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Recombination between defective tombusvirus RNAs generates functional hybrid genomes

(defective interfering RNAs/plant virus/protoplasts)

K. ANDREW WHITE AND T. JACK MORRIS*

School of Biological Sciences, University of Nebraska, Lincoln, NE 68588-0118

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ABSTRACT **The tombusviruses represent a group of small icosahedral plant viruses that contain monopartite positive-sense RNA genomes. Tombusviruses are able to generate small replicating deletion mutants of their genomes (i.e., defective interfering RNAs) during infections via RNA recombination and/or rearrangement. To further study the process of RNA recombination and to determine whether tombusviruses were capable of trans-recombination, protoplasts were coinoculated with *in vitro*-generated transcripts of a nonreplicating 3′-truncated genomic RNA of cucumber necrosis tombusvirus and either replicative or replication-defective DI RNAs of tomato bushy stunt tombusvirus. After a 48-hr incubation, two dominant replicative chimeric recombinant viral RNA populations were detected that contained various large contiguous 5′ segments of the cucumber necrosis tombusvirus genomic RNA fused to 3′-terminal regions of the tomato bushy stunt tombusvirus defective interfering RNA. Some of the larger chimeric recombinants formed in protoplasts were able to systemically infect plants and induce wild-type symptoms. In addition, a functional chimeric genome was generated *in planta* after direct coinoculation of whole plants with the defective RNA components. These results indicate that (*i*) RNA recombination can occur relatively efficiently in single-cell infections, (*ii*) trans-recombination can occur with nonreplicating viral RNA components, and (*iii*) functional chimeric genomes can be generated via recombination. Possible mechanisms for the formation of the recombinants are proposed, and evolutionary implications are discussed.**

RNA recombination has been documented for several distinct groups of RNA viruses and is now recognized to play a central role in the evolution and repair of viral genomes (1). This process has been studied in some detail in only two RNA plant virus groups, the bromoviruses (2–7) and the carmoviruses (8–10). Despite such efforts, the precise mechanisms responsible for viral RNA recombination remain poorly understood due to the largely random nature of the process and the difficulty associated with identifying primary events before selection occurs. The most widely accepted model for viral RNA recombination is one where noncontiguous RNA segments are joined by an actively copying viral RNA replicase switching from one template to another where it resumes RNA synthesis (11). It has been suggested that base pairing between participating RNA molecules may facilitate this process (3, 12–14), and Nagy and Bujarski (5) have demonstrated that base pairing between two same-sense RNAs of brome mosaic virus can direct recombination to the region of complementarity. For turnip crinkle carmovirus, a specific intramolecular secondary structure in satellite RNA C has been identified as a target site for recombination (9).

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Tombusviruses represent a group of small icosahedral plant viruses containing positive-sense RNA genomes of ≈4.8 kb. Tomato bushy stunt virus (TBSV) and cucumber necrosis virus (CNV) represent two different tombusviruses that show ≈64% identity at the ribonucleotide level (15, 16). Despite this difference, CNV can efficiently replicate TBSV defective interfering (DI) RNAs (i.e., small replicating viral RNAs representing deletion mutants of the TBSV genome) in coinfections (14). Using CNV- and TBSV-derived RNAs, we initiated studies to examine the process of RNA recombination in tombusviruses. The coinoculation of protoplasts with *in vitro*-generated transcripts of a nonreplicating 3′-truncated CNV genome and either replicative or replication-defective TBSV DI RNAs resulted in the generation of various replicative CNV–TBSV recombinants, some of which were functional *in planta*. These results demonstrate that tombusviruses are capable of trans-recombination and that functional chimeric genomes can be generated from heterologous RNA components. Furthermore, our data indicate that, in this system, viral RNA recombination can take place with nonreplicating templates.

