

Inter- and Intramolecular Recombinations in the *Cucumber Mosaic Virus* Genome Related to Adaptation to *Alstroemeria*

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In four distinct alstroemeria-infecting cucumber mosaic virus (CMV) isolates, additional sequences of various lengths were present in the 3' nontranslated regions of their RNAs 2 and 3, apparently the result of intra- and intermolecular recombination events. Competition experiments revealed that these recombinated RNA 2 and 3 segments increased the biological fitness of CMV in alstroemeria.

The genome of *Cucumber mosaic virus* (CMV), the type species of the genus *Cucumovirus* in the family *Bromoviridae* (38), consists of three single-stranded RNA molecules: RNAs 1 and 2 encode components of the viral RNA-dependent RNA polymerase (RdRp) (31, 32), while the bicistronic RNA 3 encodes the movement protein (MP) (43) and coat protein (CP) (39). The CP is translated from a subgenomic RNA, RNA 4, which is encoded by the 3' half of the RNA 3 (19). A small overlapping gene (2b), residing in RNA 2 and implicated in suppression of gene silencing (4), was discovered more recently and is most likely expressed through a second subgenomic RNA (13). Based on their genomic sequences, CMV isolates have been classified into two major subgroups, I and II (2, 32, 35). A further division of subgroup I, into IA and IB, has been proposed based on the nucleotide sequences of 5' nontranslated regions (NTRs) of RNA 3 (34, 37). With a host range of over 1,000 plant species, CMV is one of the most widespread plant viruses in the world (32). Also, the ornamental plant *alstroemeria* has been reported as a host of CMV (21), although few data on the interaction between CMV and this host are available.

RNA recombination is generally considered one of the major forces that favor the evolution and adaptation of RNA viruses (14, 24, 36, 41, 42). Most of the data collected from different experimental systems suggest that RNA recombination prefers replicase (RdRp)-driven template switching models or copy choice mechanisms (6, 30, 41). Alternatively, the breakage-and-ligation mechanism has been suggested and proven as a possible means of in vitro RNA recombination as well (12). RNA recombination has been classified according to the extent of involvement of homologous sequences (23, 24, 30).

Evidence from nucleotide sequence data suggests that recombinational events have occurred in satellite RNA associated with CMV strain Y (27) and defective RNAs of the Fny strain of CMV (20). Recombinational events have also been observed in artificial pseudorecombinant viruses composed of CMV and tomato aspermy cucumovirus (TAV) RNAs (16,

28). However, a systematic search of available CMV nucleotide sequences did not indicate that potential recombinational events among numerous analyzed CMV strains occur (7). No natural mixed infection and no genetic exchange between CMV subgroup I and II strains were found (17), indicating that most heterologous genetic recombinations seem to be at a competitive disadvantage (18), although a case of natural interspecies recombination between CMV and TAV has been reported (1). More recently, a homologous recombinational event between RNA 1 and RNA 2 or RNA 3 was demonstrated in vivo in subgroup I CMV when transgene sequences were used as donors (8).

In our previous work we described an alstroemeria-infecting subgroup II CMV isolate (ALS-CMV) that contained an additional sequence of 218 nucleotides (nt) in the 3' NTR of RNAs 3 and 4 (10). This additional sequence appeared to be identical to parts of the 3' NTRs of RNAs 1 and 2 of the homologous virus, suggesting that an RNA recombination event occurred between RNA 3 and RNA 1 or RNA 2. To investigate the commonness of additional sequences in the 3' termini of alstroemeria-infecting CMV isolates, the genomic RNAs of five distinct ALS-CMV isolates were analyzed. Furthermore the biological relevance of the recombinational events encountered are tested by competition experiments.

