Transfer of the 3' non-translated region of grapevine chrome mosaic virus RNA-1 by recombination to tomato black ring virus RNA-2 in pseudorecombinant isolates

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Transfer of the 3′ non-translated region of grapevine chrome mosaic virus RNA-1 by recombination to tomato black ring virus RNA-2 in pseudorecombinant isolates

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In grapevine chrome mosaic and tomato black ring viruses (GCMV and TBRV), as in many other nepoviruses, the 3′ non-translated regions (3′NTR) are identical between the two genomic RNAs. We have investigated the structure of the 3′NTR of two recombinant isolates which contain GCMV RNA-1 and TBRV RNA-2. In these isolates, the 3′NTR of RNA-1 was transferred to RNA-2, thus restoring the 3′ identity. The transfer occurred within three passages, and probably contributes to the spread of randomly appearing mutations from one genomic RNA to the other. The site of recombination is near the 3′ end of the open reading frame.

Nepoviruses (Harrison & Murant, 1977) are members of the family *Comoviridae* (Mayo & Martelli, 1993). They have a bipartite, single-stranded, RNA genome (Murant *et al.*, 1981). Their two genomic RNAs are polyadenylated at their 3′ ends (Mayo *et al.*, 1979) and covalently linked to a virus-encoded polypeptide (VPg) at their 5′ ends (Mayo *et al.*, 1982). They are translated into polyproteins which are further cleaved by a virus-encoded protease to yield the mature viral proteins (Fritsch *et al.*, 1980).

In all the nepoviruses where this has been investigated, the 3′ termini (and in some cases also the 5′ termini) of the two RNAs display considerable sequence homology, or even complete identity (Dodd & Robinson, 1987; Greif *et al.*, 1988; Le Gall *et al.*, 1989; Rott *et al.*, 1991; Scott *et al.*, 1992; Ritzenthaler *et al.*, 1991; Bacher *et al.*, 1994). In the case of tomato black ring virus (TBRV; Greif *et al.*, 1988) and grapevine chrome mosaic virus (GCMV; Le Gall *et al.*, 1989) for instance, the entire 3′ non-translated regions (3′NTR) of RNAs 1 and 2 are identical. In tomato ringspot virus (TomRSV), Rott *et al.* (1991) have speculated that this high level of homology is the result of RNA recombination as part of RNA-2 replication. However, the results of Bacher *et al.* (1994) showed that recombination is slower than the mutation rate in blueberry latent mosaic virus (BBLMV), a relative of TomRSV, which argues against such a model. In the case of tobacco rattle virus (TRV; a tobravirus), the 3′ ends of the two genomic RNAs are also identical in field isolates, but Angenent *et al.* (1989) showed that differences in the 3′ ends of RNAs 1 and 2 of pseudorecombinant isolates survived at least 25 successive passages in the greenhouse.

In order to assess the role of RNA recombination in the restoration of 3′-terminal identity in TBRV and GCMV, we have examined the 3′NTR of pseudorecombinant isolates obtained by mixing the genomic RNAs of GCMV and of the Scottish beet ringspot isolate of TBRV, TBRV-S (Doz *et al.*, 1980). These two viruses differ both in the sequence and size of their 3′NTRs, so that the 3′NTR of the two genomic RNAs of such a pseudorecombinant should be different in the absence of recombination. The complete nucleotide sequence of the two parental viruses is known (Meyer *et al.*, 1986; Greif *et al.*, 1988; Le Gall *et al.*, 1989; Brault *et al.*, 1989).

When they were lyophilized 16 years ago, the pseudorecombinant isolates (all independently obtained) had been propagated three times in *Chenopodium quinoa* following their initial isolation. In order to confirm their individual RNA compositions we analysed extracts of plants inoculated with the lyophilized material by dot-blot hybridization using discriminating ^{32}P-labelled RNA probes derived from the coding regions of each RNA of GCMV and TBRV (not shown). The two surviving pseudorecombinant isolates with GCMV RNA-1 and TBRV RNA-2 (GS_{21} and GS_{22}) were selected for further use. GS_{21} was passaged three more times in the greenhouse before the analysis described here. All of the 12 isolates previously described to be in the other

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Table 1. *Oligonucleotide primers used in the IC-PCR experiments**

	Sequence	RNA-2 GCMV	RNA-2 TBRV	RNA-1 GCMV	RNA-1 TBRV
P1	ATGGGAGAAGTGCTGG (+)	(4155)	4291		
P2	AATCTTTTTGTGTCCAACA (−)	4401	4623	7172	7317
P3	CACAAGTATCACCTTGG (+)			6928	(7018)
P4	GGGTAGCCTCCCTCCATG (+)		4240		
P5	GGTTAACCTCTCTAACGGTAGA (+)	4104			
P6	TGTCCAACATAGCACCACAG (−)		4613		7307
P7	TGTGTCCAACAAAGCAAACAT (−)	4393		7164	