MATERIALS AND METHODS

Materials. Plasmid K2/M5 containing an infectious cDNA copy of CNV genomic RNA (gRNA) was provided by D. Rochon (Agriculture Canada Research Station, Vancouver) (17). DI-73 has been described (14). The following deoxyoligonucleotides were used in this study: 9, GGCGGCCCGCATGCCCGGGCTGCATTTCTGCAATGTTCC; 12, GATGGTCCGGAACGTCAGAACAGCC; 15, GGCGTCTAGATAATACGACTCACTATAGGGTGACTTGCGCTACCGTTGCTT; and 19, GGCGTCTAGACTTCAGCGTCTCATTGAGATGG (see Fig. 1).

Plasmid Construction and *in Vitro* Transcription. CNVΔ3′ was constructed by digesting K2/M5 with *Apa* I and *Sma* I, rendering the termini blunt with T4 DNA polymerase and religating the larger product to generate a 3′-terminal deletion of 257 nt in the viral sequence (see Fig. 1). DI-73Δ5′ was created by PCR amplification (14) of a portion of DI-73 using deoxyoligonucleotides 15 (complementary to residues 39–61 of DI-73 and containing a 5′-terminal T7 RNA polymerase promoter and *Xba* I site) and 9 (containing 5′-terminal *Sma* I and *Sph* I sites) (see Fig. 1). After digestion with *Xba* I and *Sph* I the amplified products were ligated into *Xba* I/*Sph* I-linearized pUC19. *In vitro* transcription was carried out on *Xba* I-digested CNVΔ3′, or *Sma* I-digested DI-73 or DI-73Δ5′, as described (14).

Protoplast and Plant Inoculation. Cucumber (var. Straight 8) cotyledon protoplasts were prepared and inoculated with

Abbreviations: TBSV, tomato bushy stunt virus; CNV, cucumber necrosis virus; DI, defective interfering; ORF, open reading frame; p.i., postinoculation; RCD, CNV–TBS VDI RNAs; sg, subgenomic; gRNA, genomic RNA.

*To whom reprint requests should be addressed.

viral RNA transcripts synthesized *in vitro* as described (14). Systemic host plants (*Nicotiana benthamiana*) were inoculated with *in vitro*-generated viral RNA transcripts or aliquots of total nucleic acid preparations from protoplasts as described (18).

RNA Analysis. Total nucleic acids were harvested from protoplasts (14) or plants (18) and separated in neutral 1.4% agarose gels. Northern blotting was performed by using randomly primed ^{32}P-labeled probe A (see Fig. 1; ref. 14) or ^{32}P 5′-endlabeled deoxyoligonucleotides (19).

Cloned cDNAs corresponding to junctions in recombinant CNV–TBS VDI RNAs (RCD)-2 (see *Results* section) were prepared by reverse transcription-PCR amplification (14) with deoxyoligonucleotides 9 and 12 (containing *Sph* I and *Xba* I sites, respectively). The products were subsequently digested with *Xba* I and *Sph* I and ligated into *Sph* I/*Xba* I-linearized pUC19. Junctions in RCD-1 recombinants (see *Results*) were cloned in a similar manner by using deoxyoligonucleotides 9 and 19. Cloned cDNAs were sequenced by the dideoxynucleotide chain-termination method. Some sequencing was performed by the Center of Biotechnology DNA Sequencing Facility (University of Nebraska, Lincoln).

RESULTS

Recombination Between a Nonreplicating Viral RNA Genome and DI RNAs Generates Replication-Competent Hybrid RNA Molecules. We wanted to determine whether a TBSV DI RNA, DI-73 (Fig. 1) could recombine with a nonreplicating 3′-truncated CNV viral genome, CNVΔ3′ (Fig. 1) to form an autonomously replicating hybrid RNA. *In vitro*-synthesized transcripts of CNVΔ3′ were inoculated either singly or together with DI-73 transcripts into protoplasts. Total nucleic acid was prepared 48 hr postinoculation (p.i.) and examined by RNA blot analysis (Fig. 2) with the 3′-terminal DI RNA probe A (see Fig. 1). A dominant viral RNA population of ≈3.0 kb (denoted RCD-2) and less abundant near-genome-length viral RNA species of ≈4.8 kb (denoted RCD-1) were identified in CNVΔ3′/DI-73 coinfections (Fig. 2, lanes 8–10) but were absent in single inoculations (Fig. 2, lanes 2 and 4). The recombinants could also be detected after several serial passages in protoplasts (data not shown), indicating that they were capable of sustaining RNA replication and suggesting

