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A map of the diversity of RNA3 recombinants appearing in plants infected with *Cucumber mosaic virus* and *Tomato aspermy virus*

Mélissanne de Wispelaere[1], Stéphane Gaubert[1,2], Séverine Trouilloud, Christophe Belin, Mark Tepfer*[,3]

Laboratoire de Biologie Cellulaire, INRA-Versailles, 78026 Versailles cedex, France

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Abstract

In order to better understand the role of recombination in creating the diversity of viral genomes that is acted on by selection, we have studied in detail the population of recombinant RNA3 molecules occurring in tobacco plants coinfected with wild-type strains of *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV) under conditions of minimal selection pressure. Recombinant RNA3s were observed in 9.6% of the samples. Precise homologous recombination predominated since it was observed at 28 different sites, primarily in six hot spots. Imprecise homologous recombination was observed at two sites, particularly within a GU repeat in the 5′ noncoding region. Seven of the eight aberrant homologous recombination sites observed were clustered in the 3′ noncoding region. These results have implications on the role of recombination in host adaptation and virus evolution. They also provide essential baseline information for understanding the potential epidemiological impact of recombination in transgenic plants expressing viral sequences.

Keywords: RNA virus; Recombination; Hot spot; *Cucumovirus*; CMV; TAV; Virus evolution

Introduction

Mutation, reassortment (in multipartite viruses), and recombination are considered to be the major sources of variability in RNA viruses (García-Arenal et al., 2001; Worobey and Holmes, 1999). Recombination can result in insertion of unrelated sequence elements, as well as in exchange, duplication, or deletion of existing viral sequence elements. RNA viral replicases apparently lack proof-reading ability, and as a consequence the frequency of mutations is much higher than in organisms with a DNA genome (Drake and Holland, 1999; Malpica et al., 2002). Within the population of sequence variants that constitute a viral quasi-species, recombination between molecules bearing deleterious mutations can thus play an essential role in creating subpopulations of viral RNAs bearing fewer mutations. In this sense, intra-quasi-species recombination contributes to viral genome stability (García-Arenal et al., 2001; Worobey and Holmes, 1999). Understanding the role of recombination in generating and eliminating variation in viral sequences is thus essential to understanding viral evolution and host adaptation.

The viral species studied here, *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV), belong to the genus *Cucumovirus*, family *Bromoviridae*. Like all members of this family, their genome is composed of three (+) sense RNAs, designated RNAs 1, 2, and 3 in decreasing order of size (for a review, see Palukaitis and García-Arenal, 2004). RNAs 1 and 2 code for subunits of the viral replicase (Hayes and Buck, 1990), while RNA3 encodes the movement protein (MP), and from its 3′-coterminal RNA4, the coat protein (CP). RNA4A, which corresponds to the 3′-terminal part of

* Corresponding author. Fax: +33 1 30 83 30 99.
E-mail address: Mark.Tepfer@icgeb.org (M. Tepfer).
[1] These authors have contributed equally to this work.
[2] Current address. Laboratoire de pathologie végétale, UMR 217, 16 rue Claude Bernard, 75005 Paris, France.
[3] ICGEB Biosafety Outstation, Via Piovega 23, 31050 Ca'Tron di Roncade, Treviso, Italy.

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RNA2, encodes a multifunctional protein implicated in long-distance movement of CMV in host plants, interference with posttranscriptional gene silencing, and with virus resistance mechanisms based on sequence-specific RNA degradation (Ding et al., 1995). Certain CMV and TAV strains are also thought to transcribe the additional subgenomic RNA5 from the 3′ noncoding region of RNA3 (Blanchard et al., 1996, 1997; Shi et al., 1997). Based on their serological and biological properties, as well as on their degree of sequence identity, two subgroups of CMV isolates can be clearly distinguished. The experiments described here were carried out with a subgroup II CMV strain, R-CMV (Jacquemond and Lot, 1981), and P-TAV (Salánki et al., 1994), whose RNA3 molecules share 63% sequence identity.

Although there have been several reports of CMV-TAV recombinant RNAs created in the laboratory, these have most often been observed well after the initial recombination event(s) occurred, generally after several passages (Fernández-Cuartero et al., 1994; Masuta et al., 1998; Suzuki et al., 2003). However, preliminary results have shown that recombination between wild-type strains of CMV and TAV can be observed in the inoculated leaves of plants infected with the two viruses, thus minimizing the effects of selection on the populations of recombinant molecules (Aaziz and Tepfer, 1999a). In order to better understand the role of recombination as a source of sequence variability, this strategy was extended here to map CMV-TAV recombination sites across all but the terminal nucleotides of RNA3 in doubly infected plants. This area of study is particularly pertinent for higher plants, in which infection by several viruses is frequently observed in the field and interspecific recombination is thought to have given rise to new plant virus taxa (Aaziz and Tepfer, 1999b).

Results

Specific amplification of the products of viral RNA recombination by RT-PCR

As described previously (Aaziz and Tepfer, 1999a), young tobacco plants were inoculated with P-TAV, and then 5 days later the same leaves were inoculated with R-CMV. This strategy of successive inoculation was necessary in order to consistently obtain plants that were infected with both viruses. Seven or 10 days after the second inoculation, the inoculated leaves were harvested. Recombinant RNA3 fragments were amplified by reverse transcription (RT) followed by PCR (RT-PCR) on total RNAs extracted from plants infected with both viruses using primers specific to recombinant molecules with an R-CMV 5′ region and a P-TAV 3′ region. The primers used are designated by a letter (R or T for R-CMV and P-TAV, respectively), followed by the nucleotide position on the viral genome of the 5′ end of the primer, and + or − depending on whether the primer sequence corresponds to the viral (+) or (−) sense. We have used three overlapping primer pairs (R6+/T611−, R544+/T1304−, and R1168+/T2147−) in order to detect recombinants across nearly all of RNA3. Preliminary experiments with primers R544+/T1304− had shown that this technique makes it possible to amplify rare recombinant molecules in plants infected with wild-type R-CMV and P-TAV (Aaziz and Tepfer, 1999a).