* For each primer, its denomination (P1 to P7), sequence (from 5′ to 3′), as well as the position of its 5′-most nucleotide along the sequences of the GCMV and TBRV genomic RNAs are given. A position given in parentheses indicates that the primer and the RNA have an imperfect match, and a blank that the primer will not hybridize to the RNA. The sequences are viral sense (+) or antisense (−).

polarity (SG; Doz *et al.*, 1980) were found not to be pseudorecombinants (not shown). Thus, no SG isolate could be studied. Available data do not allow us to assess whether their initial characterization by Doz *et al.* (1980) was erroneous, or whether the SG isolates did not survive their parents when mixed infections may have occurred. Both hypotheses imply that SG isolates are less viable than GS isolates. In the case of pseudorecombinants between a Scottish and a German isolate of TBRV, Randles *et al.* (1977) also showed that pseudorecombinants of only one polarity were viable.

As a first approach, we examined whether the 3′NTRs of GS are identical to those of its parents. For this purpose, we took advantage of the existence in the 3′ region of TBRV of sequences with no aligned counterpart in GCMV to design oligonucleotide primers able to allow discrimination of the RT–PCR products obtained from each virus through their electrophoretic migration. The primer pair P1/P2 (Table 1) allowed amplification of a 333 bp fragment from TBRV RNA-2, or of a 247 bp fragment from GCMV RNA-2. The TBRV product contained 33 bp in the coding region, and 53 bp in the 3′NTR, which have no counterpart in GCMV. The use of P3/P2 similarly allowed amplification of a 300 bp fragment from TBRV RNA-1, or 245 bp from GCMV RNA-1. The use of the virus-specific primers P4 to P7 allowed amplification of a 374 bp fragment from TBRV RNA-2, and of a 290 bp fragment from GCMV RNA-2.

An immunocapture (IC) step was introduced prior to the RT–PCR (Wetzel *et al.*, 1992), using a mixture of GCMV- and TBRV-specific rabbit immunoglobins to coat the test-tubes. Purified virions or extracts of infected *C. quinoa* leaves were used as the virus source for the IC–PCR. The infected plant tissues (fresh or lyophilized for up to 18 years before analysis, and from which virtually no residual infectivity on various *Chenopodium* species could be detected) were ground in 3 vols PBS–Tween buffer (Voller *et al.*, 1976) containing 2% PVP and 20 mM-sodium diethyl dithiocarbamate (PBS–TPD) in a 1·5 ml tube using a fitted pestle. Following centrifugation (1 min at 1000 *g*), 100 µl of the supernatant were incubated overnight at 4 °C in pre-coated tubes. Alternatively, 500 ng of purified virus were incubated similarly in 100 µl PBS–TPD. The extracts were then removed and the tube rinsed once with PBS–Tween; 6 µl 1·7% Triton X-100 was then added, and the tube vigorously shaken and incubated for 10 min at 65 °C. The RT–PCR mixture [final composition: 10 mM-Tris–HCl, pH 8·8, 50 mM-KCl, 1·5 mM-$MgCl_2$, 0·01% (w/v) gelatine, 0·3% (w/v) Triton X-100, 250 µM of each of the four dNTPs, 1 µM of each of the primers, 0·5 units of avian myeloblastosis virus reverse transcriptase (Pharmacia) and 1 unit of *Taq* DNA polymerase (SuperTaq; Stehelin & Cie); final volume 50 µl] was then added and the tubes were incubated for 15 min at 42 °C, 2 min at 92 °C and cycled 40 times [92 °C, 1 min, 42 °C (primers P1 to P3) or 45 °C (primers P4 to P7), 1 min, 72 °C, 1 min].

