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Changes in Locations of Crossover Sites over Time in *de Novo* Generated RNA Recombinants

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Recombinant RNAs generated in plants 3 weeks postinoculation with turnip crinkle virus (TCV) genomic RNA and an associated satellite RNA, sat-RNA D, have a majority of TCV crossover sites in a 24-nucleotide repeat (motif IIIA/IIIB) that forms part of a stable hairpin (Carpenter *et al.,* 1995, *J. Mol. Biol.* 245, 608–622). To determine if parameters other than nucleotide sequence in the crossover region affect junction site selection, recombinants were assayed at various times postinoculation of plants and protoplasts. Populations of recombinants became progressively shorter in plants and larger in protoplasts. Levels of inoculated transcript and age of the plant were not substantial factors in the shifts in crossover site locations. The two most commonly cloned recombinant species were not amplified to detectable levels in protoplasts, suggesting that these molecules are not viable templates for replication. These results suggest that recombination between sat-RNA D and TCV is a very frequent event, and populations of recombinants are likely generated *de novo* in each infected cell and represent the original recombinant molecules rather than progeny of such molecules. Therefore, factors other than simple selection for recombinants that are more fit to replicate are probably responsible for the differences in junction sites in populations of sat-RNA D/TCV recombinants.

INTRODUCTION

One of the driving forces in the evolution of viruses is RNA recombination, the replicase-mediated joining of discontiguous RNA segments during transcription of the viral genome (Holland *et al.,* 1982; Strauss and Strauss, 1988; Lai, 1992; Rao and Hall, 1993). Recombination between viral RNAs has been detected experimentally for an increasing number of plant, animal, and bacterial viruses including picornaviruses (Kirkegaard and Baltimore, 1986; Jarvis and Kirkegaard, 1992), alphaviruses (Weiss and Schlesinger, 1991), coronaviruses (Makino *et al.,* 1986; Banner *et al.,* 1990; Liao and Lai, 1992), nodaviruses (Li and Ball, 1993), pestiviruses (Meyers *et al.,* 1991), tombusviruses (White and Morris, 1994a,b), tobamoviruses (Beck and Dawson, 1990), bromoviruses (Nagy and Bujarski, 1992, 1993, 1996), and bacteriophages (Palasingam and Shaklee, 1992). Recombination is also implicated in the evolution of luteoviruses and tobraviruses, whose modern genomes appear to be an amalgam of RNAs of viral and possibly nonviral origin (Simon and Bujarski, 1994).

RNA recombination has been divided into three types, based on the analysis of junction sequences in *de novo* generated recombinants (Lai, 1992). Homologous recombination, which occurs at identical sites in homologous regions of either similar or dissimilar RNAs, is postulated to proceed by a processive, copy-choice mechanism (Jarvis and Kirkegaard, 1991). Aberrant (imprecise) homologous recombination, where the crossing over in homologous regions is inexact, has been experimentally shown to occur more frequently in brome mosaic virus (BMV) when the sites of recombination are near AU-rich sequences (Nagy and Bujarski, 1996). Nonhomologous recombination, which involves crossing over in unrelated regions, can be experimentally targeted to sequences upstream of heteroduplexes for BMV (Nagy and Bujarski, 1993) and may also involve short direct repeats near the parting and anchoring sites in poliovirus (Pilipenko *et al.,* 1995).

In addition to specific sequences and structures at the crossover site, replication competence (fitness) of the recombinant RNAs appears to play an important role in the amplification of recombinants associated with mouse hepatitis virus, BMV, and tomato bushy stunt virus (Banner *et al.,* 1990; Nagy and Bujarski, 1992; Rao and Hall, 1993; White and Morris, 1994a). In most studies of *de novo* generated recombinants, detection requires the generation of functional, amplifiable molecules, which can substantially bias the subset of recombination events subjected to analysis (Lai, 1992). For example, the location of crossover sites differed for mouse hepatitis virus recombinants depending on whether screening was based on the generation of functional genomes containing selectable markers from the parental templates, or if total recombinants in the population were assayed by polymerase chain reactions (Banner *et al.,* 1990; Banner and Lai, 1991). The location and frequency of recombination events can also be influenced by alteration of residues in the putative helicase domain of the BMV 1a protein; such alterations resulted in an increase in

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recombination frequency and imprecise crossover sites, as well as a shifting of the crossover site to weaker regions of the heteroduplex (Nagy *et al.,* 1995).

Nonhomologous recombination associated with the single-stranded, monopartite carmovirus turnip crinkle (TCV; 4054 bases) can occur between TCV-associated satellite (sat-) RNAs, and between the viral genomic RNA and the sat-RNAs (Cascone *et al.,* 1990; Zhang *et al.,* 1991). TCV supports the replication of several sat-RNAs that share either limited sequence similarity with the TCV genomic RNA such as sat-RNA D (194 bases) or are derived from past recombination between sat-RNA D and the 3′-end region of TCV (for simplicity, "TCV" will denote the genomic RNA), generating chimeric RNAs such as sat-RNA C (356 bases; Fig. 1A; Simon and Howell, 1986). Although recombinants have been found that contain sequence originating from both plus and minus strands of TCV (Carpenter and Simon, 1994), most recombination associated with TCV is thought to occur during plus-strand synthesis based on the similarity of sequences at the 3′ sides of crossover sites and the 5′ ends of the sat-RNAs, genomic RNA, or subgenomic RNAs (Cascone *et al.,* 1990; Zhang *et al.,* 1991; M. Farley and A. E. Simon, unpublished).

