Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 10487–10492, September 1998
Biochemistry

Evolution of a quadripartite hybrid virus by interspecific exchange and recombination between replicase components of two related tripartite RNA viruses

(cucumovirus/reassortment/replicase/helicase)

CHIKARA MASUTA*†, SHIGENORI UEDA*, MASASHI SUZUKI‡, AND ICHIRO UYEDA*

*Plant Virology Laboratory, Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan; and ‡Plant Pathology Laboratory, Faculty of Agriculture, University of Tokyo, Tokyo 113, Japan

Communicated by Theodore O. Diener, University of Maryland, College Park, Beltsville, MD, June 26, 1998 (received for review January 28, 1998)

ABSTRACT **Cucumber mosaic virus (CMV) and tomato aspermy virus (TAV) belong to the *Cucumovirus* genus. They have a tripartite genome consisting of single-stranded RNAs, designated 1, 2, and 3. Previous studies have shown that viable pseudorecombinants could be created *in vitro* by reciprocal exchanges between CMV and TAV RNA 3, but exchanges of RNAs 1 and 2 were replication deficient. When we coinoculated CMV RNAs 2 and 3 along with TAV RNAs 1 and 2 onto *Nicotiana benthamiana*, a hybrid quadripartite virus appeared that consisted of TAV RNA 1, CMV RNAs 2 and 3, and a distinctive chimeric RNA originating from a recombination between CMV RNA 2 and the 3′-terminal 320 nucleotides of TAV RNA 2. This hybrid arose by means of segment reassortment and RNA recombination to produce an interspecific hybrid with the TAV helicase subunit and the CMV polymerase subunit. To our knowledge, this is the first report demonstrating the evolution of a new plant or animal virus strain containing an interspecific hybrid replicase complex.**

Cucumber mosaic virus (CMV) and tomato aspermy virus (TAV) are taxonomically related virus species that can be distinguished serologically and by nucleic acid hybridization. RNAs 1 and 2 encode the 1a and 2a replicase proteins, which are required for replication and transcription. RNA 3 encodes the movement protein, and the capsid protein, which is expressed from a subgenomic messenger RNA (RNA 4) transcribed from the 3′ half of RNA 3. CMV strains are classified into two subgroups typified by CMV-Y (subgroup I) and CMV-Q (subgroup II) that have serological and sequence differences. The overall nucleotide sequence homology between CMV-Y and CMV-Q RNAs is approximately 71–76% (1). However, the level of amino acid sequence homology within the 1a and 2a replicase proteins of the CMV-Y and CMV-Q strains is much higher. For example, the methyl transferase and the helicase domains of the 1a protein, and the polymerase domain (central core region) of the 2a protein show 92, 80, and 84% homology, respectively, between CMV-Y and CMV-Q. On the other hand, the corresponding regions within the 1a and 2a proteins of a TAV strain (V-TAV) show 83, 70, and 72% homology, respectively, with CMV-Y. This degree of identity within these regions is similar to that between two *Bromovirus* species, brome mosaic virus and cowpea chlorotic mottle virus. Bromoviruses have a very similar genome organization to cucumoviruses; *Bromovirus* constitutes Class *Bromoviridae* with *Cucumovirus*, *Ilavirus*, and *Alfamovirus*.

It has been reported that between TAV and CMV, RNA 1 encoding a helicase and RNA 2 encoding a replicase were not exchangeable (2). Actually, in the case of two related bromoviruses (brome mosaic virus and cowpea chlorotic mottle virus), it has been already shown that RNAs 1 and 2 were not exchangeable because one viral replicase could not make a complex with the other viral helicase (3–5). Here we report the identification of an interspecific hybrid replicase complex between TAV and CMV, and demonstrate that a newly evolved virus by the hybrid replicase had an altered replication nature that its parent viruses did not have. Exchange of the replicase components between two different virus species has never been considered as one of the important driving forces for viral evolution.

MATERIALS AND METHODS

Virus and Plant Materials. An uncloned isolate of V-TAV, kindly supplied by Fernando García-Arenal (Department de Biotecnología, ETSI Agrónomos, Madrid, Spain), was propagated in *Nicotiana benthamiana*. V-TAV was originally isolated in Victoria, Australia, from *Chrysanthemum. sp*. CMV-Y and a satellite RNA (Y-satRNA) were obtained from Japan Tobacco (Yokohama, Japan). The plants used for this research (*N. benthamiana* and *Nicotiana tabacum* cv. Xanthi nc) were grown at 23–27°C under natural light conditions in the greenhouse.