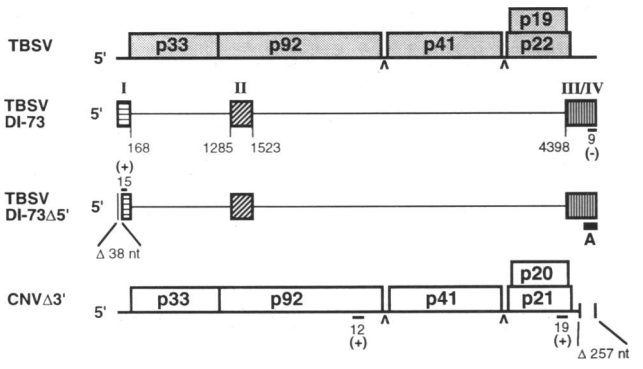

FIG. 1. Schematic representation of the viral RNAs used. TBSV gRNA is shown at top, and a nonreplicating CNV gRNA, CNVΔ3′ (containing a 3′ truncation of 257 nt) is shown at bottom. Coding regions in the gRNAs are symbolized by boxes, and the approximate molecular mass values (in kDa) of the encoded proteins are indicated (15, 16). Regions of the TBSV gRNA present in the DI RNAs (DI-73, and its 5′-truncated derivative DI-73Δ5′) are shown as hatched boxes with deleted intervening regions represented as lines. Deoxyoligonucleotides, depicted as small horizontal bars, are numbered and are of either plus (+) or minus (−) sense. The region from which probe A was derived is indicated by a thick horizontal bar, and carets indicate the initiation sites of subgenomic (sg) mRNAs.

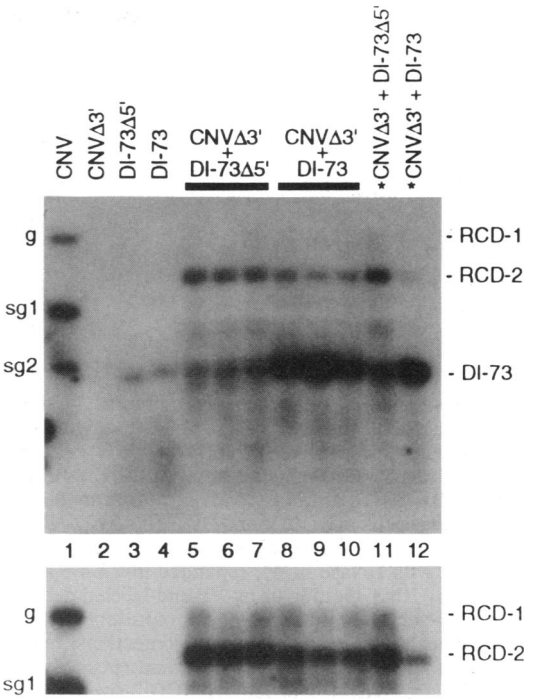

FIG. 2. RNA blot showing the accumulation of CNVΔ3′-DI RNA recombinants. Protoplasts were inoculated with various amounts of CNVΔ3′ and/or DI RNA transcripts, and total nucleic acids were harvested 48 hr p.i., separated in a 1.4% agarose gel, and analyzed by RNA blotting using ^{32}P-labeled probe A. Viral RNAs present in inoculum are indicated above lanes. Lanes: 1, inoculation with 2 μg of CNV transcript; 2–10, independent inoculations with 10 μg each of indicated transcripts; 11 and 12, inoculations with 10 μg of CNVΔ3′ and 30 μg of each DI RNA. Positions of gRNA (g), sg mRNAs, DI-73, and recombinant RNAs (RCD-1 and RCD-2) are indicated. Longer exposure of the upper portion of the blot appears below.

that some of the recombinants likely encoded the viral components (p33/92; see Fig. 1) of the RNA replicase.