Nonhomologous recombination associated with TCV does not occur in regions of heteroduplex formation but rather is associated with stable hairpins that are formed from the sequence similar to the 5′ ends of the TCV-associated RNAs (Cascone *et al.,* 1993; Carpenter *et al.,* 1995). Detection of recombinants derived from sat-RNA D and mutants of sat-RNA C containing large deletions in the sat-RNA D-similar region required the generation of functional molecules, and 100% of recombinants contained crossover sites in a 5-nucleotide region at the base of a hairpin in sat-RNA C (Cascone *et al.,* 1990, 1993). Analysis of recombinants between wild-type sat-RNA D and TCV is based on a PCR amplification assay and does not require the generation of functional molecules. About 42% of junction sites in sat-RNA D/TCV recombinants were in a 6-nucleotide region at the base of a TCV hairpin located just downstream of the coat protein open reading frame (Fig. 1B; Carpenter *et al.,* 1995). Nearly all additional junction sites were within 225 bases upstream or 80 bases downstream of this hot spot location. Mutations that disrupted the TCV hairpin strongly affected the location of junction sites. For example, deletion of 12 nucleotides comprising one-half of the stem resulted in a shift of nearly every crossover site to the base of the next downstream hairpin, while alteration of 4 nucleotides at the base of the stem resulted in 81% of the crossover sites located upstream of the hot spot region (Carpenter *et al.,* 1995).

During analysis of recombination between sat-RNA D and TCV, we found that the origin of the viral RNAs in the inoculum (*in vitro*-synthesized transcripts versus plant-derived viral RNAs) affected crossover site selection (Carpenter *et al.,* 1995). We were therefore interested in determining if parameters in addition to the sequence and structure at crossover sites could influence junction site selection. We now report that under conditions without apparent genetic selection, the locations of junction

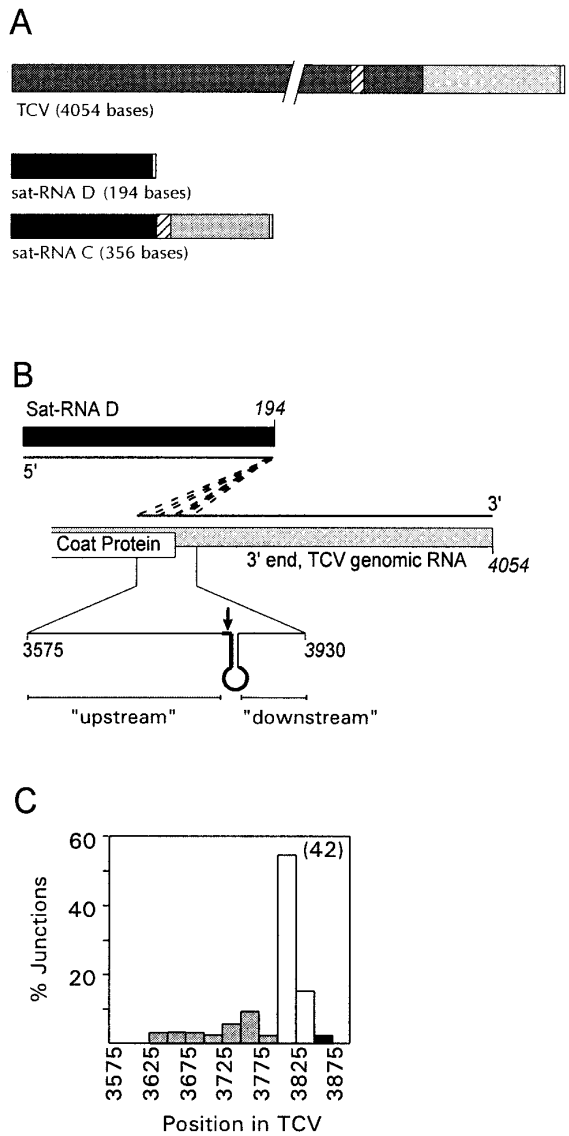

FIG. 1. Recombination between sat-RNA D and the 3′ region of TCV. (A) Schematic representation of sat-RNA D, sat-RNA C, and the 3′ end of TCV. Similar sequences are shaded alike. (B) Region of TCV involved in RNA recombination with sat-RNA D. Hatched lines denote locations of crossover sites in sat-RNA D and TCV. Thick line in the hairpin denotes the location of the motif IIIA/IIIB repeats (positions 3802–3850). Arrow denotes the location of the major hot spot region at the base of the hairpin within the motif IIIA sequence. "Upstream" junctions are crossover sites located between positions 3575 and 3800; "downstream" junctions are located between positions 3850 and 3930. (C) Junction profile of TCV crossover sites previously determined for recombinants derived from wild-type TCV and sat-RNA D transcripts 3 weeks postinoculation of turnip plants (data are from Carpenter *et al.,* 1995). Shaded bars, white bars, and black bar denote junctions that were located upstream, in motif IIIA/IIIB, and downstream, respectively. Forty-two percent of the crossover sites were between positions 3808 and 3815. Number of recombinants sequenced is shown in parentheses.

sites in TCV are affected by the stage of infection at which the recombinants are assayed.