Reverse Transcription (RT)-PCR. Viral RNAs were checked for their origins by specific amplification of PCR products to each genomic segment in Figs. 2–4. Oligonucleotide primer pairs for PCR detection, and the nucleotide positions of their annealing sites within each RNA, were 5′-CATTCGAATCACGAGTGCC-3′ (567–585) and 5′-CAACTGCTCATACTTCATGCG-3′ (1402–1382) for TAV RNA 1, 5′-GTATGTCCATGCAATCAGGC-3′ (1311–1330) and 5′-TCTCCATCAGACTTCGGACC-3′ (2133–2114) for CMV RNA 1, 5′-TGAAGAGTGTGAAGCCGACG-3′ (567–586) and 5′-TCTGGAAGCGCTCGAATAGC-3′ (1540–1521) for CMV RNA 2, and 5′-GGTGATCCAAGCTTGTTCACC-3′ (1963–1983) and 5′-TTCGGACATGACAACATCGG-3′ (2760–2741) for TAV RNA 2. All RT and PCR reactions were performed by using a Takara Shuzo (Kyoto) RNA LA PCR kit according to the manufacturer's instructions. Specifically, cDNAs were synthesized by avian myeloblastosis virus reverse transcriptase XL at 50°C for 30 min and, after a 2 min incubation at 94°C, 28 cycles of PCR amplification were conducted in programmed steps of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 90 sec.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/9510487-6$2.00/0
PNAS is available online at www.pnas.org.

Abbreviations: CMV, cucumber mosaic virus; TAV, tomato aspermy virus; RT, reverse transcription; DIG, digoxigenin.

†To whom reprint requests should be addressed. e-mail: masuta@res.agr.hokudai.ac.jp.

Northern Blot Analysis. Digoxigenin (DIG)-labeled DNA probes were prepared from the cloned cDNAs by PCR using the primers explained above. The reaction mixture (50 μl) contained a 1× labeling mixture (Boehringer Mannheim), 1.75 μl of DIG-labeled dUTP, 1 × Ex *Taq* polymerase buffer, and 2.5 units of Ex *Taq* (Takara Shuzo) in addition to template DNA and primers. PCR products were then separated and dissolved in 100 μl of water. Five microliters of DIG-labeled probes were used for each hybridization. For the C1 specific probe, a DIG-labeled RNA probe was substituted, because use of the DNA probe resulted in a high background. The cDNA fragment specific to C1 was cloned under the SP6 promoter in a plasmid vector (pGEM-T, Promega). *In vitro* transcription was performed in a 20 μl reaction mixture containing 1 mM dithiothreitol (DTT), 1 × DIG RNA labeling mixture (Boehringer Mannheim), 0.5 unit RNase inhibitor, 1 × SP6 RNA polymerase buffer (GIBCO/BRL), 15 units SP6 RNA polymerase (GIBCO/BRL), in addition to 1 μg of the recombinant template plasmid digested with *Eco*RI by incubating at 37°C for 90 min. The RNA probe was precipitated by 2 M LiCl and dissolved in 50 μl of water containing 0.5 unit RNase inhibitor and 1 mM DTT. Five microliters of the probe were used for hybridization. Viral RNAs were separated in a 1.4% agarose gel after denaturation with glyoxal and dimethyl sulfoxide, and transferred onto Hybond-N nitrocellulose membrane (Amersham) by capillary blot, and then membranes were treated with UV cross-linker. Hybridizations were performed according to Li *et al.* (6). Signals were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate or with the chemiluminescent substrate CSPD (Boehringer Mannheim).

Synthesis of Infectious Full-Length cDNA Clones and Infectious RNA Transcripts. By using infectious cDNAs and infectious RNA transcripts, pseudorecombinants were created between V-TAV(J) and CMV-Y, whose genomic RNAs are designated T1 to T3 and C1 to C3, respectively. To construct infectious cDNA clones for V-TAV(J), full-length cDNA clones representing T1, T2, and T3 were inserted downstream of a cauliflower mosaic virus 35S promoter (7), which had been cloned into a pGEM-T vector (Promega). When the recombinant plasmids were directly inoculated onto *N. benthamiana* at a concentration of 100 μg/ml after digestion with *Sac*I for T1 and T2, and *Not*I for T3, systemic symptoms appeared in 5 of 20 plants. Infectious cDNA clones for CMV RNAs under the 35S promoter had been constructed previously in a pUC119 plasmid (unpublished results). To test for infectivity of recombinant and pseudorecombinant RNAs we used infectious RNA transcripts rather than infectious cDNAs because the infectivity of the transcripts was much better (more than three times). Full-length cDNAs to T1 and T2 were created by ligating partial cDNA clones and inserted in pUC119. The oligonucleotide primer for the 3′ ends of T1 and T2 (3T2S-3) contained a *Sac*I site at the 5′ end (5′-CGAGCTTGGGACCCCTAGG-3′) and the oligonucleotide primer for the 5′ end (5T2T7P) contained a T7 RNA polymerase promoter and a *Pst*I at the 5′ end (5′-GGCTGCAGTAATACGACTCACTATAGTTTGTCTATCAAGAG-3′). Synthesis of full-length cDNA clones to C2 and C3 has been described (8). The cDNA to T1 was synthesized from progeny RNAs of T1C2(C2-T2)C3 (see *Results*) based on the results of sequencing and the two-hybrid experiments. For cloning of the recombined molecule C2-T2, the 3′-terminal region was replaced with the 3′-terminal region of T2 according to the sequence results. *In vitro* transcription from those cDNA clones were done essentially as described (8) using *Not*I-digested plasmids for C2 and C3, *Sac*I-digested plasmids for T1 and T2, and *Sac*II-digested plasmids for C2-T2. The run-off transcripts were all mixed and inoculated onto *N. benthamiana* at a concentration of 50 μg/ml.