To determine whether a high level of replication of DI-73 was essential for recombination, a replication-defective DI-73 derivative, DI-73Δ5′, was generated by deleting the 5′ terminal 38 nt (Fig. 1). RNA blot analysis of nucleic acids isolated from protoplasts coinoculated with DI-73Δ5′ and wild-type CNV gRNA detected no additional DI-73Δ5′ positive-strand accumulation over that observed in DI-73Δ5′-alone inoculations (data not shown). In some cases, however, we detected low levels of DI RNA-like molecules that were smaller than the input DI-73Δ5′, which may represent inefficiently replicating *in vivo*-modified forms of DI-73Δ5′.

Coinoculation of CNVΔ3′ and DI-73Δ5′ resulted in the accumulation of recombinant products (Fig. 2, lanes 5 to 7) similar in size to those observed in CNVΔ3′/DI-73 coinoculations, but the accumulation of the RCD-2 population was consistently greater in the former coinfections (Fig. 2; and in additional independent experiments). When higher concentrations of DI-73 or DI-73Δ5′ were used in the coinfections, the difference in recombinant accumulation between the two was even greater (Fig. 2, compare lanes 11 and 12). The higher levels of recombinant accumulation in coinoculations with DI-73Δ5′ may result from limited competition for RNA replicase by the replication-defective DI-73Δ5′. These results indicate that recombination between the two defective viral RNA components did, indeed, occur under our experimental conditions.

Recombinants Are Infectious *in Planta*. To determine whether any of the recombinants generated in protoplasts could infect whole plants, portions (one-half) of the total

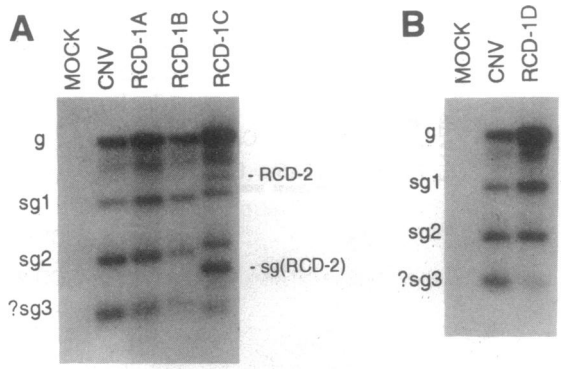

FIG. 3. RNA blots of infectious recombinant viral RNAs in plants. (*A*) Analysis of total RNA from plants that were inoculated with total nucleic acids isolated from protoplasts inoculated with either CNVΔ3′ and DI-73Δ5′ (RCD-1A and RCD-1C) or CNVΔ3′ and DI-73 (RCD-1B). (*B*) Total RNA isolated from plants directly inoculated with 10 μg each of CNVΔ3′ and DI-73Δ5′ (RCD-1D). For both *A* and *B*, total nucleic acids were prepared from apical symptomatic leaves, treated with DNase I (19), separated in a 1.4% agarose gel, and analyzed by RNA blotting using ^{32}P-end-labeled deoxyoligonucleotide 9. Lanes labeled mock and CNV represent RNAs isolated from mock-inoculated and wild-type CNV infections, respectively. Positions of authentic viral RNAs (g and sg) and a possible third small sgRNA (?sg3) are indicated at left. A RCD-2-sized RNA and its sgRNA are indicated to the right in *A*.