MATERIAL AND METHODS

Production of *in vitro* transcripts and inoculation of plants and protoplasts

The construction of full-length infectious clones of the TCV isolate TCV-M (pT7TCVms; Oh *et al.,* 1995), sat-RNA D (pT7D; Carpenter *et al.,* 1995), CX9, and CX10 (Carpenter *et al.,* 1995) have been described. Transcripts that contained the precise ends of TCV or 7 additional 3′ nucleotides for sat-RNA D were synthesized using T7 RNA polymerase as previously described (Carpenter *et al.,* 1995). Three micrograms of each transcript was inoculated onto each of three 2-week-old turnip "Just Right" plants as previously described (Li *et al.,* 1989), unless otherwise indicated in the text. For turnip protoplasts, 3×10^6 protoplasts were inoculated with approximately 27 μg each of TCV and sat-RNA D transcripts (unless otherwise noted) as previously described (Carpenter *et al.,* 1995).

Detection of recombinants between TCV and sat-RNA D

Total RNA was prepared at various times postinoculation of plants (Simon *et al.,* 1988) or protoplasts (Carpenter *et al.,* 1995) and recombinants were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers homologous to positions 104–118 of sat-RNA D and complementary to positions 3943–3962 of TCV as described (Carpenter and Simon, 1994). PCR products were not detected in control reactions using the TCV- and sat-RNA D-specific primers and approximately 1 μg of genomic and sat-RNA D transcripts (data not shown).

Preparation of protoplasts

Turnip protoplasts were prepared from 1- to 2-in. leaves of uninoculated plants as previously described (Carpenter *et al.,* 1995) with the following modifications: enzyme incubation time was decreased to 1.5 hr; the levels of cellulase (10,500 U/g) and macerase (3500 U/g) were decreased to 0.75 and 0.0375% (w/v), respectively; centrifugation times were increased to 6 min; and protoplasts were washed only three times.

RNA gel blots

Two micrograms of total protoplast RNA per lane was denatured in formamide and then subjected to electrophoresis through a formaldehyde–1.2% agarose gel, as previously described (Carpenter *et al.,* 1995). After blotting to nitrocellulose, RNAs were sequentially hybridized to ^{32}P kinase-labeled oligonucleotides complementary to the plus strands of TCV (positions 4035–4054), sat-RNA C (positions 175–199), and sat-RNA D (positions 44–59). The blot was then hybridized to a ribosomal RNA probe labeled with [α-^{32}P]dCTP using a random primer kit (Boehringer Mannheim). Hybridizations were performed in buffer containing formamide at 34° (Carpenter *et al.,* 1995) and the final wash conditions were 0.1× SSC, 34°.

RESULTS

Location of junction sites in recombinants generated in plants over time

We previously reported that plants inoculated with wild-type TCV and sat-RNA D transcripts accumulated recombinants composed of full-length or nearly full-length sat-RNA D at the 5′ end joined to the 3′-end region of TCV. For recombinants assayed at 3 weeks postinoculation, about 42% of the crossover sites in the TCV sequence were in a 6-base sequence at the base of a stable hairpin that was partly composed of two 24-nucleotide imperfect repeats (TCV positions 3802–3850; Fig. 1; Carpenter *et al.,* 1995). These imperfect repeats, designated motif IIIA and motif IIIB, were similar in sequence to the 5′ end of the 1.45-kb subgenomic RNA that encodes the TCV coat protein (Zhang *et al.,* 1991). Of the remaining recombinants, 23% had crossover sites elsewhere in the TCV motif IIIA/IIIB region (white bars in Fig. 1C), 33% had upstream junctions (gray bars in Fig. 1C), while 2% had downstream junctions (black bar in Fig. 1C; references to "upstream" and "downstream" refer to crossover site location in relation to the motif IIIA/IIIB region). Recombinants generated using TCV containing mutations in the motif IIIA/IIIB region that did not disrupt the hairpin had very similar crossover sites, demonstrating the reproducibility of junction profiles in different populations of recombinants accumulating under similar conditions (Carpenter *et al.,* 1995).

To determine if junction sites in recombinant molecules could be affected by variables other than the sequence/structure at the junction sites, transcripts of sat-RNA D and TCV were co-inoculated onto three turnip seedlings and the location of junction sites in populations of recombinants was analyzed at 1 to 6 weeks postinoculation. Recombinants were amplified from the youngest partially expanded leaf at the time on each plant using RT-PCR and oligonucleotides homologous to plus strands of sat-RNA D and complementary to plus strands of TCV. Between 40 and 52 recombinant cDNA clones were analyzed for each time point with an approximately equal number of clones sequenced from each plant. To control for possible biased amplification by RT-PCR, only unique recombinants were scored, i.e., recombinants at specific time points within individual plants were scored only if they contained unique sat-RNA D and TCV crossover sites and/or contained a unique number or composition of nontemplate nucleotides at the junctions (mainly variable numbers of uracil residues). As previously reported (Carpenter *et al.,* 1995), over 90% of recombinant clones from a leaf were unique, and duplicate clones nearly always contained a crossover site at