Two-Hybrid System. The two-hybrid vectors (pAS2–1 and pACT2) were purchased from CLONTECH. The entire 2a protein from CMV-Y RNA 2 was fused to GAL4 transcription activation domain in pACT2. T1C2(C2-T2)C3 is quadripartite with TAV RNA1 (T1), CMV RNA2 (C2), a chimeric RNA between C2 and T2 (C2-T2), and CMV RNA3 (C3); C2-T2 encodes the same 2a and 2b proteins as those of CMV. The ORFs for 1a proteins from CMV-Y RNA 1 (C1a), V-TAV(J) RNA 1 (wild-type T1a), and T1C2(C2-T2)C3 RNA 1 [T1C2(C2-T2)C3 T1a] were generated by PCR and cloned into pAS-2 to make a fusion protein with DNA binding domain after all the nucleotide sequences were confirmed. Yeast cells supplied by CLONTECH were grown by the conventional method, and transformation was performed by the lithium acetate method. After 3 days of growth, the yeast transformants containing the recombinant plasmid(s) were assayed for the β-galactosidase activity with the filter colony lift assay.

RESULTS

Generation of V-TAV(J) from V-TAV. During propagation in *N. benthamiana*, an isolate of V-TAV, which we designated V-TAV(J), was recovered that had modified biological properties. The infectivity of this isolate was very low, and although it could spread in the inoculated leaves of *N. tabacum*, it failed to move systemically into the upper uninoculated leaves. Moreno *et al.* (9) recently reported that the original V-TAV contains a defective movement protein, which affects cell-to-cell movement of the virus and alters its symptom phenotype. However, this appears not to be the case for V-TAV(J), because we found that the corresponding movement protein sequence had undergone a mutation whereby a stop codon within the gene that interrupted the original V-TAV ORF was changed to a serine (at position 4) in V-TAV(J). Sequence analysis of RNAs 1 and 2 of V-TAV(J) also revealed two amino acid changes (at positions 183 and 573, V to A and T to S, respectively) in the 1a protein and five differences (at positions 23, 65, 111, 176, and 531, T to A, D to N, I to S, S to G, and E to G, respectively) in the 2a protein, which had arisen during the transition from V-TAV.

Infectivity of Pseudorecombinants Between V-TAV(J) and CMV-Y. Synthesis of infectious cDNA clones for TAV has been recently documented (9–11). As shown in several labs (9–11), pseudorecombinants of RNA 3 are supported by both TAV and CMV RNAs 1 and 2, but viable combinations have not been recovered from reciprocal exchanges of RNAs 1 and 2 (e.g., T1C2C3, C1T2C3, T1C2T3, and C1T2T3). Surprisingly, when we inoculated combinations of C1T1T2C3 and T1T2C2C3, we observed systemic infections that differed in their symptom phenotype from CMV, V-TAV(J), or T1T2C3 infections (Fig. 1). These experiments were performed four times using five plants for each inoculation; and among these plants, we obtained two individual isolates from the C1T1T2C3 infections and five isolates from the T1T2C2C3 inoculations.

Progeny Virus Isolates from C1T1T2C3 and T1T2C2C3 Inoculations. RNAs were extracted from the progeny virions of C1T1T2C3 and T1T2C2C3, which had been purified from primarily infected tissues, and RT-PCR was conducted to identify the sources of encapsidated RNAs 1 and 2. As shown in Fig. 2*A*, the results indicate that both T1- and C1-specific sequences are present in the C1T1T2C3 progeny preparations, and conversely that both T2- and C2-specific sequences are present in the T1T2C2C3 preparations. To analyze further the nature of the reassortments, the progeny virion RNAs were subjected to Northern hybridization with probes corresponding to the sequences used for RT-PCR. Fig. 2*B* shows that both C1 and T1 were amplified and encapsidated in the C1T1T2C3 virions, and both C2 and T2 were also present in the T1T2C2C3 preparations. To determine whether each RNA fragment in the pseudorecombinants could be maintained through several host passages, inoculation experiments were

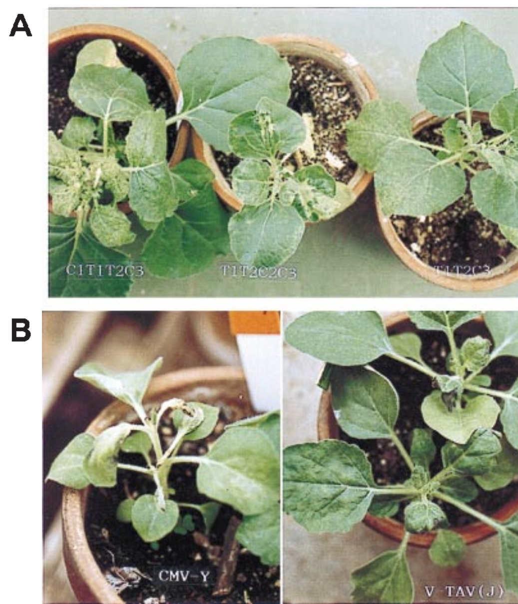

FIG. 1. (*A*) Symptoms induced on *N. benthamiana* plants inoculated with pseudorecombinants C1T1T2C3 (*Left*), T1T2C2C3 (*Center*), T1T2C3 (*Right*). (*B*) Symptoms induced on *N. benthamiana* plants inoculated with CMV-Y (*Left*) and V-TAV(J) (*Right*).