nucleic acids isolated from coinoculated protoplasts were inoculated onto systemic host plants (*Nicotiana benthamiana*). Apical necrosis, typical of wild-type infections, developed in 3 of the 10 inoculated plants (one from a CNVΔ3′/DI-73 infection and two from CNVΔ3′/DI-73Δ5′ infections) ≈8–10 days p.i. Viral RNAs with sizes similar to those of wild-type gRNA and sg mRNAs were identified in Northern blots of RNA isolated from the systemically infected plants (Fig. 3*A*), suggesting the presence of functional recombinant genomes. There were, however, noticeable decreases in the electrophoretic mobilities of sg mRNA 2 for RCD-1A–RCD-1C (Fig. 3*A*), indicative of 3′ modifications. In one of the infections (RCD-1C), a RCD-2-sized RNA and a smaller viral RNA, likely representing a sg transcript from the RCD-2-sized RNA, could also be identified (Fig. 3*A*). No viral RNAs could be detected in plants inoculated with a mixture of nucleic acids isolated from protoplasts that were inoculated singly with either CNVΔ3′ or DI-73Δ5′ (data not shown), suggesting that the infectious viral RNAs were generated in the protoplasts.

To test whether functional recombinants could be formed *in planta*, 14 *N. benthamiana* plants were each directly coinoculated with *in vitro*-generated transcripts of CNVΔ3′ (10 μg) and DI-73Δ5′ (10 μg). Seven days p.i. one of the inoculated plants showed systemic symptoms typical of a CNV infection. The standard viral RNA complement was identified in a total RNA preparation from the infected plant (RCD-1D; Fig. 3*B*). In addition, virus particles isolated from tissue infected with any of the recombinants (RCD-1A through RCD-1D) were infectious when inoculated onto healthy plants.

Structure of RCD-1 and RCD-2 Recombinant RNA Molecules. The size of the RCD-1-like recombinants detected in plants and their ability to spread and be encapsidated suggested that they represented hybrids containing a contiguous 5′ portion of the CNV genome encoding functional coat (p41) and movement (p21) proteins (17) joined to a 3′ portion of the TBSV DI RNA. Reverse transcriptase-PCR using deoxyoli-