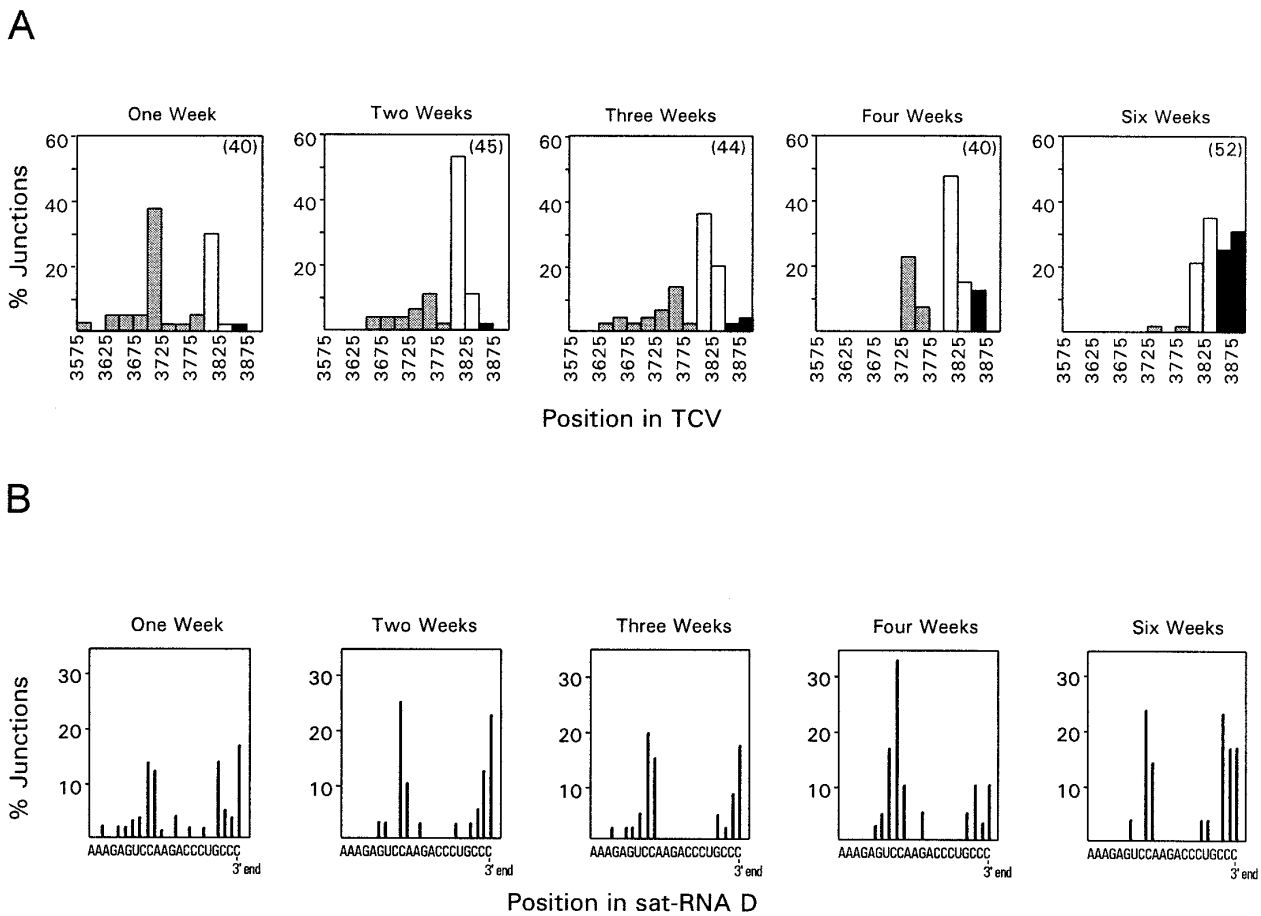

FIG. 2. Junction profiles for recombinant molecules analyzed at various times postinoculation of turnip plants. (A) Location of crossover sites in TCV. Shaded bars, white bars, and black bars denote junctions that were located upstream, in motif IIIA/IIIB, and downstream, respectively. Recombinants from three plants (inoculated at the same time) were analyzed for each time point. Number of recombinants sequenced for each time point is shown in parentheses. (B) Corresponding location of junctions in sat-RNA D. The 3′-end sequence of sat-RNA D is shown below each profile.

the major hot spot nucleotides in sat-RNA D and TCV (D_{181}/TCV_{3811}; subscript numbers denote junction points in the respective parental molecules).

TCV and sat-RNA D crossover sites found in recombinants isolated at different times postinoculation are shown in Fig. 2. The TCV crossover sites in recombinants assayed at 1 week postinoculation differed substantially from crossover sites in recombinants amplified later from the same plants (Fig. 2A). For example, 35% of the crossover sites at 1 week postinoculation were located between positions 3701 and 3725, about 100 bases upstream of the motif IIIA/IIIB region, while only 7% of recombinants had junctions in this region at 2 weeks postinoculation. This difference was considered to be significant by Fisher's exact test ($P = 0.001$). Only 26 of the nearly 1200 recombinants (1.9%) analyzed previously at 3 weeks postinoculation had junctions between positions 3701 and 3725 of TCV (Carpenter *et al.,* 1995). In addition, no recombinants recovered at 1 week postinoculation had junctions between positions 3735 and 3757, a region where between 15 and 25% of crossover sites were found between 2 and 4 weeks postinoculation.

Recombinants assayed at later times postinoculation had progressively fewer upstream crossover sites and an increasing number of downstream junction sites. The crossover site profile of recombinants isolated 3 weeks postinoculation was very similar to that previously found for wild-type sat-RNA D and TCV transcripts inoculated in a similar fashion and analyzed at 3 weeks postinoculation (see Fig. 1C). At 6 weeks postinoculation, 54% of the crossover sites were downstream from the motif IIIA/IIIB region compared with just 2% at 1 or 2 weeks postinoculation. Unlike the junction sites for TCV, the junctions in sat-RNA D did not differ substantially over time (Fig. 2B). Sat-RNA D junctions were mainly either 0–3 or 12–14 bases upstream from the 3′ end, as previously found (Carpenter *et al.,* 1995).