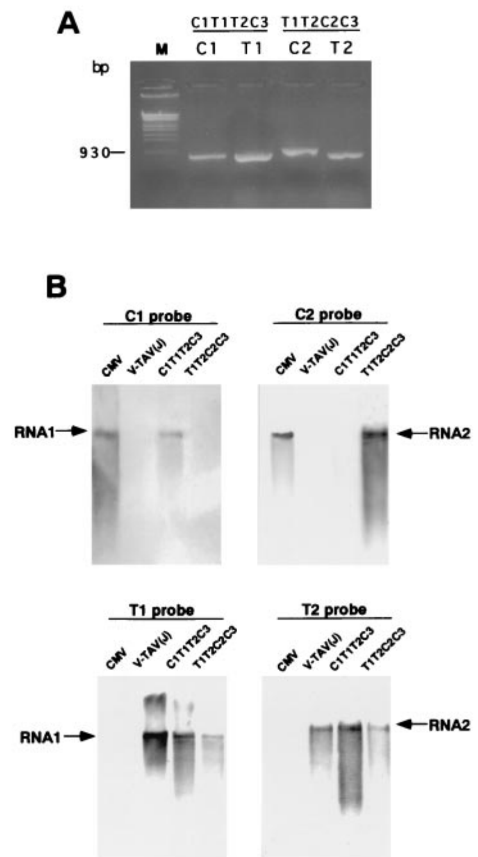

FIG. 2. Detection of each RNA of the progeny viruses after inoculations of C1T1C2C3 and T1T2C2C3. Viruses were purified from primarily infected tissues of *N. benthamiana*. (*A*) Agarose gel electrophoresis of segment-specific PCR products amplified from encapsidated viral genomic RNAs. Lane M contains marker DNA species, Lane C1 was loaded with the PCR product from C1T1T2C3 for detection of CMV RNA 1, Lane T1 shows the PCR product from C1T1T2C3 for detection of TAV RNA 1, Lane C2 contains the PCR product from T1T2C2C3 for detection of CMV RNA 2, Lane T2 illustrates the PCR product from T1T2C2C3 for detection of TAV RNA 2. (*B*) Northern blot analysis of encapsidated viral RNAs from pseudorecombinants. RNAs (0.4 μg), extracted from purified CMV-Y, V-TAV(J), C1T1T2C3, and T1T2C2C3, were hybridized with the probes specific for each RNA segment, as indicated at the top of the blots.

performed on *N. benthamiana* and *N. tabacum*. C1T1T2C3 and T1T2C2C3 could both infect *N. benthamiana* systemically. However, both sets of pseudorecombinants produced distinct chlorotic spots on the inoculated leaves of *N. tabacum*, but the isolates failed to move systemically. One T1T2C2C3 isolate out of five obtained was inoculated onto *N. tabacum*, and six single chlorotic spots were isolated and this isolation was repeated twice. Then those six isolates were subjected to three passages through *N. benthamiana* before the analysis. Total RNAs were extracted from the infected leaves and used for RT-PCR and Northern blot experiments. Fig. 3 shows one of the representative RT-PCRs, indicating that C2, but not T2 existed in the RNA preparations. Therefore, it is likely that the T2 sequence was eliminated to establish an apparent T1C2C3 pseudorecombinant. On the other hand, C1 has disappeared from C1T1T2C3 after lesion isolation followed by serial passages through *N. benthamiana*, because any sequence of C1 was not detected by RT-PCR (Fig. 4) using RNAs prepared from the *N. benthamiana* tissues infected with one C1T1T2C3 isolate out of two.

Evolution of Possible Quadripartite Hybrid Virus. We subsequently decided to focus on the nature of the T1C2C3 pseudorecombinant. Six isolates recovered after two passages of single lesion transfer were maintained in *N. benthamiana*, and RNAs 1 and 2 from those isolates were cloned by using a CapFinder cDNA library construction kit (CLONTECH). The complete sequences of each RNA from four isolates and partial sequences of the 3′ regions of RNA 2 from two other isolates were determined. At least three independent cDNA clones for each RNA were sequenced. The sequence analyses revealed that the 3′-terminal 140 nt of the C2 RNA from each of the T1C2C3 isolates was replaced by the corresponding T2 sequences (320 nt), and thus an RNA recombination event had occurred at the 3′ end of C2 (Figs. 5 and 6). Surprisingly, intact wild-type C2 always coexisted with the recombinant C2 containing the 3′ end of T2 RNA (designated C2-T2). It turns out that all six isolates contained C2-T2 and its T2 3′-terminal region had the same sequence. Therefore, it appeared that the apparent T1C2C3 isolate actually had become possible quadripartite, with four RNAs, T1, C2, C2-T2, and C3, constituting the hybrid virus (Fig. 6*A*). Hereafter, to be specific, we use T1C2(C2-T2)C3 to describe the hybrid virus instead of T1C2C3. The sequencing results revealed that RNA–RNA recombination had occurred at a very precise position, so that the 5′ end (nt 1–2,908) of C2 RNA had recombined with the 3′-terminal 320 nt of the T2 RNA (positions 2754–3074), with a two-base (UA) insertion separating the chimeric regions. To confirm that T2 is not detected and C2-T2 was newly generated, we performed extensive RT-PCR experiments and Northern blot analyses using viral RNAs from the purified virus of one T1C2(C2-T2)C3 isolate, which had been obtained by single lesion transfer. As shown in Fig. 5*A*, combination of a primer specific to the 3′ end of T2 (3T2S-3) and a primer specific to the 5′ end of C2 (CY2T7) or to the internal sequence of C2 (C5–2068) amplified DNA fragments with expected sizes for C2-T2, which are about 180 nt longer than the corresponding C2 fragments. On the other hand, combination of the primers for the T2 5′ region resulted in no DNA amplification, whereas the 289-nt long 3′-terminal region was amplified with the two primers for the T2 3′-terminal region, suggesting that for T2 only the 3′-terminal region exists in the RNA preparation (Fig. 5*A*). Furthermore, T2 was not detected in T1C2(C2-T2)C3 by Northern blot, and thus it is likely that T2 was eliminated from the hybrid virus (Fig. 5*B*). To test for