The CMV strains used in this study, designated ALS-0 through -4 (Table 1), were all independently isolated from alstroemeria plants (ALS-CMV) and were stock material of the Bulb Research Center, Lisse, The Netherlands, the Inspection Service for Floriculture and Arboriculture (NAKtuinbouw), Roelofarendsveen, The Netherlands, and Plant Research International, Wageningen, The Netherlands. ALS-0, although originally isolated from alstroemeria, had been maintained on tobacco for an extended period (at least 5 years); the others were exclusively passaged on alstroemeria. For short-term propagation, all five ALS-CMV isolates were mechanically inoculated onto *Nicotiana benthamiana*, while alstroemeria plants (clone VV024-6) (25) were used for pathogenicity assays. Purifications of virions, viral RNA, and total RNA were carried out by using published protocols (26, 33, 40). Reverse transcriptase (RT)-PCR amplification of CMV genomic RNAs was performed as previously described (9) with degenerate primer sets for RNA 1 (corresponding to nt 1 to 23 and 1098

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TABLE 1. Lengths and accession numbers of genomic RNAs of ALS-CMV isolates

Isolate	RNA 1			RNA 2			RNA 3		
	Length (nt)		Accession no.	Length (nt)		Accession no.	Length (nt)		Accession no.
	Full RNA	3' NTR		Full RNA	3' NTR		Full RNA	3' NTR	
ALS-0	3,391	317	AJ304405	3,039	424	AJ304396	2,204	321	AJ304399
ALS-1	3,442	317	AJ304393	3,334	719	AJ304394	2,419	539	AJ304397
ALS-2	3,391	317	AJ304404	3,289	674	AJ304395	2,419	539	AJ304398
ALS-3	ND ^a	ND	ND	1,398 ^b	719	AJ304400	1,413 ^b	616	AJ304402
ALS-4	ND	ND	ND	1,261 ^b	582	AJ304401	1,415 ^b	616	AJ304403

^a ND, not determined.

^b Partial sequence.

to 1108, nt 1074 to 1087 and 2238 to 2255, and nt 2238 to 2248 and 3307 to 3391), RNA 2 (corresponding to nt 1 to 22 and 1105 to 1118, nt 1105 to 1118 and 2062 to 2075, and nt 1937 to 1951 and 3025 to 3039), or RNA 3 (corresponding to nt 1 to 34 and 1120 to 1131 and nt 1087 to 1111 and 2195 to 2204). RT-PCR products were cloned into pGEM-T Easy (Promega), and inserts were sequenced in both directions. Programs of the University of Wisconsin Genetics Computer Group package were used for sequence analysis (Table 1).

The sequences obtained from all ALS-CMV genomic RNAs appeared to be highly homologous (>97%) to those of reported subgroup II CMV strains. Whereas no large differences were found in the RNAs 1 of the three ALS-CMV isolates tested (ALS-0, ALS-1, and ALS-2), the 3' NTRs of both RNA 2 and RNA 3 were found to vary significantly in length (Table 1). The RNA 2 molecules did not differ in their 5' NTR (92 nt), 2a open reading frame (ORF) (2,523 nt), or 2b ORF (303 nt) but varied considerably in their 3'-NTR length (Table 1). Also, the RNA 3 molecules had similar lengths in their 5' NTRs (96 nt), 3a ORF (840 nt), intergenic regions (287 to 290 nt), and CP ORF (657 nt) but were notably different in 3'-NTR length (Table 1). The additional sequences were not PCR artifacts, as upon electrophoresis RNAs 3 and 4 of ALS-CMV strains 1 to 4 were visibly larger (Fig. 1) than those of a wild-type subgroup

II CMV isolate. Furthermore, the size addition to RNA 2 made this RNA indistinguishable in size from RNA 1 in Northern blots (Fig. 1). ALS-0 had RNAs of wild-type size and sequence, and strikingly, this was the only alstroemeria strain maintained (for several years) on *N. benthamiana*, instead of its original host. Hence, it cannot be excluded that it originally also contained additional sequences.