A serious potential technical problem when using RT-PCR to detect viral recombinants is the possibility for recombination to occur in vitro either by the reverse transcriptase (RTase), which is known to be able to change templates during cDNA synthesis (Negroni and Buc, 2001), or by the *Taq* polymerase, which can also produce recombinant DNA (Bradley and Hillis, 1997). In order to test for artefactual recombination in vitro by either enzyme, we have used as a negative control mixtures of total RNAs extracted from plants infected individually with only R-CMV or P-TAV. It has been proposed that degradation of the template RNA strand by the RNaseH makes it possible for the neoformed cDNA to base pair with another RNA template and thus favors a template switch by the RTase (Negroni and Buc, 2001). Consistent with the hypothesis that the RNaseH plays an important role in recombination, using all three primer pairs we have amplified in vitro recombination products from the negative control with certain RT-PCR protocols using an RTase that is RNaseH+, such as the “OneStep RT-PCR kit” by Qiagen (Fig. 1A). The amplification product was due to recombination by the RTase since when cDNA synthesis was performed separately on the two RNA samples, which were then mixed before PCR, no amplification product was observed (Fig. 1A, lane cCMV + cTAV). Surprisingly, this problem is not always associated with use of an RNaseH + RTase, since as described previously (Aaziz and Tepfer, 1999a) we have observed no recombination in vitro using a native RTase in a two-step RT-PCR protocol with primers R544 + and T1304−. With the other two primer pairs, we were able to overcome the problem of recombination in vitro by using the Invitrogen Superscript™ One-Step RT-PCR kit with Platinum® Taq, whose RTase has been mutated in order to inactivate its RNaseH activity (Fig. 1B). The propensity of an RNaseH + RTase to recombine in vitro has recently been exploited as a tool to create a collection of recombinant cucumoviral RNA3 clones (Fernandez-Delmond et al., 2004).

RNA3 recombination sites identified in plants coinfected with R-CMV and P-TAV

Three series of plants coinfected with R-CMV and P-TAV have been studied. Fig. 2 shows typical amplification profiles with the three primer pairs. Bands resulting from nonspecific amplification from the viral RNAs in singly infected plants were observed in all cases. Bands amplified from doubly infected plants that were not amplified from singly infected ones were considered to be due to potential recombinants, which was confirmed by sequencing clones

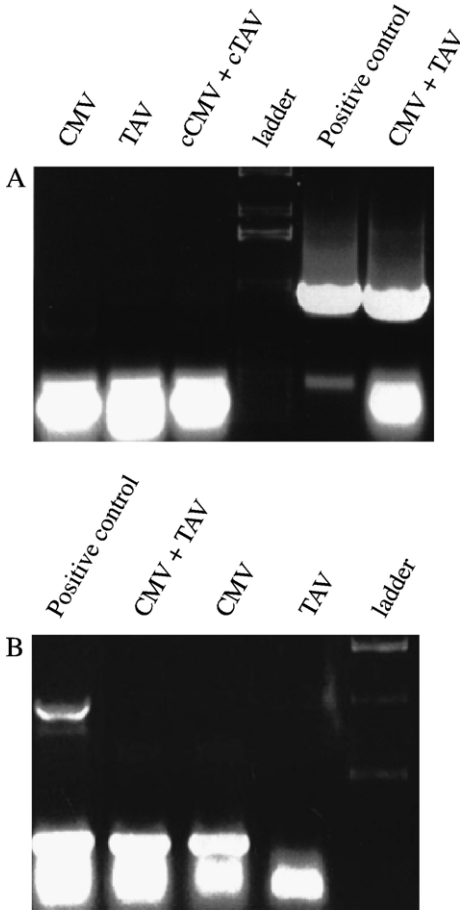

Fig. 1. RT-PCR amplification products using primers R1168+ and T2147− and a reverse transcriptase (RTase) with or without RNaseH activity. In panel A, the RTase had native RNase H activity (Qiagen); in panel B, the RTase was RNase H− (Invitrogen). Lanes labeled CMV or TAV were produced using total RNAs extracted from leaves of plants infected singly with either CMV or TAV, respectively. In lanes labeled CMV + TAV, the CMV and TAV total RNA samples were mixed prior to RT-PCR. In the lane labeled cCMV + cTAV, reverse transcription was carried out on the individual total RNA samples, which were then mixed prior to PCR. The positive controls were appropriate recombinant RNA synthesized in vitro.

obtained from the bands. Table 1 presents the type and number of crossover sites observed across RNA3. In most cases, inoculated leaves were analyzed in order to minimize counterselection of recombinants affected in long-distance movement. In one experiment, systemically infected leaves were also harvested 30 days after the second inoculation. Spearmann rank correlation analysis showed no significant differences between the populations of recombinants in inoculated and systemically infected leaves in the central region of RNA3 (r_s = 0.550, P = 0.0474; see Table 2). We observed recombinant RNAs in 6–12% of the samples tested, which is similar to what was observed previously in the central region of RNA3, in which recombinants were observed in 4% of the samples tested (Aaziz and Tepfer, 1999a). Eleven, 14, and 13 crossover sites were observed in the 5′, central, and 3′ regions, respectively. It is of interest to note that in most of the 20 samples in which recombinants were observed, more than one crossover site was detected. In one sample, 19 different crossovers were found (not shown).