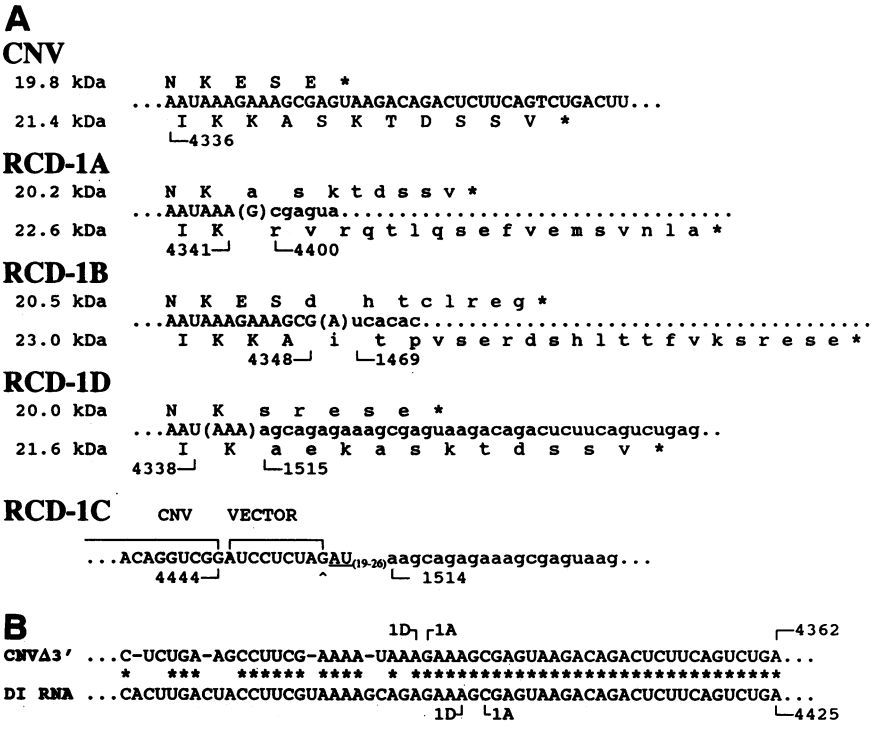

FIG. 4. Junctions in RCD-1 recombinants. (*A*) For comparison, a portion of the wild-type CNV nucleotide sequence and its encoded amino acids for p20 (19.8 kDa) and p21 (21.4 kDa) is shown at top. In the recombinant junctions below, nucleotides and encoded amino acids contributed by CNVΔ3′ are in uppercase letters, whereas those contributed by the DI RNA are in lowercase letters. The new predicted molecular masses of the proteins encoded by the recombinants are shown at left. The nucleotides in parentheses could be contributed by either CNVΔ3′ or the DI RNA, and junction coordinates in CNVΔ3′ (*Left*) and the DI RNA (*Right*) are given under the sequences (15, 16). In RCD-1C the caret indicates the predicted 3′ end of CNVΔ3′ (which includes nine vector-derived nucleotides), and the underlined residues represent nontemplated nucleotides. The sequences shown for RCD-1A–RCD-1D were each determined from 14 to 16 individual cloned cDNAs. (*B*) Alignment of plus-sense sequences of CNVΔ3′ and the DI RNA in the region of crossovers. Positions of identical composition are indicated by asterisks, and left and right junctions in RCD-1A and RCD-1D (1A and 1D) are indicated above and below the sequence, respectively.

Table 1. Junctions in RCD-2 recombinants

CNVΔ3′+DI-73		CNVΔ3′+DI-73Δ5′	
5′	3′	5′	3′
2637*	4402	2610	1499
2616	1500	2625	1515
2608	1500†	2627*	4403
2605	4403	2623*	1515†
2633*	1513	2626*	1518
2615	1499	2626	1479
2628	4398	2623*	1513
2625*	1513	2640*	1513
2624	1465	2630*	1501
2629	4398	2627*	4399
2633*	1523	2618*	1476
2650*	4407	2621	4399
2626	1469	2621*	1502
2643	1469	2628*	4399
2625*	4399†	2630	1467
2624	1521	2626	1472
		2627*	4399
		2626	1511
		2621	4399

Junctions were determined by sequencing cloned cDNAs from four independent coinfections of either CNVΔ3′+DI-73 or CNVΔ3′+DI-73Δ5′. The coordinates given correspond to published gRNA sequences (18, 19) and define the CNVΔ3′ residue at the left junction (5′) and the DI RNA residue at the right junction (3′).
*Ambiguous residues at the junction were placed with the 5′ junction.
†Two clones contained this junction.

gonucleotides 9 and 19 amplified products of ≈500–600 nt that were not amplified from mock-inoculated plants. Sequence analysis of these cloned partial cDNAs mapped the upstream junction sites to the 3′ end of the p20/p21 overlapping open reading frames (ORFs) in CNVΔ3′ [RCD-1A, -1B, and -1D; Fig. 4A] or at the predicted 3′ terminus of the CNVΔ3′ transcript [RCD-1C; Fig. 4A]. All of these CNV segments were joined to 3′ regions of DI RNA. The sequences at the junctions in RCD-1A, -1B, and -1D predict modifications of the ORFs encoding p20 and p21 such that authentic amino acids at their C terminus would be (*i*) deleted and replaced by different residues (RCD-1A and RCD-1B) or (*ii*) interrupted by a 2-aa insertion followed by resumption of the normal C-terminal sequence [RCD-1D; Fig. 4A]. The nucleotide sequence of RCD-1A predicted replacement of the p20 C terminus of CNV with that of p22 of TBSV. For RCD-1C there was no predicted modification of the C termini of p20 or p21, and the sequence indicated an upstream junction site at the predicted 3′ end of the CNVΔ3′ transcript, followed by an insertion of an adenosine residue and a poly(U) tract of 19–26 residues (Fig. 4A). The presence of nontemplated nucleotides at junctions in TBSV DI RNAs has been reported (14, 20) and may result from replicase stuttering (5, 8). The sequence data confirmed that the infectious viral RNAs represented derivatives of a recombination event between CNVΔ3′ and the DI RNAs. These junction sites are consistent with a proposed structure for the RCD-1 population, in which the 5′ terminal region of CNVΔ3′ is joined to a 3′ portion of the DI RNA (Fig. 5A).

