The nucleotide sequence of the RNA-2 of an isolate of the english serotype of tomato black ring virus: RNA recombination in the history of nepoviruses

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The nucleotide sequence of the RNA-2 of an isolate of the English serotype of tomato black ring virus: RNA recombination in the history of nepoviruses

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The RNA-2 of a carrot isolate from the English serotype of tomato black ring nepovirus (TBRV-ED) has been sequenced. It is 4618 nucleotides long and contains one open reading frame encoding a polypeptide of 1344 amino acids. The 5′ non-coding region contains three repetitions of a stem–loop structure also conserved in TBRV-Scottish and grapevine chrome mosaic nepovirus (GCMV). The coat protein domain was mapped to the carboxy-terminal one-third of the polyprotein. Sequence comparisons indicate that TBRV-ED RNA-2 probably arose by an RNA recombination event that resulted in the exchange of the putative movement protein gene between TBRV and GCMV.

The genome of tomato black ring nepovirus (TBRV; Murant, 1970) consists of two single-stranded RNAs (RNA-1, M_r ca. $2{\cdot}7 \times 10^6$; RNA-2, M_r ca. $1{\cdot}5 \times 10^6$: Murant *et al.*, 1981). As in other members of the family *Comoviridae* (Mayo & Martelli, 1993), these RNAs are translated into polyproteins which are further processed by an RNA-1-encoded protease to yield the mature viral proteins (Fritsch *et al.*, 1980; Demangeat *et al.*, 1990, 1991, 1992). Both RNAs are 3′-polyadenylated (Mayo *et al.*, 1979) and linked to a small peptide (VPg) at their 5′ end (Mayo *et al.*, 1982). Studies involving pseudorecombinants (viruses with their two genomic components derived from two different strains) and protoplast inoculations have shown that the RNA-2 of TBRV carries the determinants for systemic movement in infected plants and for vector specificity, as well as for the serological properties (Harrison & Murant, 1977; Robinson *et al.*, 1980).

Two serotypes of TBRV have been described (Harrison, 1958). One, called 'English' or 'German', contains most isolates including the type strain whereas the other, 'Scottish', contains TBRV-S and the pseudo-aucuba isolate. The genomic RNAs of TBRV-S and of a related nepovirus, grapevine chrome mosaic virus (GCMV; Martelli & Quacquarelli, 1972) have been sequenced in their cloned cDNA form (Meyer *et al.*, 1986; Greif *et al.*, 1988; Le Gall *et al.*, 1989; Brault *et al.*, 1989). In this study, we have determined the nucleotide sequence of the RNA-2 of a TBRV isolate belonging to the English serotype.

TBRV-ED (Murant, 1982) was originally isolated from carrot in Norfolk, UK, and kindly provided by Dr A. F. Murant (Scottish Crop Research Institute, Dundee, UK). The virus was propagated in the greenhouse in *Nicotiana clevelandii* and purified as described (Doz *et al.*, 1980). A mixture of gradient-purified RNAs 1 and 2 was inoculated onto *Chenopodium murale* plants to establish, after two local-lesion passages, a satellite-free virus line called TBRV-ED(−). The RNA-2 of TBRV-ED(−) was used to prepare a double-stranded cDNA according to Gubler & Hoffman (1983) using pdT_{12-18} (Pharmacia) as the primer. This cDNA was ligated into the *Eco*RV site of pBluescript KS(+) (Stratagene) after two cycles of size selection by precipitation in 625 mM-NaCl containing 6·7% PEG 8000, and the ligation mixture used to transform *Escherichia coli* (strain DH5α, BRL).

The nucleotide sequence was determined as described (Le Gall *et al.*, 1989). Most (93·4%) of the TBRV-ED RNA-2 sequence was determined from at least two overlapping independent cDNA inserts (on average, each nucleotide was sequenced 3·34 times from 3·09 independent cDNAs). The sequence of the entire cloned region was determined on both cDNA strands. No sequence heterogeneity was found between the cDNA clones sequenced. The 20 5′-terminal nucleotides were determined by chemical sequencing (Maxam & Gilbert, 1980) of a primer extension product obtained using the synthetic oligonucleotide 5′ TGAACTTCGATG 3′

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† The TBRV-ED RNA-2 sequence has been deposited in EMBL/GenBank/DDBJ under accession number X80831.