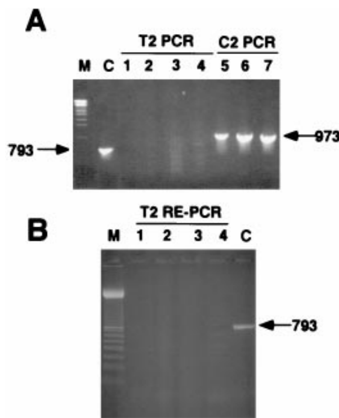

FIG. 3. Detection of T2- and C2-specific sequences from the progeny T1T2C2C3 isolated after single lesion transfer. (*A*) Agarose gel electrophoresis of segment-specific RT-PCR products amplified from viral RNAs isolated after two passages of single lesion transfer in *N. tabacum*. Lanes 1–4 contain RT-PCR products from four independent isolates of T1T2C2C3 for detection of TAV RNA 2 (T2 PCR). Lanes 5–7 contain PCR products for CMV RNA 2 (C2 PCR) from the RNAs used for lanes 2–4, respectively. Lane C contains a positive control of a 793-bp T2-specific DNA fragment. (*B*) Reamplification of PCR products (RE-PCR) using the same primer pair as in *A* and each DNA (2 μl out of 100 μl) from the samples of lanes 1–4 in *A*. Lane C contains a positive control for T2 as used in *A*. Note that there is no amplification of T2-specific DNA even after reamplification of PCR products generated in *A*. Lane M is a 100-bp DNA ladder.

evolution of T1C2(C2-T2)C3, we conducted inoculation experiments using infectious transcripts synthesized from the cDNA clones of the progeny RNAs of T1C2(C2-T2)C3. The plants (*N. benthamiana*) inoculated with the transcripts of T1+C2+(C2-T2)+C3 showed systemic symptoms in 3 of 10 plants. One of the three progeny isolates was purified after three passages through *N. benthamiana*. Full-length clones were obtained for the viral RNAs 1 and 2 and the sequences of four independent clones for each RNA were confirmed. The results showed that the four kinds of RNAs of T1, C2, C3, and C2-T2 existed in the preparation, but that the 3′-terminal region of C2-T2 contained two point substitutions and one deletion (Fig. 6*B*); the other progeny viral RNAs had the sequence of the transcripts from which they were derived. During the viral evolution, the 3′-terminal region may still undergo sequence changes.

New Biological Property of Evolved Virus. We also found that the new hybrid replicase complex acquired new properties, which we think are significant in the evolution process. Among these we determined that T1C2(C2-T2)C3 is able to support efficient replication of a CMV satellite RNA (Y-satRNA) that cannot be replicated by V-TAV (Fig. 7). This demonstrates that the interspecific hybrid replicase has been altered in its

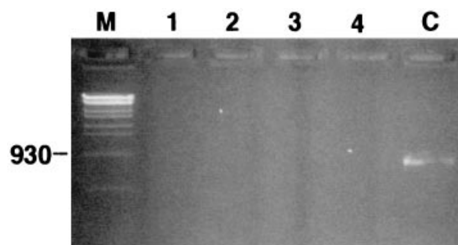

FIG. 4. Detection of C1-specific sequence by RT-PCR from the progeny C1T1T2C3 isolated after single lesion transfer. Lane C contains a positive control for C1 (822 bp). Lane M is the *Sty*I-cut λ DNA. Lanes 1–4 contain the RT-PCR products prepared from four independent isolates. Note that no DNA amplification was detected in the sample lanes.