The wild-type (and ALS-0) 3' NTR of RNA 2 can be arbitrarily divided into five regions (A through E) (Fig. 2A), and that of RNA 3 can be divided into four regions (A, B, C, and F) (Fig. 2B). Regions A through F are 13, 109, 16, 170, 116, and 183 nt long, respectively. Regions A, B, and C are common to all reported CMV segments and can be folded into a tRNA-like structure that is involved in viral minus-strand synthesis (3). In ALS-0, secondary structures consisting of stem-loop configurations are predicted between regions C and D (positions 2868 to 2906) and between regions D and E (positions 2698 to 2731) (Fig. 2C). Interestingly, in all four other ALS-CMV isolates the additional sequences were located in the central region of the 3' NTRs of both RNA 2 and RNA 3. In the RNA 2 of both ALS-1 and ALS-3, the extra sequences were 295 nt long and represented an exact duplicate of regions D, C, and B (Fig. 2A). Shorter additional sequences of 250 and 158 nt were found in the RNAs 2 of ALS-2 and ALS-4, respectively, and seemed to have arisen from deletions following the original duplication that resulted in the RNAs 2 of ALS-1 and ALS-3.

In the 3' NTR of RNA 3, the ALS-CMV isolates harbored an additional sequence of 218 nt (ALS-1 and -2) or 295 nt (ALS-3 and -4) (Fig. 2B). Strikingly, the additional 295 nt inserted in the RNA 3 of ALS-3 were completely identical to those of the homologous RNA 2, both in length and in sequence (Fig. 2).

Their presence in the genome of four ALS strains maintained in their original host, and their absence in the only ALS strain not maintained in this host, suggests that the additional sequences may play a role in host adaptation. Indeed, based on visual observations, all isolates containing recombinant RNAs 2 and 3 (ALS-1 through -4) were more virulent than isolates lacking these repetitions, when inoculated into alstroemeria (data not shown), suggesting a greater fitness on this host. To verify this possibility, the ALS-CMV strains were tested in competitive inoculation assays. Tobacco and alstroemeria plants were inoculated with mixtures of various ratios of purified virions of wild-type ALS-0 and one of the recombinant isolates (ALS-2). In systemically infected leaves of tobacco

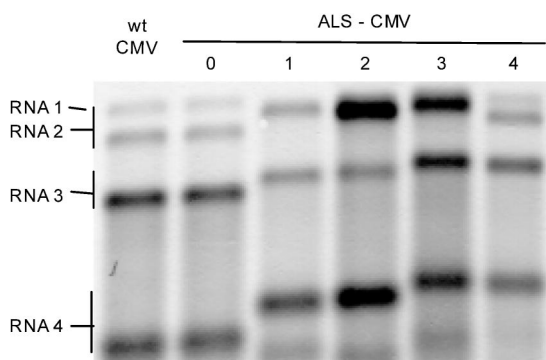


FIG. 1. RNA patterns of five alstroemeria-infecting CMV strains (ALS-0 to -4). Northern blot analysis used a mixture of two digoxigenin-labeled cDNA probes specific for the RNA 2-derived 218 nt in RNA 3 of ALS-1 and ALS-2 (10) and for the CP ORF of subgroup II CMV. The CP-specific probe was PCR amplified with a pair of degenerate primers corresponding to positions 1227 to 1240 and 1866 to 1883 on RNA 3 of ALS-0. A subgroup II isolate from *Crocus* (9) was used as a wild-type (wt) reference isolate. Size ranges of the genomic RNAs (and subgenomic RNA 4) are indicated on the left.