RNA blot analysis and the ability of the recombinants to be serially passaged in protoplasts suggested that the smaller RCD-2 recombinants contained the 3′ terminus of DI-73 fused to a 5′ portion of CNVΔ3′ encoding the p33/92 replicase components. In an attempt to identify junction sites, reverse transcriptase-PCR was performed on total nucleic acid isolated from coinoculated protoplasts with primers corresponding to the 3′ end of the ORF encoding p92 (deoxyoligonucleotide 12; Fig. 1) and the 3′ terminus of DI-73 (deoxyoligonucleotide 9; Fig. 1). A prominent product of

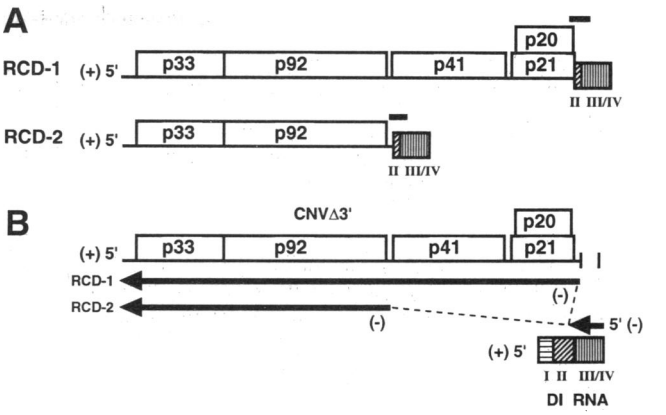

FIG. 5. (*A*) Schematic representations of the predicted structures of recombinant RCD-1 and RCD-2 molecules. The thick horizontal bars span the regions in which the junctions vary in different recombinants. (*B*) A possible replicase-driven mechanism to describe the formation of RCD-1 and RCD-2 recombinants. During synthesis of the DI RNA negative strand the replicase may switch from copying the plus-sense DI RNA template to copying the plus-sense CNVΔ3′ (as denoted by the bold arrows). The resulting negative-sense hybrid RNA could then serve as a template for the production of positive-sense recombinants that could be further amplified.

≈600 nt was amplified from coinoculations of either CNVΔ3′/DI-73 or CNVΔ3′/DI-73Δ5′, but no similar-sized products were amplified from mock or single inoculations or from mixtures of *in vitro* transcripts of the viral RNAs (data not shown). Sequence analysis of cloned cDNAs mapped the left junction sites to positions just downstream of the p92 ORF in CNVΔ3′ and mapped the right junction sites to either the 5′ portion of region III/IV or the 3′ portion of region II in the DI RNAs (Table 1). The predicted size of recombinants containing these junctions (≈3.0 kb) was consistent with the estimated size of the RCD-2 recombinant population identified by RNA blot analysis (Fig. 2). The positions of upstream junction sites in the RCD-2 populations were further confirmed by RNA blot analysis using CNVΔ3′-specific deoxyoligonucleotide probes flanking the predicted sites (data not shown). Taken together, these results agree with a predicted structure for the RCD-2 recombinants in which a contiguous 5′ segment of CNVΔ3′, which includes the p33/92 ORFs, is joined to various 3′ regions of DI-73 (Fig. 5A). The RCD-2 type recombinants were also capable of autonomous replication because *in vitro*-synthesized RNA transcripts corresponding to two different RCD-2 recombinants, as well as a gel-purified native RCD-2 population isolated from protoplasts, were able to accumulate when inoculated individually into protoplasts (data not shown). The RCD-2 transcripts were not systemically infectious when inoculated onto plants.