Overall, precise homologous recombination was the most widespread type observed since it was observed at 28 sites widely scattered across RNA3 (Table 1). In contrast, imprecise homologous recombination was observed at only two sites, one in the 5′ region where numerous recombination events were observed, and the other was at a site in the 3′ region, where three clones produced by imprecise recombination were obtained. Aberrant homologous recombination occurred predominantly at a tight cluster of seven sites in the 3′ region, and a single aberrant clone was also observed using the 5′ primer pair.

Molecular characteristics of the recombinant molecules observed

The 28 precise homologous recombination sites observed in RNA3 are shown in Fig. 3 and Table 2. The majority of the frequently observed sites of precise recombination correspond to the larger blocks of sequence identity. However, there are large blocks of sequence identity in which recombination was not observed, and also two sites of frequent precise homologous recombination with only two identical nucleotides, but that were immediately adjacent to larger blocks of identity. We have considered that a hot spot for recombination exists when recombination was observed at a given site in at least half of the RNA samples in which recombinants were observed, grouping together blocks of identity separated by only a single nucleotide that differs. Using these criteria, we consider that there were six hot spots for precise homologous recombination in RNA3. Two were observed in the 5′ region (CMV 47–63/TAV 41–57 and CMV 130–149/TAV 123–142), two were observed in the central region (CMV 616–642/TAV 609–635 and CMV 647–673/TAV 640–666), and two in the 3′ region (CMV 1899–1922/TAV 1900–1923 and CMV 1940–1975/TAV 1945–1980). The frequency of recombination at these hot spots was above the upper limit of the 95% confidence interval of 100,000 Monte Carlo simulations under the hypothesis that all recombinants occurred with identical frequencies.

We have observed two sites of imprecise homologous recombination. At the site in the 3′ noncoding region (Table 2), which was observed in two samples, variable numbers of nontemplate U and A residues were inserted at the site of recombination. This site (R-CMV 1896/P-TAV 1902) is one where precise and aberrant homologous recombination were also frequently observed. In all samples in which recombination was observed with the 5′ primer pair, imprecise homologous recombination was observed in the largest of the blocks of repeated GU residues in the 5′ noncoding region (Table 2 and Fig. 4). This GU block is considerably longer in P-TAV than in