Sat-RNA D/TCV recombinants are not amplified to detectable levels in turnip protoplasts

Since populations of recombinants in plants contained progressively shorter TCV-derived sequence over time (i.e., the junction sites were further downstream), the

larger recombinant molecules produced early in the infection could have been parental templates for further recombination events, with selection accounting for the accumulation of smaller recombinants at later stages of infection. If selection for recombinants more fit to replicate or move through plants was responsible for the temporal differences in the locations of crossover sites in sat-RNA D/TCV recombinant molecules, then recombinants should have been able to multiply in whole plants and protoplasts, with smaller recombinants being more capable of accumulation than larger recombinants.

As previously reported, turnip plants inoculated with transcripts of two recombinant molecules, CX9 (D_{181}/TCV_{3811}) and CX10 (D_{179}/TCV_{3902}), did not accumulate either molecule to levels detectable by RNA gel blot analysis (Carpenter *et al.,* 1995). These two recombinants were selected for this analysis for the following reasons: (i) CX9 was the most prevalent chimeric RNA found in plants assayed at 2 to 3 weeks postinoculation; (ii) CX9 was found in all plants at 6 weeks postinoculation (Carpenter *et al.,* 1995, this report); (iii) CX9 was the most common molecule found in duplicate clones from the same leaf; (iv) 57% of the clones sequenced from plants inoculated with TCV containing a U to C alteration at position 3834 were CX9 (Carpenter *et al.,* 1995); (v) CX10 was the most prevalent recombinant RNA cloned from plants at 6 weeks postinoculation and was not detected at early times postinoculation. CX9 and CX10, therefore, should represent the most "fit" of the sat-RNA D/TCV recombinants if selection is a determinant in the accumulation of these molecules during infection.

To determine if CX9 and CX10 could be amplified to detectable levels in single cells, transcripts of CX9, CX10, and sat-RNA C (a recombinant molecule associated with our original TCV preparation that is able to be amplified in plants and protoplasts) were inoculated with wild-type TCV transcripts onto turnip protoplasts. RNA was extracted at 16 and 44 hr postinoculation based on previous results indicating that minimal levels of subviral RNA transcripts are present at 16 hr postinoculation with RNA levels increasing thereafter until at least 50 hr postinoculation (Zhang and Simon, 1994). As shown in Fig. 3, levels of sat-RNA C increased between 16 and 44 hr postinoculation. However, CX9 and CX10 levels decreased between 16 and 44 hr postinoculation, probably due to ongoing degradation of inoculated transcripts. These results indicate that CX9 and CX10 were amplified little, if at all, in protoplasts, suggesting that the appearance of these recombinants was not due to selection for molecules that were preferentially amplified. Therefore, the junction profiles shown in Fig. 2 likely represent populations of recombinants generated *de novo* in the leaves assayed.

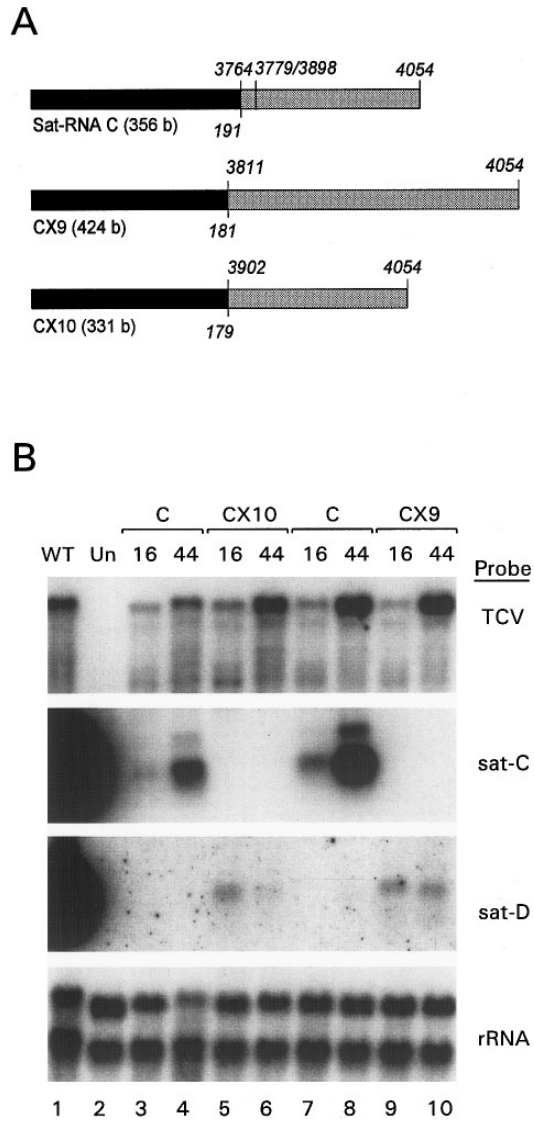

FIG. 3. Inefficient amplification of CX9 and CX10 in turnip protoplasts. (A) Schematic diagram of the recombinant RNAs. Similar regions are shaded alike. The regions in black and gray in sat-RNA C are 90 and 88% similar, respectively, to the corresponding region in CX9 and CX10. (B) Turnip leaf protoplasts were inoculated with full-length transcripts of TCV and sat-RNA C (C), CX9, or CX10, and RNA was extracted at 16 and 44 hr postinoculation. The sat-RNA D probe used for the detection of CX9 and CX10 does not crossreact with sat-RNA C because of sequence divergence in the region. WT, RNA extracted from turnip plants infected with wild-type TCV isolate TCV-M (contains the TCV genomic RNA and sat-RNAs C and D; Li *et al.,* 1989). Un, RNA from uninfected turnip protoplasts. Lanes 3–6 and 7–10 represent independent experiments.