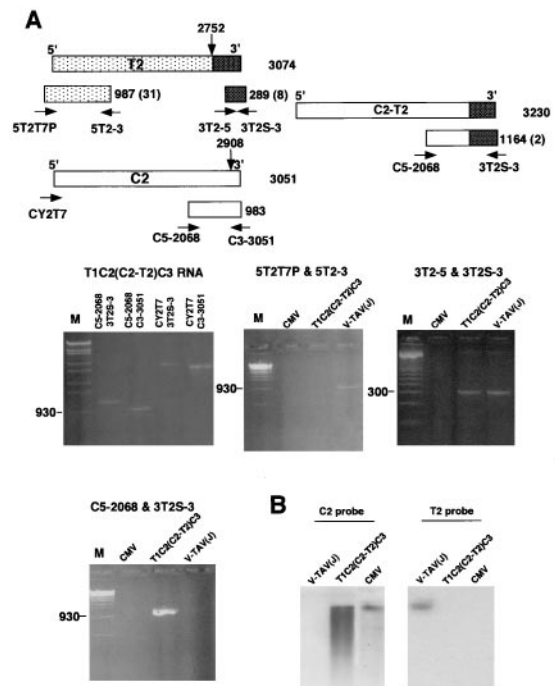

FIG. 5. Detection of C2-T2 and T2 by RT-PCR from the progeny RNAs after T1T2C2C3 inoculation. (*A*) RT-PCR products using primer pairs shown at top. Primers are: CY2T7 (5′-CCGGATCCATTAATACGACTCACTATAGTTTATTTACAAGAGCG-3′, corresponding to T7 promoter + nt 1–17 of C2), C3–3051 (5′-TGGTCTCCTTTTGGAGGCC-3′, hybridizing to nt 3051–3033 of C2), 5T2–3 (5′-GCTTGTAGTAGATCTACAGTC-3′, hybridizing to nt 956–936 of T2), C5–2068 (5′-GTTCCAGATCCATTGCG-3′, corresponding to nt 2068–2084 of C2), and 3T2–5 (5′-GTCTGAGTTGGTAGTATTGC-3′, corresponding to nt 2793–2812 of T2). The primers of 5T2T7P and 3T2S-3 have been described. The estimated sizes of the PCR products are indicated at the right of each box representing the PCR products. The number of nonviral bases derived from the primers is indicated in parentheses. The cross-hatched box represents the 3′-terminal region that was fused to C2. Marker lanes (M) contain a DNA ladder of *Sty*I-digested λ DNA or a 100-bp DNA ladder (Right, middle row). (Left, middle row) RT-PCR products that were amplified from T1C2(C2-T2)C3 RNAs prepared from the purified virus with the primer pairs indicated for each lane. The three remaining figures show RT-PCR products that were amplified from CMV, T1T2(C2-T2)C3, and V-TAV(J) RNAs by using the primer pairs as indicated. (*B*) Northern blot analysis of C2 and T2. Extracted RNAs from the purified viruses were subjected to electrophoresis in a 1.5% denaturing agarose gel, blotted onto a nitrocellulose membrane, and hybridized either with C2 probe or T2 probe.

recognition specificity for cis-acting elements required for replication of the CMV satRNA.

Interaction Between TAV 1a Protein and CMV 2a Protein. To determine whether the 1a protein from TAV and the 2a protein from CMV can interact, we performed yeast two-hybrid experiments. Since several experiments suggested that 1a and 2a interactions are required for RNA replication (5), we expected some interaction to occur between the 1a and 2a proteins of T1C2(C2-T2)C3. Fig. 8 shows that the polymerase (2a) and the helicase (1a) subunits from the T1C2(C2-T2)C3 isolate could interact and make a hybrid complex, whereas the 1a subunit from V-TAV(J) failed to interact with the CMV 2a subunit. Nevertheless, as estimated from the β-galactosidase activity, the degree of interaction between 1a from T1C2(C2-T2)C3 and CMV 2a was only one-half to one-third that of the homologous CMV 1a and 2a interaction (Fig. 8). Sequence analyses revealed two amino acid changes (at positions 342 and 521, V to I and G to V, respectively) in the 1a subunit from the T1C2(C2-T2)C3 isolate, suggesting that during T1C2(C2-

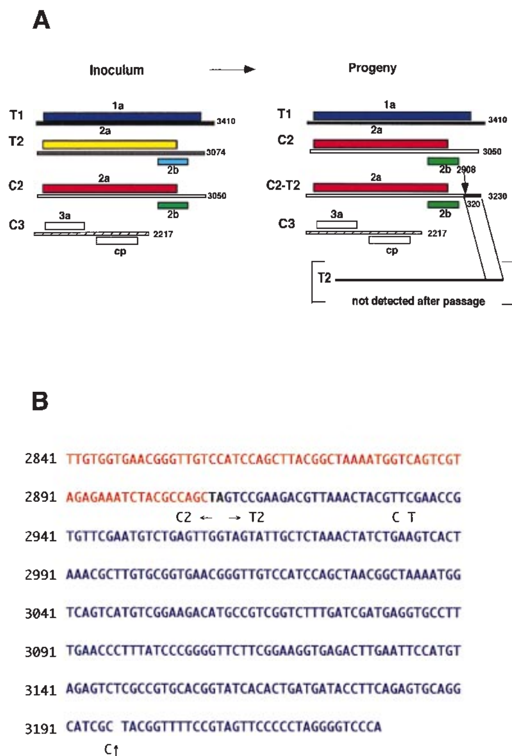

FIG. 6. Genome organization of pseudorecombinants. (*A*) Schematic representation of the genomic structure of T1C2(C2-T2)C3, which is thought to have been generated after several host passages or single lesion transfers. Solid boxes indicate 1a, 2a, or 2b proteins. The 3′-terminal 320 bases of T2 were recombined with C2 at position 2908 of C2 (with two inserted intervening bases separating the C2-T2 segments) to generate the 3,230 nt long C2-T2 segment. (*B*) Nucleotide sequence around the junction between C2 and T2 in the C2-T2 cDNA clone that was obtained from the progeny viral RNAs after inoculation of the infectious transcripts of T1+C2+(C2-T2)+C3. (Top line) New sequence of the 3′-terminal region of the progeny T2-C2; nucleotide substitutions and deletions were indicated related to the original sequence.