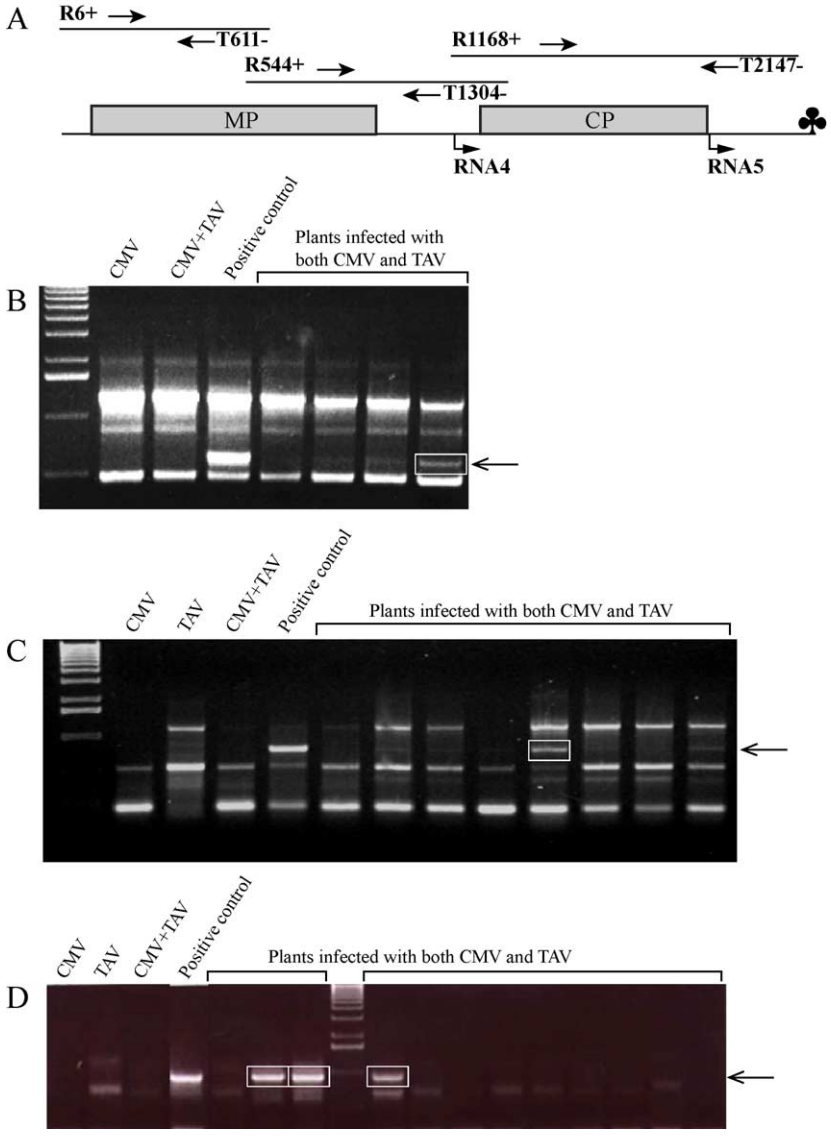

Fig. 2. Typical RT-PCR results obtained with the three primer pairs. RT-PCR was carried out with total RNAs from infected plants. In panel A, below the primers are shown the ORFs encoding the MP and the CP. The sites of initiation of subgenomic RNAs 4 and 5 are shown by a bent arrow, and that of the 3′-terminal tRNA-like structure by ♣. In panel B, primers R6+/T611−; in panel C primers R544+/T1304−; and in panel D primers R1168+/T2147− were used, respectively. In lanes indicated CMV, TAV, or CMV + TAV, total RNA from plants infected with R-CMV, with P-TAV, and with a mixture of total RNA from singly infected plants was used, respectively. The positive controls were the appropriate recombinant transcripts synthesized in vitro. RT-PCR products from samples of doubly infected plants are also shown. The arrows on the right indicate the expected size of a homologous precise recombinant amplification product. Amplified bands corresponding to potential recombinants of sufficient intensity to be cloned are boxed.

R-CMV, and in the 12 clones of imprecise homologous recombination products, the GU block was of intermediate length compared to the parental viruses.

In addition, a single clone due to aberrant homologous recombination was obtained with the 5′ primers, and numerous recombinant clones from three out of five samples were the result of aberrant homologous recombination events in the 3′ noncoding region of RNA3 (Table 2 and Fig. 5). Among the sites observed, the one at CMV 2066/TAV 1902 was found in all three samples, whereas all the other sites were observed in a single sample. In all of these cases of aberrant recombination in the 3′ noncoding region, the result was the duplication of part of this region of RNA3. In all cases, aberrant recombination involved sites between CMV 2046 and 2108, and in all but two cases, crossover on P-TAV occurred at positions 1901–1902.

Discussion

One of the objectives of this study was to have a complete overview of the sequence variants created by recombination between related wild-type viral RNAs. However, although many RNAs bearing deleterious mutations would be expected to be replicated in trans, as in any

Table 1
Number of plants tested, number, and types of recombination sites amplified with the three RNA3 primer pairs

Primer pair	Plant set			Total plants tested	Plants with recombinants (%)	Number of recombination sites			
	Set A (93)[a]	Set B (9)	Set C (76)			Precise	Imprecise	Aberrant	Total crossover sites
R6+/T611−	0	1	76	77	5 (6.5)	9	1	1	11
R544+/T1304−	93	0	0	93	10 (10.7)	14	0	nd	14
R1168+/T2147−	0	9	30	39	5 (12.8)	5	1	7	13
Total				209	20 (9.6)	28	2	8	38

[a] Numbers of plants in each set between parentheses.

Table 2
Number of different types of recombination events at all RNA3 sites observed

Recombination site			Recombinants observed		
Primers	CMV	TAV	Number of samples with recombinant RNA3		Number of recombinant clones
			Inoculated	Systemic	
R6+ and T611−	47–63[a]	41–57	5/5	–	12
	47–63	41–57	4/5	–	7
	115–117	108–110	1/5	–	1
	130–140	123–133	1/5	–	1
	142–146	135–139	3/5	–	7
	148–149	141–142	4/5	–	16
	232–242	225–235	1/5	–	1
	268–275	261–268	1/5	–	1
	382–386	375–379	1/5	–	1
	439–446	432–439	1/5	–	1
	565	518	1/5	–	1
					49
R544+ and T1304−	565–569	558–562	0/3	1/7	1
	583–584	576–577	0/3	1/7	2
	586–588	579–581	0/3	1/7	1
	592–602	585–595	1/3	3/7	6
	616–617	609–610	1/3	1/7	3
	619–620	612–613	3/3	2/7	6
	622–642	615–635	3/3	6/7	50
	647–656	640–649	1/3	3/7	9
	658–673	651–666	3/3	5/7	14
	688–698	681–691	1/3	1/7	2
	709–710	702–703	0/3	1/7	2
	713–720	706–713	0/3	1/7	1
	1102–1112	1083–1093	1/3	0/7	1
	1146–1157	1126–1137	1/3	0/7	1
					99
R1168+ and T2147−	1899–1900	1900–1901	3/5	–	15
	1896	1902	2/5	–	3
	1902–1922	1903–1923	1/5	–	3
	1940–1965	1945–1970	3/5	–	9
	1967–1975	1972–1980	1/5	–	1
	1998–2018	1999–2019	1/5	–	1
	2046	1839	1/5	–	2
	2066	1902	3/5	–	8
	2081	1902	1/5	–	1
	2082	1902	1/5	–	1
	2082	1944	1/5	–	1
	2094	1902	1/5	–	1
	2108–2109	1901–1902	1/5	–	1
					47

[a] Site of precise, imprecise, and aberrant homologous recombination are shown on a white, a light grey, and a medium grey background, respectively.