RNA-1-specific IC–PCR products of the expected electrophoretic mobilities were observed for both parental viruses as well as for GS_{21} (not shown). However, the mobility of the RNA-2-specific product obtained from GS_{21} was intermediate between those expected and observed from GCMV and TBRV (Fig. 1*a*). Only three passages after its primary isolation, GS_{22} yielded a product with the same electrophoretic mobility as that of GS_{21} (Fig. 1*a*, lane 6). When extracts from plants infected with GCMV and TBRV were mixed and used as a control, only the two bands corresponding to GCMV and to TBRV were seen (Fig. 1*a*, lane 3), indicating that the product of intermediate mobility is not an IC–PCR artefact. The use of virus-specific primers showed that whereas each parental virus gave the expected IC–PCR product when the proper pair of primers was used, only the pseudorecombinant isolate gave a signal with a pair composed of a TBRV-specific 5′-primer and a GCMV-

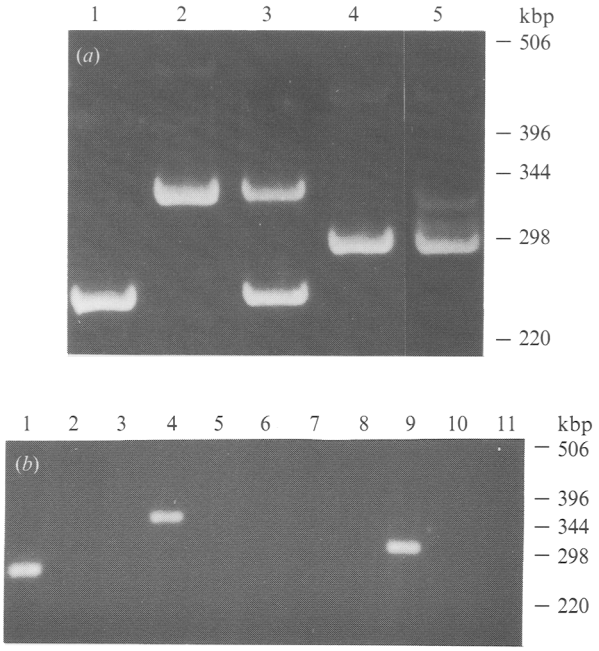

Fig. 1. (*a*) Electrophoretic mobilities, in a 6% polyacrylamide gel, of the IC–PCR products obtained from various RNA-2 templates using the primer pair P1/P2. Lane 1, GCMV; 2, TBRV; 3, a mixture of GCMV and TBRV; 4, GS_{21}, 5, GS_{22}. The mobilities of DNA molecules of known sizes (1 kb DNA ladder; BRL) are indicated. (*b*) IC–PCR amplification of the RNA-2 of GCMV, TBRV and GS_{21} using primers P4 to P7. Lanes 1–3, P5/P7; lanes 4–6, P4/P6; lanes 7–11, P4/P7. Templates are: lanes 1 and 7, GCMV; lane 2, a mixture of GS_{21} and TBRV; lanes 3, 6 and 11, no template added; lanes 5 and 10, a mixture of GCMV and TBRV; lanes 4 and 8, TBRV; lane 9, GS_{21}. The mobilities of DNA molecules of known sizes (1 kb DNA ladder) in the 1·5% agarose gel used are indicated.

specific 3′-primer, and only with this primer pair (Fig. 1*b*). Taken together, these results indicate that the RNA-1 of the pseudorecombinant isolate is, as expected, similar to that of GCMV at its 3′ end, but that the 3′ end of its RNA-2 is also derived from GCMV and not from TBRV.

Since recombination seemed to happen so soon, we tried to detect its occurrence during mixed (GCMV+TBRV) infections of *C. quinoa*, using virus-specific primers. We always failed in this attempt (not shown). Thus, if recombination occurs in this case, it is either at an undetectable level, or the recombinants are outcompeted by the more efficient replication of the two parents.

To investigate the structure of the 3′NTRs of GS_{21}, they were sequenced by the chain termination method using purified RNAs (Fichot & Girard, 1990). The RNA-1 of GS_{21} has a 3′NTR similar to that of GCMV (not shown), confirming the IC–PCR results described above. On the other hand, the sequence of the 3′ end of the RNA-2 is also identical to that of GCMV (Fig. 2), and thus of RNA-1, whereas the sequence of its coding region is identical to that of TBRV. The A/G transition present in this region of GS_{21} as compared to TBRV (Meyer *et al.*, 1986) is probably the result of a mutation since the TBRV isolate present in our laboratory has an A at this position, like GS_{21} (not shown). The identity between the two genomic RNAs of GS_{21} starts at heptanucleotide UCUUAGG, which is conserved between GCMV and TBRV and contains the translation termination codon (except for GCMV RNA-1; Le Gall *et al.*, 1989). This heptanucleotide is the starting point of the identity in the two parental viruses as well (Greif *et al.*, 1988; Le Gall *et al.*, 1989). Thus, in the pseudorecombinant isolate obtained by mixing the genomic RNAs of GCMV and TBRV, the identity of the 3′NTR is restored by incorporation of RNA-1 sequences into RNA-2. It is interesting to note that, in the case of TomRSV where parts of the coding region are involved in the strong homology between the two viral RNAs, it also seems that sequences derived from RNA-1 are present in RNA-2 (Rott *et al.*, 1991).