Location of junction sites in recombinants generated in protoplasts over time

The above results suggested that factors other than genetic selection were responsible for the generation of different populations of recombinants over time. Since such factors might vary in plants and protoplasts over time, we determined the distribution of junctions in populations of recombinants accumulating in protoplasts. Turnip protoplasts were prepared from two-leaf turnip seedlings and then inoculated with sat-RNA D and TCV transcripts. Aliquots of the inoculated protoplasts were removed at 16 to 50 hr postinoculation, the RNA was extracted, and recombinants were amplified by RT-PCR,

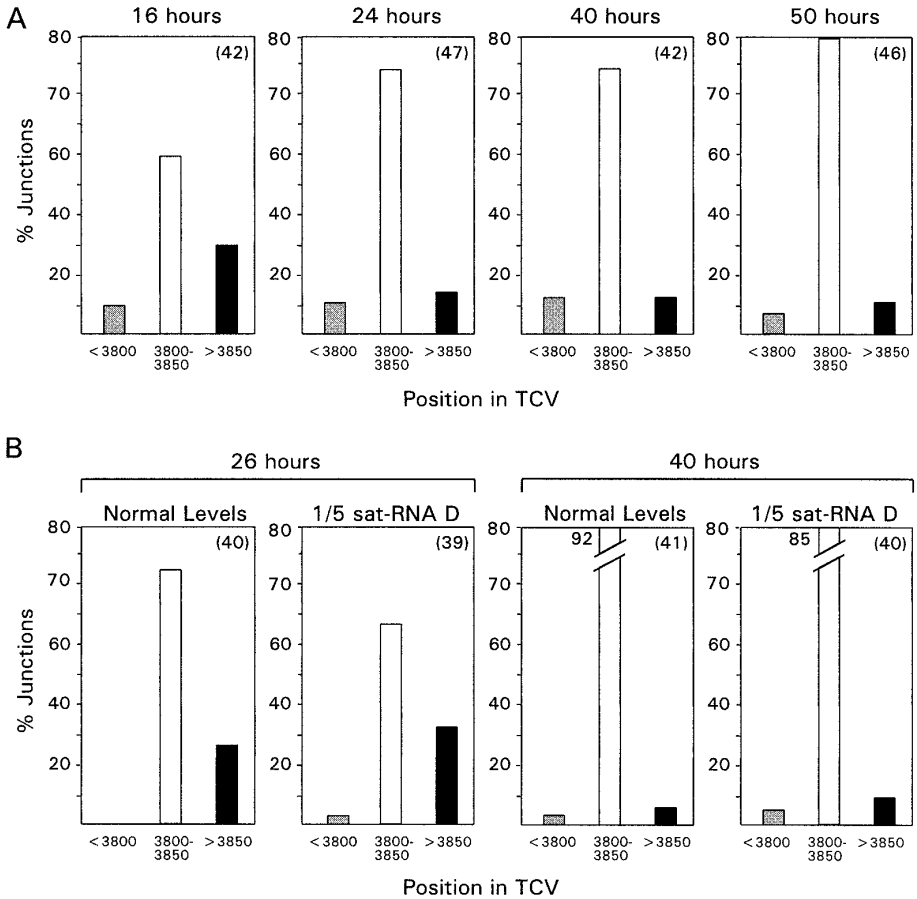

FIG. 4. Location of crossover sites in protoplasts over time. Protoplasts were inoculated with (A) normal levels (27 μg each) of transcripts of sat-RNA D and TCV or (B) 27 μg of TCV transcripts and 5.4 μg of sat-RNA D transcripts (1/5 sat-RNA D). Aliquots of protoplasts were removed at the time points indicated and recombinants cloned and sequenced. Shaded bars, white bars, and black bars denote junctions that were located upstream, in motif IIIA/IIIB, and downstream, respectively. Number of recombinants sequenced for each time point is shown in parentheses.

cloned, and sequenced. As with the recombinants isolated from plants, greater than 90% represented unique molecules based on the location of the junction site and the presence of nontemplate nucleotides (data not shown). Between 42 and 47 unique recombinants were analyzed for each time point. As shown in Fig. 4A, 30% of the recombinants had junctions downstream from the motif IIIA/IIIB region at 16 hr postinoculation, whereas only 11% of recombinants had downstream junctions at 50 hr postinoculation. This difference is considered significant by Fisher's exact test ($P = 0.032$). The decrease in the number of downstream junctions for recombinants accumulating in protoplasts over time is in contrast to our finding for whole-plant infections, where the percentage of downstream junctions increased over time (Fig. 2A). The decrease in the number of recombinants with downstream junctions at later times in protoplasts was accompanied by an increase in the number of junctions in the motif IIIA/IIIB region. The number of upstream junctions in the protoplast-derived recombinants did not vary significantly over time (9–12% for the four time points).

