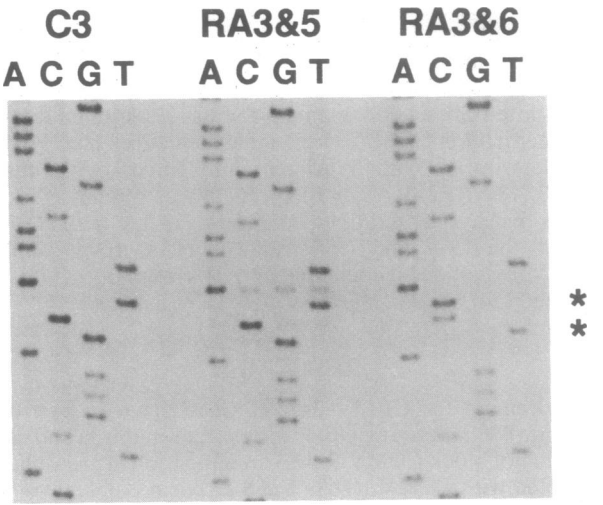

FIG. 4. Recovery of parental sequence markers in recombinant RNA3s. Sequence of polymerase chain reaction-amplified cDNA generated from full-length virion RNA3 molecules recovered from systemically infected leaves. Plant inocula included C1, C2, and RNA3 component(s) as indicated (C3, RA3 and RA5, or RA3 and RA6). Individual reaction mixtures contained the indicated dideoxynucleotide. The sequence shown is complementary to RNA3 and extends from the end of the 3a initiation codon (base 241 at the bottom of the gel) to base 219 of the 5′ untranslated region (3). The asterisks at right highlight the positions at which nucleotide differences between starting parents RA5 (equivalent to wt) and RA6 are recovered in the recombinant progeny (see text).

from wt due to nonhomologous recombination, although similar variations have sometimes been seen with nonrecombinant virus samples prepared by the same small-scale extraction procedure. To determine whether the recombination events were predominantly homologous or nonhomologous, the sequence of the complete overlap region common to RA3 and RA5/6 was determined for five independent recombinants by using polymerase chain reaction-amplified cDNA. In all cases, the sequences obtained were identical to wt RNA3, indicating that all of these RNAs were derived from homologous recombination between the two deletion mutants.

DISCUSSION

Involvement of 3a and Coat Proteins in Systemic Infection. The individual behavior of deletion mutants RA3 and RA5 shows that both the 3a and coat genes of CCMV are involved in systemic infection of cowpea. The mechanisms by which these genes contribute to systemic infection remain uncertain: they may provide active functions for transport of infection between cells, assist in avoiding or suppressing active host defenses, or both (27). As for the similarly implicated TMV 30-kDa nonstructural protein, the biochemical function(s) of the 3a protein are not yet known.

While this work was in progress, it was established that functional coat protein is also required for systemic infection of barley by BMV (28). These BMV experiments showed that systemic infection was dependent on both sufficient coat gene expression and the ability of the coat protein to interact with viral RNA. RNA encapsidation may contribute to movement of infection in a variety of ways, such as protecting the genome from degradation or facilitating entry into uninfected cells, particularly during vascular transport between leaves. The need to transport three separate genomic RNA species to new sites might make bromovirus infection more susceptible to coat-protein loss than the single-component RNA viruses for which infection spread in the absence of coat protein has been reported (12–15). Coat protein might also have direct or indirect effects on systemic infection beyond encapsidation. Failure to express functional coat protein in BMV infections is associated with increased nonstructural protein expression (28) and increased (−)-strand RNA accumulation (P. Kroner and P.A., unpublished results). Relative to wt, the RA5 coat deletion induced a much more severe reduction in viral RNA accumulation in inoculated cowpea leaves than in barley protoplast infections, suggesting that the contribution of coat protein to virus spread may not be limited to vascular transport.

Coinoculation and Recombination Results. Complementation between mutants RA3 and RA5 appeared to be inefficient and provided no discernible ability to support effective systemic spread, since the parental RA3 and RA5 RNAs were not easily detected in coinoculated leaves, approximately half of the coinoculated plants failed to develop systemic infection, and all systemic infections were associated with recombinants. It is possible that in the absence of recombination low-level systemic complementation might be detected or that some RA3 or RA5 features, such as RA3 expression of a truncated 3a protein, interfere with complementation. It should be possible to use additional mutants to address these issues.