T2)C3 evolution of 1a from V-TAV(J), compensatory amino acid changes occurred in T1a that permitted a productive interaction with C2a. In contrast, we detected no differences between the 2a subunit of CMV and that of T1C2(C2-T2)C3. Our observations on the 1a–2a interactions are in good agreement with data presented for the bromoviruses, brome mosaic virus, and cowpea chlorotic mottle virus (4, 5). In these viruses, heterologous RNA 1–RNA 2 combinations failed to mediate RNA replication, suggesting that they are similar to CMV and TAV in that 1a and 2a interactions are required for RNA synthesis.

DISCUSSION

In this report we demonstrate that the genome structure of one of the hybrid viruses between V-TAV(J) and CMV-Y was probably quadripartite; the virus consists of T1, C2, C3, and C2-T2, a rearranged RNA between C2 and T2. We speculate that the appearance of C2-T2 is an initial event and is necessary to establish the quadripartite system. As indicated in Figs. 3, 5, and 6, T2 is selectively eliminated from the progeny pseudorecombinants because all of the virus preparations analyzed (a total of six) were quadripartite and each contained the same version of C2-T2. Because a recombinant RNA segment sometimes can outcompete authentic parental segments (14), C2-T2 must have been well adapted to the hybrid replicase complex. To our knowledge, interspecific exchanges

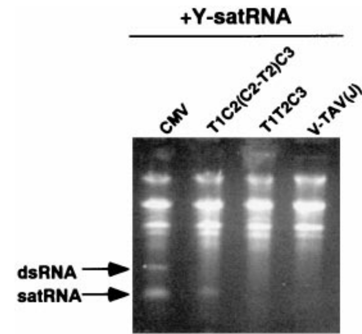

FIG. 7. Agarose gel electrophoresis of total RNA extracted from *N. benthamiana* inoculated with CMV-Y + Y-satRNA, T1C2(C2-T2)C3 + Y-satRNA, T1T2C3 + Y-satRNA, or V-TAV(J) + Y-satRNA. Total RNAs were extracted from the upper leaves for detection of Y-satRNAs as described by Masuta *et al.* (12). The positions of the single- and double-stranded Y-satRNAs are indicated. A discrete band of single-stranded Y-satRNA was observed in coinoculations with T1C2(C2-T2)C3, but the double-stranded Y-satRNA (dsRNA) was difficult to visualize in the same preparations. This phenomenon is similar to the observation of Moriones *et al.* (13), who found that in contrast to CMV, V-TAV does not induce accumulation of large amounts of double-stranded RNA.

of complete replicase components have not been reported in any virus, including animal viruses.

Based on the results we have presented, we propose a model whereby the evolution of T1C2(C2-T2)C3 occurred in several steps as follows. After inoculation of T1T2C2C3, a replicase complex initially supplied from T1 and T2 amplified small amounts of C2, as well as T1, T2, and C3. Then, a primary recombinant appeared that generated a precursor to C2-T2. In the 3′-terminal sequence, there is a well-conserved region (positions 2795–2889) between T2 and C2 (93% homology). Actually, crossovers occurred around this region, and the recombinant C2-T2 contains a sequence duplication of this region. Therefore, we think that aberrant homologous recombination by the template switching mechanism took place between T2 and C2 (15). As C2-T2 began to supply increasing amounts of C2a, the probability for productive interactions between C2a and T1a increased. Subsequently, T1 mutated to produce a 1a protein with the ability to bind more specifically and with higher affinity to C2a than to T2a. Then, the heterogeneous T1a-C2a replicase became dominant in the viral replication system, and T2 was eliminated after several host passages, whereas C2 survived. These events or permu-

Binding domain	Activation domain	Filter assay
C1a	none	-
C1a	C2a	+++
none	C2a	-
wt T1a	C2a	-
T1C2(C2-T2)C3 T1a	C2a	+
T1C2(C2-T2)C3 T1a	none	-

FIG. 8. Interactions between the V-TAV 1a protein and the CMV-Y 2a protein. Detection of positive blue colonies indicates *in vivo* protein–protein interactions. Note that the 1a protein from V-TAV(J) (wild-type, wt T1a) was not bound to the 2a protein from CMV-Y RNA 2 (C2a), whereas the 1a protein from T1C2(C2-T2)C3 was bound to C2a.

tations thereof led to stable establishment of the quadripartite virus, T1C2(C2-T2)C3.