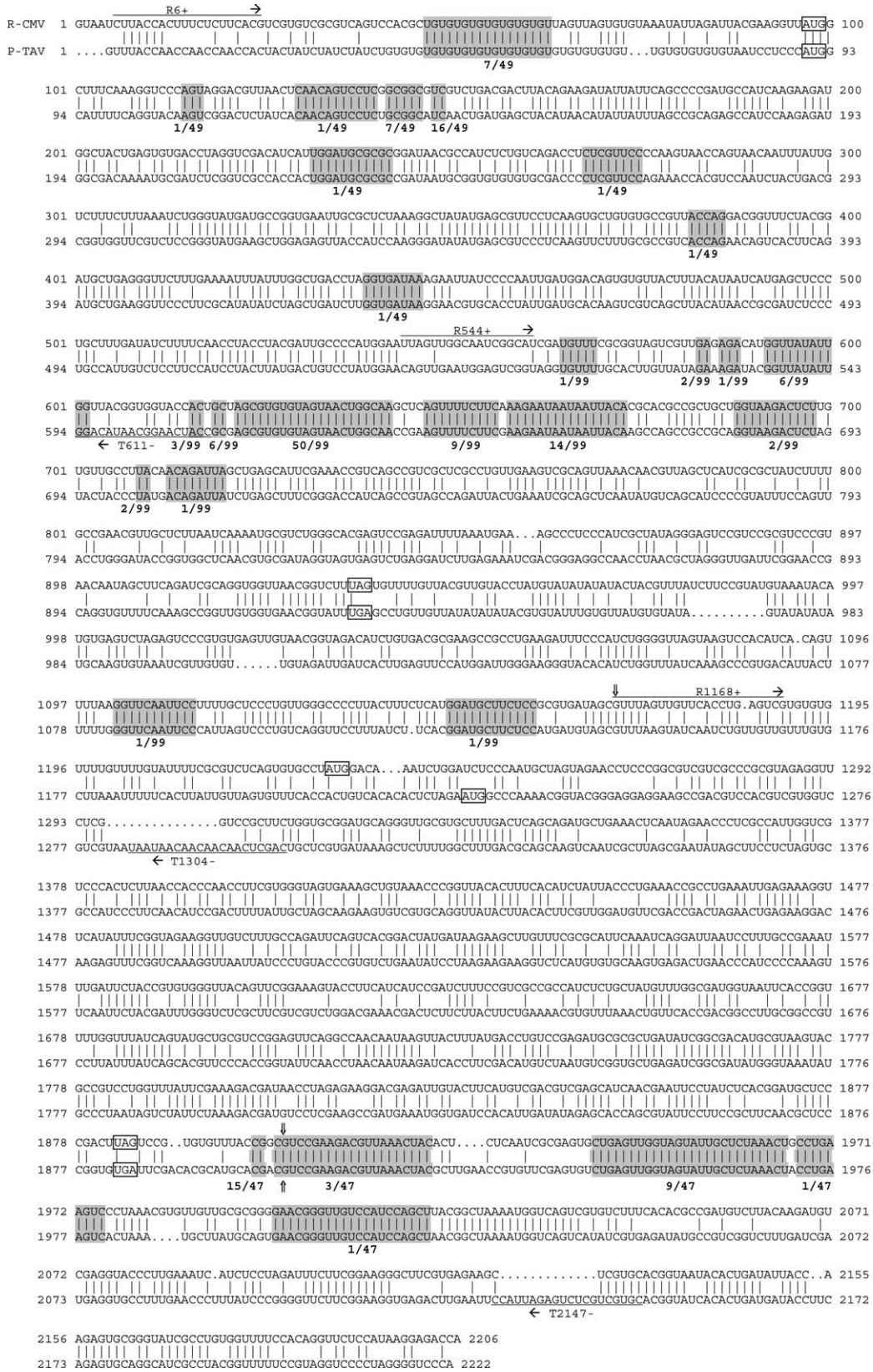

Fig. 3. Distribution across RNA3 of sites of precise homologous recombination. The sequences of RNA3 of R-CMV and P-TAV were aligned with GCG GAP. Start and stop codons of the MP and CP ORFs are boxed, and the first nucleotide of RNAs 4 and 5 are indicated by a vertical double arrow. The positions of primers used for RT-PCR are indicated by horizontal arrows. Blocks of sequence identity in which precise homologous recombination was observed are shown on a grey background. Below each site is indicated the number of clones obtained that had recombined there over the total number of recombinants for a given primer pair (49 with R6+/T611−, 99 with R544+/T1304−, 47 with R1168+/T2147−).

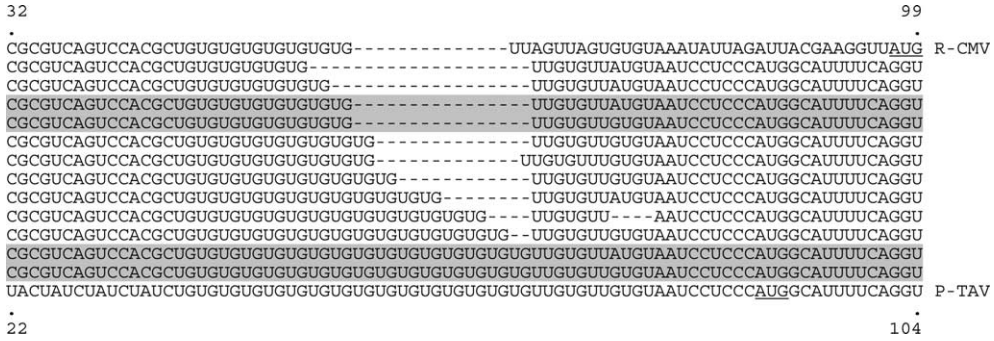

Fig. 4. Hot spot for precise and imprecise homologous recombination within the longest GU repeat in the 5′ noncoding region of RNA3. The top and bottom lines are the sequences of R-CMV and P-TAV, respectively; the AUG start codon of the MP ORF is underlined. Between the reference sequences are those of the observed CMV-TAV recombinants. Cases of homologous precise recombination (in which the GU repeat is of the same length as in one of the parental viruses) are shown on a light grey background.

viral quasi-species, certain recombinants would be expected to have been counterselected. These would include recombinants modified at a site for interaction of the viral RNA with essential proteins, such as those required for virus replication, encapsidation, or cell-to-cell movement. Thus, although the experiments were carried out under conditions of minimal selection pressure, the population of recombinant RNA3 molecules described here may not be entirely representative.