DISCUSSION

Formation of Recombinants. No evidence exists for the spread of viral infections between protoplasts; therefore, the detection of recombinants at 48 hr p.i. indicates that their generation must have occurred in a significant proportion of the individual protoplasts, suggesting that, in this system, the process may be a relatively frequent event. The RCD-2 recombinants that accumulated contained what could be considered the minimal sequence elements required for autonomous replication. Their high level of accumulation in protoplasts may, therefore, be primarily due to selection for efficient replication. As expected, the RCD-1 recombinants detected in plants maintained the virus-encoded proteins important (e.g., coat protein, p41) or critical (e.g., movement

protein, p21) for systemic accumulation. The sequences of the RCD-1 recombinants do suggest, however, that some modification of the 3′ terminus of the CNV genome and of the C termini of p20 and p21 can be tolerated.

The RCD-2 hybrids, as well as RCD-1B and RCD-1C, appeared to be generated by nonhomologous recombination (1) because no significant sequence identity was observed at the junctions. For RCD-1A and RCD-1D, the junctions mapped to a region of identity between CNVΔ3′ and the DI RNAs, but the crossovers were not precisely at comparable positions (Fig. 4*B*), indicative of aberrant homologous recombination (1). If a replicase-driven mechanism (11) is functioning in this system, the tracts of identity between CNVΔ3′ and the DI RNA, located to the right of the junctions, could act as guides for repriming of the incomplete nascent negative-strand DI RNA–replicase complex on CNVΔ3′ (Fig. 4*B*). Smaller discontinuous tracts of identity could also be identified to the right of most RCD-2 junctions (data not shown). A similar guide sequence-mediated mechanism has been implicated in the deletion of 3′ segments of TBSV DI RNAs (14).

Although CNVΔ3′ is nonreplicative, it could still act as a mRNA to allow for the production of the viral components (p33/92) of the RNA replicase (as shown for brome mosaic virus RNA 2; ref. 21) at levels sufficient to direct DI RNA replication. In a replicase-mediated event, the formation of recombinants would most likely involve template switching during negative-strand DI RNA synthesis (Fig. 5*B*). This mechanism is also consistent for recombinant formation involving the replication-defective DI-73Δ5′, which likely contains a functional negative-strand promoter. More recently, we have demonstrated that similar replicative recombinants are formed in coinoculations of CNVΔ3′ and nonreplicating transcripts corresponding to only region III/IV of DI-73 (our unpublished data). Therefore, although replicating molecules might be predicted to have a higher probability of participating in replicase-mediated recombination events, these results imply that replication of either participating RNA is not strictly required.

Evolutionary Implications. The theory of modular evolution suggests that RNA virus genomes can evolve through the exchange of blocks of genetic information (for review, see ref. 22). An essential element of this theory would be the ability of viral genomes to recombine in trans. We have demonstrated that functional monopartite plant virus genomes can be generated via trans-recombination between RNA components from two different viruses. The event led to the union of heterologous cis-acting and trans-encoded elements and resulted in the formation of functional chimeric viral genomes. This finding suggests that tombusviruses may be able to evolve via recombination and illustrates the potential for similar types of recombination events *in planta* in which replication-defective RNAs may be rescued via recombination with other defective RNAs containing the missing element(s). These data, therefore, provide additional support for the proposal that RNA plant viruses are able to evolve via RNA recombination. In addition, the observation that recombination can occur with nonreplicating RNAs also suggests that legitimate nonreplicating viral RNAs such as sg mRNAs, as well as cellular RNAs and transgenically expressed viral RNAs, may represent potential participants in RNA virus recombination.

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