The complete nucleotide sequence of GCMV (Le Gall *et al.*, 1989; Brault *et al.*, 1989) has been determined from the same virus source as the one used in this work, but after a further 10 years of successive passages in the greenhouse. Beside showing the restoration of the 3′

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GCMV   // AGGAGACACAAGUAUC-ACCUUGGUUUGGUACAAAAU
GS21   // ACCAGCUACUGUCGCCAAUGUUAGUGCGGUAU-CAGG
TBRV   // ACCAGCUACUGUCGCCGAUGUUAGUGCGGUAU-CAGG

GCMV   UUCUUAGGCAUUUCUUGAAGAGAAUAUCCAUCCCGCUU
GS21   CUCUUAGGCAUUUCUUGAAGAGAAUAUCCAUCCCCACUU
TBRV   CUCUUAGGUAUUUCUUAUAGAGAAUAUCCCUCCCUGGU

GCMV   GACAGGGAUUU----------CUGUUUGUCAAGCUA- //
GS21   GACAGGGAUUU----------CUGUUUGUCAAGCUA- //
TBRV   GAUCUGGAGUAAAAAGAACCAUUGGUGUUGAGAUAA //
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Fig. 2. Alignment of the sequences of GCMV RNA-1 (positions 6922–7020), TBRV RNA-2 (positions 4319–4429) and GS_{21} RNA-2 around the recombination site. The translation termination codons are in bold, dashes denote gaps, nucleotides different from GS_{21} are shadowed and the recombination site is framed.

identity in the GS isolates, in the present study five differences between the 3′NTRs of GS_{21} and the published GCMV RNA-2 sequence were detected: G/A_{4220} (Fig. 2), C/U_{4275}, U/C_{4284}, C/U_{4322} and G/A_{4357}. These transitions must have arisen during the 10 year period separating the virus isolations for the two studies. Interestingly, all of these variations were found in both genomic RNAs of GS_{21}, indicating that, unlike in BBLMV (Bacher *et al.*, 1994), the rate of recombination between RNA-1 and RNA-2 is higher than the rate of mutation in RNA-2.

We have recently described another instance of presumed RNA recombination that may have resulted in the transfer of an entire domain of the coding region from GCMV to the ED strain of TBRV (accompanying paper: Le Gall *et al.*, 1995). However, this was probably the result of an isolated and rather ancient event in the history of TBRV-ED since there is already sequence divergence between the regions involved. We now describe the apparently rapid (less than three passages in GS22) and systematic (observed in both independent isolates in which it was investigated) replacement of RNA-2 sequences by RNA-1-derived sequences, a phenomenon that can be linked with the 3′ identity of TBRV and GCMV. Thus, the genome of nepoviruses could be subject to 'accidental' natural recombination, as are the genomes of other plant viruses (Robinson *et al.*, 1987; Allison *et al.*, 1989; Edwards *et al.*, 1992; Cervera *et al.*, 1993; Fernández-Cuartero *et al.*, 1994; Rathjen *et al.*, 1994; Gibbs & Cooper, 1995), but, in addition, these viruses could also use such a mechanism in their replication, as suggested by Rott *et al.* (1991). This model predicts that the replication of RNA-2 happens after a copy choice during the negative strand synthesis, initiated on RNA-1. The data presented here on the GCMV/TBRV pair are in agreement with such a model, unlike those of Bacher *et al.* (1994) with BBLMV.

The common occurrence of RNA recombination and the resulting identity of the termini of the two genomic RNAs could be a characteristic of nepoviruses. According to the occurrence and extent of terminal identity, at least three sets of nepoviruses can be distinguished. In the first set, illustrated by TomRSV (Rott *et al.*, 1991) and perhaps CLRV (Scott *et al.*, 1992) and BBLMV (Bacher *et al.*, 1994), both the 5′ (including part of the coding regions) and the 3′ ends are strongly homologous between the two genomic RNAs for over 1000 nucleotides, but a few differences exist and persist after several passages. The second set contains GCMV and TBRV, and is characterized by a strict identity of about 300 nucleotides only in the 3′NTRs. The homology in the NTRs is strong but not strict in viruses belonging to the third set, exemplified by GFLV. If the transfer of terminal sequences reflects a replication strategy specific to nepoviruses, then differences in the extent of this transfer might reflect differences in replication mechanisms between these subgroups. Interestingly, a similar clustering of nepoviruses was obtained by Martelli (1975) using unrelated criteria.

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