These results indicate that populations of recombinants in protoplasts increased in size over time. For the larger recombinants to be derived from the smaller recombinants (i.e., evolving to molecules more fit to accumulate), two crossover events would be required: one between a new sat-RNA D molecule and new upstream region of TCV and a second between the two TCV-derived sequences, generating the larger molecule. Since double recombination events leading to the generation of a recombinant are very rare (Carpenter *et al.,* 1995), it is more likely that the larger recombinants represent new single recombination events between sat-RNA D and TCV. Furthermore, differences in junction profiles are probably due to factors unrelated to the sequence/structure at the crossover site, since the TCV sequence is unlikely to vary significantly in the motif IIIA/IIIB region during the infection in protoplasts. To determine whether junction site selection could be altered by decreasing the amount of sat-RNA D transcripts used to inoculate the protoplasts, protoplasts were inoculated with normal levels of TCV transcripts and either normal levels of sat-RNA D or $\frac{1}{5}$ the normal levels of sat-RNA D. At 26 hr postinoculation, recombinants accumulating in protoplasts inoculated with normal levels of transcripts contained 28% downstream junctions and 72% motif IIIA/IIIB

junctions (Fig. 4B). At 40 hr postinoculation, the number of recombinants with downstream junctions decreased to 6%, with 91% of junctions in the motif IIIA/IIIB region and 3% upstream. The shift in junction sites from downstream to within the motif IIIA/IIIB region over time was similar to the shift found in the independent experiment shown in Fig. 4A. The junction profile for recombinants from protoplasts inoculated with $\frac{1}{5}$ the normal amount of sat-RNA D transcripts at the two time points did not vary significantly (according to Fisher's exact test) from protoplasts inoculated with higher levels of sat-RNA D. Recombinants at 26 hr postinoculation contained 31% downstream junctions, 66% motif IIIA/IIIB junctions, and 3% upstream junctions ($P = 0.8076$), while recombinants at 40 hr postinoculation contained 10% downstream junctions, 85% motif IIIA/IIIB junctions, and 5% upstream junctions ($P = 0.252$). Statistical analysis did suggest that the number of downstream junctions at 26 hr postinoculation varied significantly from the number of downstream junctions at 40 hr postinoculation for both inoculums ($P < 0.001$ for normal sat-RNA D levels and $P = 0.0254$ for $\frac{1}{5}$ sat-RNA D levels). These results suggest that the amount of input RNA used in this experiment did not alter the junction profile of the recombinants.

Effect of plant age on junction profiles

One possible factor that could influence junction site selection is a difference in replication-associated host factors present in leaves of older plants compared with younger plants. To examine this possibility, plants were either inoculated at the normal two-leaf stage with both TCV and sat-RNA D transcripts and assayed 6 weeks later (analogous to conditions used for the 6-week time point shown in Fig. 2) or inoculated with TCV and sat-RNA D transcripts 5 weeks after the two-leaf stage, and recombinants assayed 1 week later (Fig. 5A). These conditions result in recombinants being assayed in leaves of plants of the same age. As shown in Fig. 5B, 25% of recombinants assayed 6 weeks postinoculation had downstream junctions while 17% had upstream junctions. However, only 13% of recombinants assayed in plants of the same age at 1 week postinoculation had downstream junctions while 44% contained upstream junctions. The difference in the percentage of upstream junctions in recombinants generated by the two inoculation conditions was considered significant by Fisher's exact test ($P = 0.0037$). The junction profile of recombinants assayed 1 week postinoculation of the older plants more closely resembled the junction profile of recombinants assayed at 1 week postinoculation of younger plants (see Fig. 2A), indicating that the length of time of infection rather than plant age is influencing junction site selection.

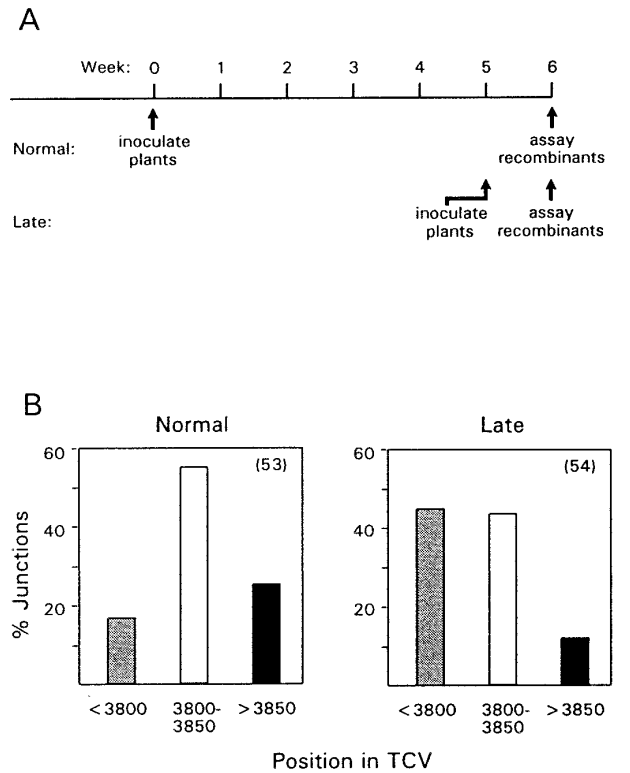

FIG. 5. Effect of plant age on junction site selection. (A) Plants were inoculated either using normal conditions (at the two-leaf stage; Week 0) and recombinants assayed in the youngest partially expanded leaf 6 weeks later or under late conditions, where plants were inoculated 5 weeks after the "normal" plants and recombinants assayed 1 week later. (B) Junction site profiles for recombinants generated under normal and late conditions described in (A). Shaded bars, white bars, and black bars denote junctions that were located upstream, in motif IIIA/IIIB, and downstream, respectively. Numbers of recombinants sequenced for each time point are shown in parentheses.