Precise homologous recombination

Most of the hot spots for precise homologous recombination corresponded to sites where there is a block of at least 15–20 nucleotides that are identical between R-CMV and P-TAV (Table 2). However, in the 3′ noncoding region, there were blocks of sequence identity in which recombination was not observed. This region includes the highly structured tRNA-like domain that is recognized by the viral replicase for initiation of synthesis of (−) strand RNA (Boccard and Baulcombe, 1993; Sivakumaran et al., 2000), as well as other possible structural features (Suzuki et al., 2003). Thus, recombinants with crossovers at these sites could be strongly counterselected. It may also be that secondary structures interfere with recombination at these sites. However, a larger number of clones would need to be analyzed in order to determine if recombination is indeed statistically underrepresented at these sites. In contrast, a site where two nucleotides of sequence identity immediately 5′ to a larger block of sequence identity was a distinct hot spot for precise homologous recombination (CMV 1899–1900, adjacent to CMV 1902–1922; Fig. 3). This was the most important site for this type of recombination observed with the 3′ primers. In the 5′ region, a less extreme but perhaps similar situation was observed, with the preponderance of precise homologous recombination at a dinucleotide identity immediately 3′ to a larger block of sequence identity (CMV 148–149, adjacent to CMV 142–146; Fig 3). At these sites, the large block of adjacent sequence identity may serve as a landing site for reinitiation at the time of the template switch. However, this does not explain why recombination at these sites occurs preferentially at the dinucleotide rather than within the larger block of sequence identity.

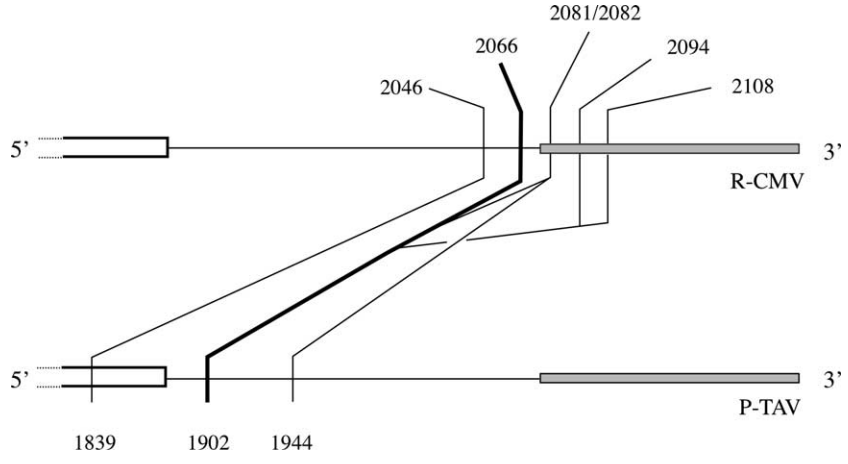

Fig. 5. Hot spot for aberrant homologous recombination in the 3′ noncoding region of RNA3. The donor and acceptor sites of recombination are joined by a line. The most frequently observed site (CMV 2066/TAV 1902), which was observed in 3 of 5 plants, is indicated by a thicker line. The position of the end of the CP ORF is indicated by an open box, and the part of the 3′ terminus that can adopt a tRNA-like structure is indicated by a grey box.

Imprecise homologous recombination

We have observed numerous cases of a previously undescribed type of imprecise homologous recombination within the largest of the blocks of repetition of the dinucleotide GU, which are present in the 5′ noncoding regions of cucumoviral RNAs 3, but not in RNAs 1 and 2. It has been suggested that the GU repeat plays a role in destabilizing secondary structure in the 5′ noncoding region of RNA3 that is involved in regulation of translation (Kwon and Chung, 1999, 2000). Polymerases often are slowed by such repeats, and consistent with this, we have observed a clear decrease of peak height when sequencing across the GU repeat (not shown). Thus, recombination at this site may be favored if the viral replicase pauses and stutters within the GU repeat. In phylogenetic studies of cucumoviruses, Roossinck et al. (1999) have shown that one of the most prominent features discriminating among CMV subgroups is differences in the position and length of the blocks of GU residues in the 5′ noncoding region, and that within subgroups these features are highly conserved. They suggested that rearrangements in this region may be at the root of divergence between the major CMV subgroups. This would be somewhat different from the recombination events that we have observed, which resulted in changes in the length of the GU blocks, while their position was unchanged.

Aberrant homologous recombination

Within the 3′ noncoding region of RNA3, we have observed a major hot spot for aberrant homologous recombination (Fig. 5). In nearly all cases, position 1902 was the site involved on TAV RNA3. Remarkably, this was also the site of frequent precise homologous recombination, and also of occasional imprecise homologous recombination. To the best of our knowledge, this is a unique example of a site where all three types of RNA recombination occur under experimental condition. In the majority of cases, aberrant homologous recombination at this site involved CMV position 2066, or to a lesser frequency, CMV positions 2081–2082. This type of recombination event results in the duplication of a region of approximately 160–180 nucleotides of the 3′ noncoding region just upstream from the tRNA-like structure.