Prior to this study the only demonstrated bromovirus recombinants were exchanges between BMV 3′ noncoding regions. A large percentage of these involved nonhomologous recombination events, many creating duplications or insertions large enough to be detected by electrophoresis of intact genomic RNA (19). Although most of the overlap region between the RA3 and RA5/RA6 deletions in this study was also noncoding sequence (Fig. 1), the recombinants displayed wt electrophoretic mobility, and the five recombinants for which the crossover region was sequenced all involved homologous exchanges. Recently, we have observed a possible nonhomologous recombinant from a systemically infected leaf from a plant inoculated with transcripts C1, C2, RA3, and RA5. This particular virion RNA sample contained an RNA3 species with slightly lower electrophoretic mobility than wt C3. Screening larger numbers of RA3–RA5 recombinants may also reveal additional nonhomologous recombinants. The predominance of homologous recombinants suggests, however, that there may either be preferential generation or selection of homologous crossovers in the overlap region between the RA3 and RA5–RA6 deletions. Most nonhomologous recombinants in this region might be selected against because of constraints on the function of the intercistronic region, which contains sequences essential for production of the subgenomic coat protein mRNA (6, 21) and possibly for other unidentified functions.

Alternatively, the overlap region may contain sites facilitating homologous exchange. An obvious candidate is the variable length, intercistronic oligo(A) segment (Fig. 1), which averages 40 residues in length (2). Under the generally favored copy-choice model for RNA virus recombination (29), the weak pairing in this oligo(A) region would provide a favorable site for separation of nascent and template strands, while the minimal sequence alignment required would facilitate subsequent reinitiation of RNA synthesis at the equivalent position on a new template. Because oligo(A) length is heterogenous even in CCMV populations derived from *in vitro* transcripts (2), the oligo(A) length distribution in the RA3–RA5 recombinant populations does not meaningfully test the possible role of this region as a recombination site. However, varying the deletion boundaries in the starting parents will allow such questions to be addressed with additional mutants. In contrast to the limited numbers of selectable markers available for most viruses, deletion mutants provide total flexibility in defining the region within which recombination must occur to regenerate a complete genome. The demonstration that such mutants can reproducibly give rise to recombinants should thus provide useful opportunities in further recombination studies.

Evolutionary Implications. The results presented here demonstrate that recombination not only can modify infection-competent RNA virus genomes but also can rescue functional virus when plants are coinoculated with viruses that are defective for systemic infection. The rapidity and frequency of this recombination show that such genome rescue is potentially significant in natural infections as well as laboratory experiments. Since plant RNA viruses can generally replicate in primarily inoculated cells of most plants but have practical host ranges determined at the level of systemic spread, such recombination has important implications for virus survival and evolution. Recombinational assembly of functional genomes from independently defective RNAs should enhance the infectivity of RNA virus populations by compensating for the high substitution rate associated with RNA-dependent RNA replication (30, 31). This effect should allow RNA viruses to emphasize the genetic advantages of their extreme quasispecies population variance over the associated liability of deleterious mutations (32–34).

The results presented here also support the concept that *in planta* recombination between a virus not properly adapted to the host and a coinfecting virus could give rise to a new virus strain with an altered host range. Remarkable sequence similarities among plant and animal RNA viruses suggest that recombinational reassortment of core RNA replication genes with capsid and accessory genes that provide varied host and vector adaptations has been a major pathway for divergence during previous evolution of many (+)-strand RNA viruses

(7, 35, 36). Among existing (+)-strand RNA viruses, active recombination is particularly well documented in animal picornaviruses and coronaviruses (37–39). Direct evidence for homologous and heterologous recombination has also been found for several other plant-virus families in addition to bromoviruses: mosaic-type defective-interfering RNAs have been identified in tombusvirus infections (40, 41); an apparent intramolecular recombination event in TMV leading to the loss of an inserted gene has also been described (42); and natural recombinants between tobacco rattle virus strains have been isolated (43, 44). Sequence results also show that recombination events occurred during the divergence of CCMV and BMV (3).

Evidence for recombination during previous evolution of distinct strains has also been presented for the animal alphaviruses (45), which share extensive similarity in RNA replication genes with CCMV, BMV, and other plant viruses (7, 35, 36). Although previous studies failed to reveal active homologous recombination in alphavirus infections (37, 46), genome rescue similar to that seen here with CCMV was observed after coinoculation of cultured cells with alphavirus deletion mutants (S. Schlesinger, personal communication). This further suggests that the results described here are relevant to other RNA viruses and constitutes yet another similar characteristic between the animal alphaviruses and bromoviruses. The earlier finding that cellular RNA sequences were incorporated in some Sindbis virus defective-interfering RNAs suggests that recruitment of cellular sequences might also assist virus adaptation to new hosts (47).

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