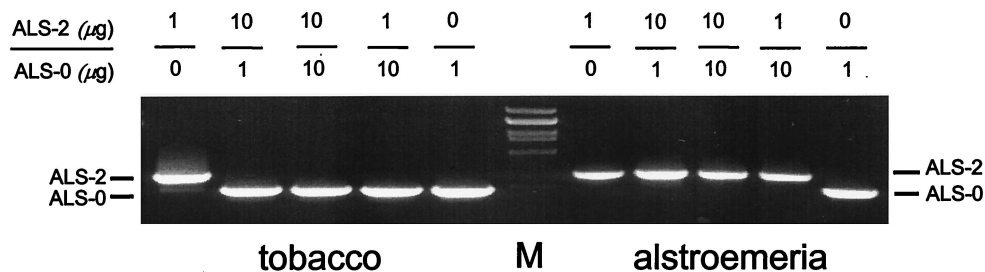


FIG. 3. Agarose gel electrophoresis of RT-PCR fragments amplified from total RNA of tobacco or alstroemeria leaves infected with CMV isolates ALS-0 and ALS-2 in various ratios. The degenerate primers used amplified the intercistronic region, CP ORF, and 3' NTR of RNA 3, resulting in cDNA fragments of 1.1 kb (ALS-0; wild type) or 1.3 kb (ALS-2) (9). Lambda DNA digested with *Pst*I was used as a size marker (M).

To further delineate the difference in replication speed, tobacco and alstroemeria plants were inoculated with mixtures of virus isolates in which the least fit virus was added in 10-fold excess. Viral RNA replication was monitored in the inoculated leaves by Northern blot analyses and RT-PCR assays (Fig. 4). In tobacco, the enlarged RNAs 3 and 4 of ALS-2 could be detected only until the fourth day postinoculation, while those of ALS-0 could be detected from the first day postinoculation, with the signals further increasing over time. However, on alstroemeria, ALS-0, even though inoculated in 10-fold excess, remained undetectable on inoculated leaves. This experiment indicated that ALS-0 and ALS-2 are outcompeted during the early stages of infection on alstroemeria and tobacco, respectively, in spite of the initial molecular excess over the competitor. Therefore, the additional sequences in the 3' NTRs of RNAs 2 and 3, which were the only differences observed between these isolates, were responsible for the enhanced fitness of the virus in alstroemeria but produced an adverse effect in tobacco plants. The significance of this finding is further underscored by the fact that four different alstroemeria isolates have these extra sequences, indicating that the occurrence of such recombination is not a single incidence.

Though many cases of recombination events between RNA segments of viruses or between viral RNAs and their satellite RNAs have been described (e.g., see references 5, 28, and 29),

naturally occurring stable recombinations are rare (17). An increase in the biological fitness of a virus by recombination within or between its segments has not been reported previously without applying selection pressure in favor of the recombinant virus. However, our conclusion that the 3' NTRs of the CMV genome are important for viral fitness is confirmed by previous observations that an artificial pseudorecombinant of CMV and TAV, having RNAs 1 and 2 from CMV and RNA 3 from TAV, gained in fitness upon a recombinational event in which CMV RNA 2-derived 3'-NTR sequences were inserted in its TAV-derived RNA 3 (16).

How the recombinant RNAs in the alstroemeria CMV isolates may have arisen remains subject to further study. Some clues, however, can be obtained from the positions of two stem-loop structures (Fig. 2), in combination with the previously implicated involvement of the CMV RdRp in template switching (22), a mechanism commonly accepted as a major means of generating recombinant RNAs (6, 11, 30, 41, 44). The RNAs 2 of the recombinant isolates show the duplication of a contiguous internal 3'-NTR segment. The same RNA 2-derived sequence was found to be incorporated in the 3' NTR of the RNAs 3 of the recombinant alstroemeria CMV isolates, some of which seem to have undergone further deletions. A complex secondary structure consisting of a stable hairpin (Fig. 2C, panel II) may have caused the RdRp to halt and disengage