It is interesting to note that RNA3s produced by aberrant homologous recombination at the sites observed here have been described in atypical wild-type cucumoviral isolates. This is the case of the V strain of TAV, compared to other wild-type TAV strains (Moreno et al., 1997). Similarly, the *Alstroemeria* isolates of CMV described by Chen et al. (2002) resulted from recombination at this site. The latter authors showed that the recombinant had a selective advantage on this host species but was counterselected in tobacco, showing that this type of recombinant can play a significant role in host adaptation. Recombination at this site has also been observed on several occasions under laboratory conditions (Fernández-Cuartero et al., 1994; Masuta et al., 1998; Suzuki et al., 2003).

The block of approximately 20 conserved nucleotides at position TAV 1903–1923 corresponds to a putative subgenomic promoter that in CMV subgroup II and TAV strains are believed to be responsible for synthesis of an RNA5 of approximately 200 nucleotides (Blanchard et al., 1996, 1997; Shi et al., 1997). Using MFOLD (Zuker, 2003), we have found that this region is consistently folded into a stem-loop structure (Fig. 6), with the hot spot for recombination at position TAV 1900–1902 in a predicted bulge. The corresponding sequence of RNA3 of R-CMV is predicted to adopt a similar secondary structure (Fig. 6). Since sites of initiation of RNA synthesis can serve as hot spots for aberrant recombination, as was clearly shown for BMV (Olsthoorn et al., 2002), for *Turnip crinkle virus* (TCV) (Nagy et al., 1999) and also for tombusviruses (Cheng and Nagy, 2003), it is tempting to hypothesize that the putative RNA5 promoter may serve as a site of reinitiation when template switching occurs. This is also coherent with the suggestion of Suzuki et al. (2003) that the double stem loop at approximately CMV position 2060 could serve as the donor in this type of recombination. The fact that we have never observed recombinants involving a site corresponding to position 1902 on R-CMV or position 2066 on P-TAV suggests that this type of recombination

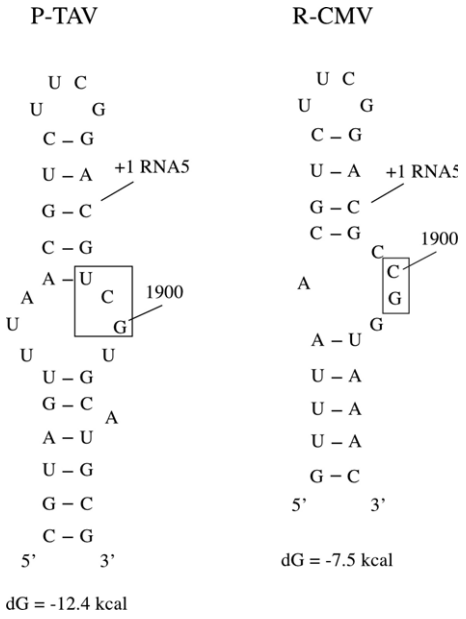

Fig. 6. Predicted secondary structure of the region of P-TAV RNA3 (−) strand involved in the hot spot for aberrant homologous recombination and the corresponding region of R-CMV. Predicted secondary structures of the (−) strand RNA3 of P-TAV positions 1893–1924 and R-CMV positions 1893–1919 were analyzed using MFOLD (41). The stem-loop structures shown were consistently predicted. Nucleotides involved in the hot spot for recombination at positions TAV 1899–1900 and CMV positions 1900–1901 are boxed. The residue corresponding to the first nucleotide of CMV RNA5 according to Blanchard et al. (1996) or TAV RNA5 according to Shi et al. (1997) is indicated. Nucleotide positions are according to (+) strand numbering.

occurs only upon synthesis of either (+) or (−) strand RNA, but not both.

However, it should be noted that the well-characterized subgenomic promoter responsible for synthesis of cucumoviral RNA4, which has an element of stem-loop structure at position CMV 1140–1162 (Chen et al., 2000), was clearly not a hot spot for recombination in the experiments described here, and thus not all cucumoviral subgenomic promoters are recombination hot spots. In contrast, when the BMV subgenomic promoter responsible for synthesis of RNA4 was moved to an ectopic position, it was clearly a hot spot for homologous recombination (Wierzchoslawski et al., 2003). However, there are major differences between the RNA4 promoters of CMV and BMV (Kao, 2002), and in particular the CMV promoter lacks the poly(U) tract that plays a key role in recombination in BMV (Wierzchoslawski et al., 2004).

The results presented here show that the three major types of homologous recombination (precise, imprecise, and aberrant) can be detected under experimental conditions in plants infected with both CMV and TAV. Particularly in the case of imprecise and aberrant homologous recombination, little is known about the mechanism(s) involved, at least within the *Bromoviridae*. Studies currently underway should help clarify the roles of RNA sequence and/or secondary structure in determining the sites where these types of recombination were observed. Among the recombination sites identified, several may have played a significant role either in the evolution of large groups of cucumoviruses (Roossinck et al., 1999), or in the adaptation of certain isolates to specific hosts (Chen et al., 2002).

The host range of CMV is much broader than that of TAV, and thus one could expect that plants infected with both plants would occur in nature. Although it has been reported that one CMV-TAV recombinant RNA3 created in the laboratory had a selective advantage when it was co-inoculated with parental strains (Fernández-Cuartero et al., 1994), the absence of naturally occurring CMV-TAV recombinants suggests that they are generally counterselected. The abundance of recombinant RNA3 molecules described here, and the rarity of recombinants in the field, even between extremely closely related CMV haplotypes (Fraile et al., 1997), further supports that there is generally strong counterselection of cucumoviral recombinants.