Our results provide convincing evidence that pseudorecombination and recombination are important mechanisms of speciation in cucumoviruses, and these evolutionary mechanisms undoubtedly extend to other viruses. In this regard, it has been proposed that multipartite RNA viruses generally have an evolutionary advantage because of their ability to facilitate genetic exchange by reassortment (15). For example, in segmented animal RNA viruses, it is well known that pandemic strains of influenza A virus arise by genetic reassortment between avian and human viruses, although this does not involve interspecific reassortment (16). Numerous experimental findings favor the hypothesis that recombination between related viruses has played a major role in the evolution of plant and animal viruses (17–19). Recombination of bromovirus sequences observed under stringent selection pressure in several different experimental systems (20) provides additional support for this hypothesis. Sackey and Francki (21) extensively investigated the interactions between CMV and TAV in mixed infections and found that replication of RNAs 1 and 2 of each virus could occur in dual infections. Such coinfections could provide an opportunity for pseudorecombinants to survive for some period until RNA recombination generates chimeras with a selective advantage. Similar events could have occurred during evolution of a natural cucumovirus pseudorecombinant that was recently found to consist of genomic RNAs 1 and 2 from peanut stunt virus, another *Cucumovirus* member, and CMV RNA 3 (22).

In summary, our observations suggest that pseudorecombination and recombination between genomic RNAs of different virus strains, followed by segment reassortment, can favor maintenance of variability in viral genomes and facilitate evolution of new viruses with altered biological properties. During mixed infections of different viruses, genetic material may be rearranged or exchanged between species, as well as within species. Considering that the high error rate of viral replicase promotes potential recombination events, the appearance of hybrid replicase complexes between different virus species, as we have demonstrated in this report, could lead to the acquisition of new and unexpected virulence potential. However, as suggested by Fraile *et al.* (23), productive natural recombination events that could result in evolution of new virus derivatives would be expected to be relatively rare over limited periods of time in local populations. Nevertheless, over longer periods of time in intensively cropped agricultural areas that provide the potential for high levels of natural coinfection, new virus species should arise that could adapt to special environments and distinctive selection pressures.

We thank Dr. F. Garcia-Arenal for providing us with the uncloned V-TAV isolate and Dr. A. O. Jackson for his advise and critical reading of the manuscript. We also appreciate the competent technical assistance of Ms. M. Nishimura.

1. Palukaitis, P., Roossinck, M. J., Dietzgen, R. G. & Francki, R. I. B. (1992) *Adv. Virus Res.* **41,** 281–348.
2. Rao, A. L. N. & Francki, R. L. B. (1981) *Virology* **114,** 573–575.
3. Ahlquist, P. (1992) *Curr. Opin. Genet. Dev.* **2,** 71–76.
4. Dinant, S., Janda, M., Knorer, P. A. & Ahlquist, P. (1993) *J. Virol.* **67,** 7181–7189.
5. Smirnyagina, E., Lin, N.-S. & Ahlquist, P. (1996) *J. Virol.* **70,** 4729–4736.
6. Li, S.-F., Onodera, S., Sano, T., Yoshida, K., Wang, G.-P. & Shikata, E. (1995) *Ann. Phytopathol. Soc. Jpn.* **61,** 381–390.
7. Takahashi, Y., Takahashi, T. & Uyeda, I. (1997) *Virus Genes* **14,** 235–243.
8. Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N. & Takanami, Y. (1991) *Virology* **183,** 106–113.
9. Moreno, I. M., Bernal, J. J., de Blas, B. G., Rodríguez-Cerezo, E. & García-Arenal, F. (1997) *Mol. Plant-Microbe Interact.* **10,** 171–179.
10. Shi, B.-J., Ding, S.-W. & Symons, R. H. (1997) *J. Gen. Virol.* **78,** 1181–1185.
11. Salánki, S., Carrère, I., Jacquemond, M., Balázs, E. & Tepfer, M. (1997) *J. Virol.* **71,** 3597–3609.
12. Masuta, C., Hayashi, Y., Wang, W. Q. & Takanami, Y. (1990) *Ann. Phytopathol. Soc. Jpn.* **56,** 207–212.
13. Moriones, E., Díaz, I., Rodrígues-Cerezo, E., Fraile, A. & García-Arenal, F. (1992) *Virology* **186,** 475–480.
14. Fernández-Cuartero, B., Burgyán, J., Aranda, M. A., Salánki, K., Moriones, E. & García-Arenal, F. (1994) *Virology* **203,** 373–377.
15. Lai, M. M. C. (1992) *Microbiol. Rev.* **56,** 61–79.
16. Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K. F., Kawaoka, Y. & Webster, R. G. (1994) *J. Gen. Virol.* **75,** 2183–2188.
17. Robinson, D. J., Hamilton, W. D. O., Harrison, B. D. & Baulcombe, D. C. (1987) *J. Gen. Virol.* **68,** 2551–2561.
18. Strauss, J. H. & Strauss, E. G. (1994) *Microbiol. Rev.* **58,** 491–562.
19. Gibbs, M. J. & Cooper, J. I. (1995) *Virology* **206**, 1129–1132.
20. Bujarski, J. J., Nagy, P. D. & Flasinski, S. (1994) *Adv. Virus Res.* **43,** 275–302.
21. Sackey, S. T. & Francki, R. I. B. (1990) *Physiol. Mol. Plant Pathol.* **36,** 404–419.
22. White, P. S., Morales, F. & Roossinck, M. J. (1995) *Virology* **207,** 334–337.
23. Fraile, A., Alonso-Prados, J. L., Aranda, M. A., Bernal, J. J., Malpica, J. M. & García-Arenal, F. (1997) *J. Virol.* **71,** 934–940.