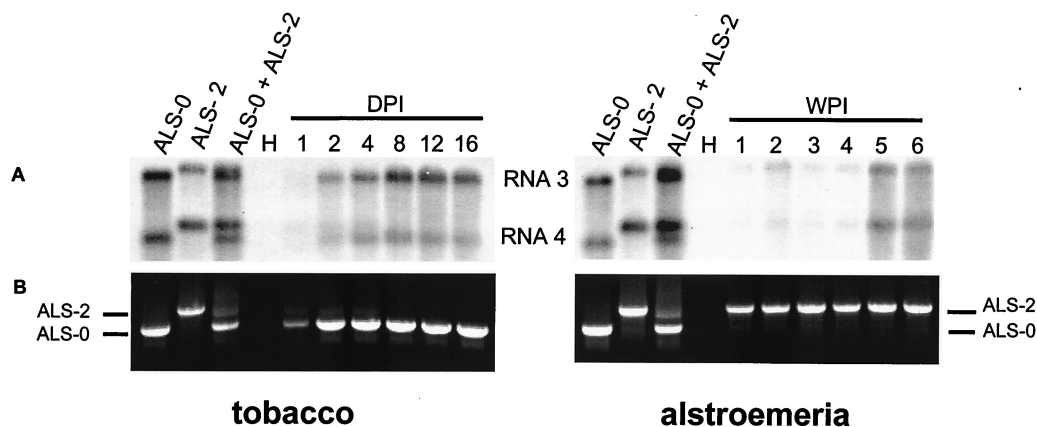


FIG. 4. Competition between ALS-CMV recombinant (ALS-2) and nonrecombinant (ALS-0) isolates upon mixed infection of tobacco and alstroemeria. Total RNA was extracted at various times in days (DPI) or weeks (WPI) postinoculation. (A) RNA gel blot assay using the probes used to produce Fig. 1; (B) products of RT-PCR of the isolated RNA with the primers described in the legend to Fig. 3.

during minus-strand synthesis of the recombinant RNA 2. Subsequent restarting of replication at the 3' end of a second RNA 2 template resulted in the initial duplication (Fig. 2A). While replicating the 3' end of the recombinant RNA 2, the viral replication complex may have halted and separated at hairpin loop I (Fig. 2C). Subsequently, the RdRp continued replication on the highly homologous 3' NTR of RNA 3, resulting in an RNA 3 molecule with a duplicated 3' NTR of RNA 2.

Recombinational events leading to mutant viruses as observed here may be commonplace for CMV, as suggested by Canto and coworkers (8). These authors were readily able to generate recombinant CMV RNAs in a transgene setting, selecting for such events. Nonetheless, recombinant viruses do not occur frequently in nature, most likely due to reduced fitness (17). Our observations with the mixed infections of tobacco plants are in agreement with that notion. *Alstroemeria* plants, however, seem to be an exception to that rule, sustaining the presence of specific recombinant viruses. Additional sequences observed in the ALS-CMV isolates are all duplicated 3' NTRs. These 3' ends of positive-strand viral RNAs contain the origin of minus-strand synthesis and are the presumed site of the promoter elements that control this synthesis in *cis* (15). It can be hypothesized that a duplication within the NTRs enhances the recruiting of host proteins involved in the viral replication complex. If binding of these components is less efficient in *alstroemeria* than in other host plant species, it may be beneficial to duplicate the regions involved in recruiting these factors. Although the inserted sequences might interrupt the sequence and secondary structure of the 3' termini, the alteration apparently offers biological advantages to *alstroemeria*-infecting CMV isolates. In addition, the slow infection process of CMV in *alstroemeria* and low accumulation of viral RNA (results not shown) could indicate that duplications in viral sequences are tolerated in this host, while in other hosts these viruses are increased in their sensitivity to host defense responses, such as gene silencing. The competitive inoculation tests demonstrated the merit of these recombination events, which enhance the fitness of ALS-CMV infecting *alstroemeria*.

Nucleotide sequence accession numbers. The sequences of the genomic RNAs of ALS isolates used here have been made available in GenBank (accession numbers AJ304393 through AJ304405).

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