One of the main purposes of this study was to provide baseline information for evaluating the potential impact of recombination in transgenic plants expressing viral sequences, such as those that are rendered resistant to certain viruses by expression of a viral CP gene (for a review, see Aaziz and Tepfer, 1999b; Tepfer, 2002). In order to evaluate the potential epidemiological impact of recombination in transgenic plants, it is essential to be able to compare what occurs in transgenic plants with what occurs in nontransgenic plants, since only if novel recombinants occur in transgenic plants can they be thought to present risk. We are currently using the strategy described here to study the RNA3 recombinants occurring in transgenic plants expressing a cucumoviral CP gene when infected with another cucumovirus.

Materials and methods

Virus strains and plant inoculation

The strains used were R-CMV, a subgroup II strain originally isolated from *Ranunculus asiaticus* in France (Jacquemond and Lot, 1981), and P-TAV, isolated from pepper in Hungary (Salánki et al., 1994). The sequences of their RNA3 have been published (R-CMV: Carrère et al., 1999; accession no. Y18138) (P-TAV: Salánki et al., 1994; accession no. L15335). We used an R-CMV that was cloned and initially propagated from infectious transcripts (Salánki et al., 1997). The first expanded leaf of young tobacco plants (two or three leaf stage) was mechanically inoculated using Carborundum (Prolabo, France) and leaves from infected tobacco plants (*Nicotiana tabacum* cv Xanthi XHFD8) ground in 50 mM KH_2PO_4. Plants were grown in an S3 biosafety greenhouse (7000 lx, 18 h light, 70% humidity, 20–27 °C).

Extraction of total leaf RNA

Total RNA was extracted from the co-inoculated leaf of individual plants as described by Goormachtig et al. (1995) with minor modifications. Briefly, 200 mg of leaf tissue were ground in 400 μl of buffer solution (200 mM Tris–Cl, 100 mM LiCl, 5 mM EDTA, 1% SDS) and then 400 μl of water-saturated phenol was added. The aqueous phase was reextracted with phenol–chloroform–isoamyl alcohol (25:24:1∷v/v/v). Total RNA was precipitated for 2 h at 4 °C in 3 M LiCl, washed twice with 70% ethanol, and resuspended in 10 μl of sterile deionized water.

RT-PCR and cDNA cloning

Primers R6+ (5′-CTTACCACTTTCTCTTCACG-3′) and T611− (5′-GGTAGTTCCGTTATGTCC-3′) covered the 5′ region; primers R544+ (5′-TTAGTTGGCAATCGGCAT-3′) and T1304− (5′-GTCGAGTTGTTGTTGTTATTA-3′) covered the central region; primers R1168+ (5′-GTTTAGTTGTTCACCTGAGTCG-3′) and T2147− (5′-GCACGACGAGACTCTAATGG-3′) covered the 3′ region of RNA3. For primers R544+/T1304−, first-strand cDNA was synthesized as recommended by the manufacturer (Invitrogen) in a 20-μl reaction mixture containing 1 μg of total RNA and 20 pmol of the specific primer, which is complementary to P-TAV RNA3. After incubation at 37 °C for 50 min, 5 μl of the cDNA mixture was submitted to a 35-cycle PCR according to the manufacturer's instructions (Invitrogen).

The PCR mixture contained 20 pmol of sense primer (R544+) and 20 pmol of antisense primer (T1304−). Each PCR cycle was 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 45 s. For primers R6+/T611− and R1168+/T2147−, synthesis and amplification of cDNA using Superscript™ One-Step RT-PCR with Platinum® Taq were carried out according to the manufacturer's instructions (Invitrogen). cDNA was synthesized at 50 °C for 30 min, followed by a 10-min denaturing step. The amount of template RNA, $MgSO_4$, and PCR steps differed with each primer pair. Two hundred nanograms of total RNA plant was used with primers R6+/T611− with 3.2 mM final concentration of $MgSO_4$. Each of the 35 cycles of PCR was 94 °C for 20 s, 54 °C for 30 s, and 72 °C for 30 s. Five hundred nanograms of total plant RNA was used with primers R1168+/T2147− with 1.2 mM final concentration of $MgSO_4$. Each of the 35 cycles of PCR was 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 60 s.

Positive controls for RT-PCR were constructed by fragment exchange between plasmids pT3 and pR3, which bear cDNAs corresponding to full-length RNA3 of P-TAV and R-CMV, respectively, under the control of the T7 promotor (Carrère et al., 1999; Salánki et al., 1997). Chimeric recombinants pRTR3 and pTRT3 were constructed by using the common unique *Bpl*I and *Bbs*I sites, respectively, at the 5′ part of MP gene and at the beginning of the 3′ noncoding region. Transcripts of positive controls were synthesized in vitro using T7 RNA polymerase (Invitrogen) according to the manufacturer's instructions. Transcripts of pRTR were used as positive control for RT-PCR with R6+/T611− and transcripts of pTRT were used for R1168+/T2147−. For R544+/T1304−, the previously described pRT3 (Salánki et al., 1997) was used to synthesize the positive control.

The resulting amplified cDNAs were analyzed by electrophoresis on a 1% agarose gel. cDNA bands corresponding to potential recombinants were purified from agarose gel fragments using the GeneClean II Kit (BIO 101 Inc.), cloned into a pGEM-T vector (Promega) in *Escherichia coli* strain DH10B (Invitrogen) and sequenced by Genome Express (Meylan, France). Essentially all clones obtained were sequenced (average 9.75 clones/sample, range 1–29).

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