DISCUSSION

In this report, we demonstrate that factors in addition to nucleic acid topology can influence the choice of junction sites in sat-RNA D/TCV recombinants. In infected plants, the population of recombinants gets progressively shorter over a 6-week period while in protoplasts, the population of recombinants over a 50-hr period gets larger. These opposite trends argue against selection for molecules more fit to replicate as the cause of the differences in junction site preference. In addition, the following current and previous results argue against selection pressure as a factor in the accumulation of a particular population of sat-RNA D/TCV recombinants: (i) the parental templates are fully functional wild-type viral RNAs and there is no apparent advantage for the generation of functional recombinants; (ii) virtually every recombinant generated in a single leaf from wild-type sat-RNA D and TCV contained a unique crossover site and these recombinants cannot all be the products of selection (Carpenter *et al.,* 1995; this report); (iii) inoculation of plants or protoplasts with transcripts of the most commonly generated recombinant species did not result in any detectable amplification of those species (Carpenter *et al.,* 1995; this report). Taken together, these results suggest that the generation of sat-RNA D/TCV recombi-

nants is a very frequent event leading to numerous molecules with unique junction sites. In addition, since the recombinants are apparantly not amplifiable, populations of recombinants are likely generated *de novo* in each infected cell and represent the original recombinant molecules rather than progeny of such molecules.

What possible factors could be influencing the changes in junction profiles? Using an *in vitro* system, DeStefano *et al.* (1992) demonstrated that the ratio of donor and acceptor templates, the level of deoxyribonucleotides, and the ratio of reverse transcriptase to template can affect the amount of strand transfer (i.e., processivity) of reverse transcriptase using templates derived from human immunodeficiency virus (HIV). Recombination frequency in poliovirus-infected HeLa cells was also influenced by the concentration of the acceptor template (Jarvis and Kirkegaard, 1992). While these factors may affect junction site selection in the TCV system, HIV and poliovirus recombine in a homologous fashion, unlike the nonhomologous recombination associated with TCV, and variations in crossover sites under different cellular or *in vitro* conditions were not determined for these viruses. Our results using $\frac{1}{5}$ of the normal level of sat-RNA D transcripts to inoculate protoplasts did not significantly affect the junction profile of the recombinants (Fig. 4B), although these results do not rule out a role for differences in template levels as a factor in crossover site selection. The junction profiles of recombinants from protoplasts at 26 hr postinoculation with either normal (0% upstream, 28% downstream) or $\frac{1}{5}$ of the normal (3% upstream, 31% downstream) level of sat-RNA D were more similar to each other than they were to the junction profile for recombinants generated in separately prepared protoplasts at the nearly analogous time point of 24 hr (11% upstream, 15% downstream; Fig. 4A). It is possible that different preparations of protoplasts have different metabolic states, which might affect such factors as the levels of ribonucleotides or uptake of RNA, which could be influencing junction site selection.

Junction profiles for TCV differed substantially between 1 and 6 weeks postinoculation (Fig. 2). However, the age of the plant was not the only factor contributing to the difference; the junction profile for recombinants at 1 week postinoculation of older plants was more similar to the junction profile for 1 week postinoculation of young plants than it was to the junction profile for recombinants at 6 weeks postinoculation but assayed in plants of the same age (Fig. 5). This result suggests that the stage of the infection is more important than the absolute age of the plant. We previously found that plants inoculated with total RNA from TCV-infected plants, instead of TCV transcripts synthesized *in vitro,* had a substantial and reproducible alteration in junction profiles at 3 weeks postinoculation (about 4% upstream, 30% downstream; Carpenter *et al.,* 1995). These junction profiles were more similar to those from transcript-inoculated plants at 6 weeks postinoculation than 3 weeks postinoculation (see Fig. 2A). Inoculation of plants with *in vivo*-synthesized TCV RNA normally results in faster symptom production (C. D. Carpenter and A. E. Simon, unpublished), which at 3 weeks may establish conditions similar to those at 6 weeks postinoculation of plants with transcripts. It is possible that different stages of infection may have different amounts of replicase and/or ratios of acceptor and donor templates, factors that may vary during a quicker establishment of infection, thereby influencing either the processivity of the TCV RNA-dependent RNA polymerase (if TCV is the donor template) or the selection of the internal reinitiation site (if TCV is the acceptor template).

It is not known why CX9 and CX10 are not amplified to detectable levels in plants and protoplasts, whereas the similar recombinant sat-RNA C is an excellent template in both systems. Studies using active RNA-dependent RNA polymerase extracts from infected plants have identified a stable hairpin in the 3′-terminal 29 bases of sat-RNA C as responsible for minus-strand synthesis (Song and Simon, 1995). While the TCV 3′ end contains a larger hairpin in this location, sat-RNA C with the 3′-end 100 bases of TCV is a viable molecule *in vivo* (H. Guan and A. E. Simon, unpublished). Minus strands of sat-RNA C contain two independent promoters for plus strand synthesis *in vitro* (C. Song *et al.,* unpublished), and both promoters are represented in CX9 and CX10. However, the 5′ region of sat-RNA C is only 90% similar to sat-RNA D and the 3′ region is only 88% similar to the corresponding region of TCV. Therefore it is possible that specific nucleotide differences impart biological activity on sat-RNA C. In addition, previous results have shown that the size of the subviral RNA associated with TCV plays an important role in ability to be amplified in plants and protoplasts (Li and Simon, 1991; Zhang and Simon, 1994). The larger and smaller sizes, respectively, of CX9 and CX10 when compared with sat-RNA C may therefore also negatively affect the activity of the templates.

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