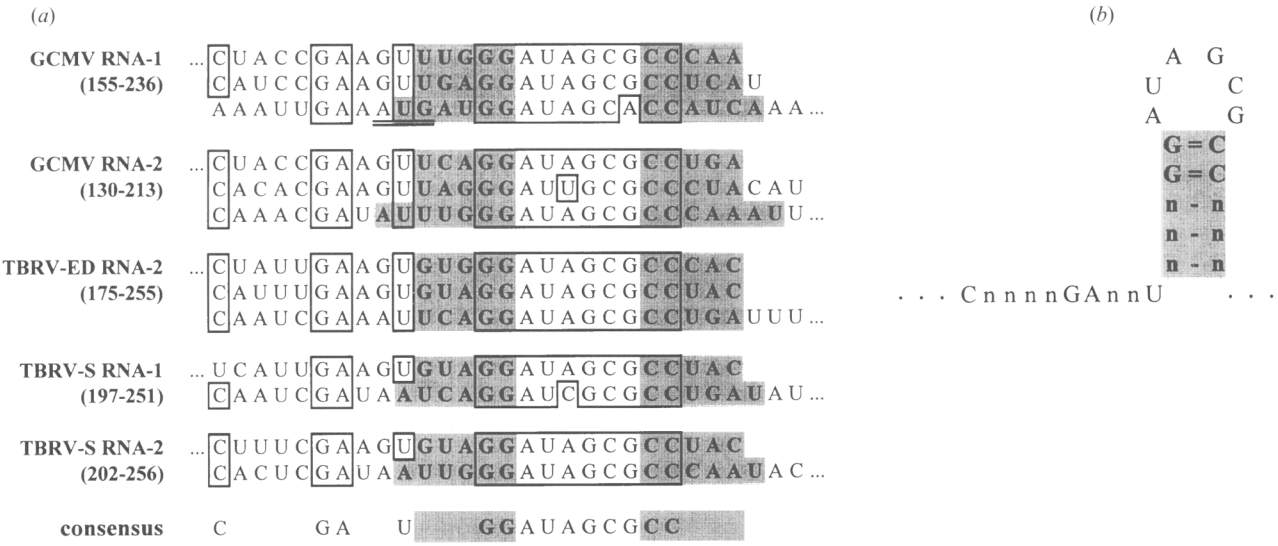

Fig. 1. Repeated and conserved stem–loop in TBRV and GCMV 5′ non-coding regions. (*a*) Alignment of the GCMV, TBRV-ED and TBRV-S sequences (positions indicated in parentheses; for TBRV-ED RNA-1, no information is available). The nucleotides conserved in more than 80% (> 11/13) of the sequences are boxed and reported in the consensus line. Those predicted to be paired have a grey background. The initiation codon of GCMV RNA-1, present in the region considered, is underlined. (*b*) Predicted folding of a 'consensual' repeat unit. The paired nucleotides of the stem are on a grey background. The third repeats of GCMV and TBRV-S RNAs have slightly longer predicted stems (see *a*).

(complementary to positions 50–61), and confirmed by dideoxynucleotide sequencing using the same oligonucleotide primer (Fichot & Girard, 1990). The sequence compilation and analysis were made using the packages PC/Gene 6.01 (Genofit), Microgenie 7.0 (Beckman) and GCG version 7.1 (Devereux *et al.*, 1984).

The TBRV-ED RNA-2 sequence comprises 4618 nucleotides, excluding its 3′ polyadenylate (EMBL, GenBank and DDBJ accession number X80831). It is 68·3% and 66·1% identical with TBRV-S and GCMV RNA-2 respectively. A large ORF extends from the second AUG triplet from the 5′ end (position 300) to position 4331, potentially encoding a polypeptide of 1344 amino acids. The first AUG at the 5′ end of TBRV-ED RNA-2, at position 273, at the start of an ORF only 18 nucleotides in length and in a context (UUAUU-UU**AUG**UCU) predicted to be unfavourable for translation initiation (Lütcke *et al.*, 1987), is unlikely to be used as an initiation codon.

The 5′ non-coding region of TBRV-ED RNA-2 contains four distinct domains: a terminal hairpin (positions 1–18), a 156 nucleotide pyrimidine-enriched stretch (63·5% vs 51·5% in the whole RNA), a region with three repeats, and a 47 nucleotide domain containing 28 (56·8%) Us. Three direct repeats are present between positions 175 to 252. Strikingly, the same repeated motif is found in the 5′ non-coding regions of TBRV-S (two repeats) and GCMV (three repeats) (Fig. 1*a*), and is able to form a stable hairpin whose apical sequences (a decanucleotide) are remarkably conserved (Fig. 1*b*). In each of these 13 repeats, the various nucleotide changes found around the conserved decanucleotide do not alter the possibility of forming the hairpin. In the five RNAs considered in Fig. 1, the distance between the region containing these repeats and the initiation codon is greatly variable, ranging from −20 (in GCMV RNA-1 where the initiation codon is within the repeats) to 44 nucleotides (in TBRV-ED RNA-2), which argues against a role of these structures in translation.

The 3′ non-coding region of TBRV-ED RNA-2 contains 287 nucleotides. The last 151 nucleotides are identical to those of another English isolate of TBRV, TBRV-C, with the exception of an A–G transition at position 4525 (Dodd & Robinson, 1987). The 3′ non-coding region can be separated into three domains according to their level of identity with TBRV-S (Meyer *et al.*, 1986): 93% in the region 4337–4390, 67% in the region 4391–4450 and 90% in the region 4391–4618. The percentage identities between TBRV-ED and GCMV in these three domains are 86%, 54% and 79% respectively. The third region defined here is almost exactly that where Dodd & Robinson (1987) described a high level of homology between strains of TBRV.

The sequence of the predicted 1344 amino acid long polypeptide potentially encoded by TBRV-ED RNA-2 (P2) was compared with those of TBRV-S, GCMV as well as of other proteins of viral or non-viral origin. The amino acid sequence identity between TBRV-ED and TBRV-S is 68·5%, and 61·4% between TBRV-ED and

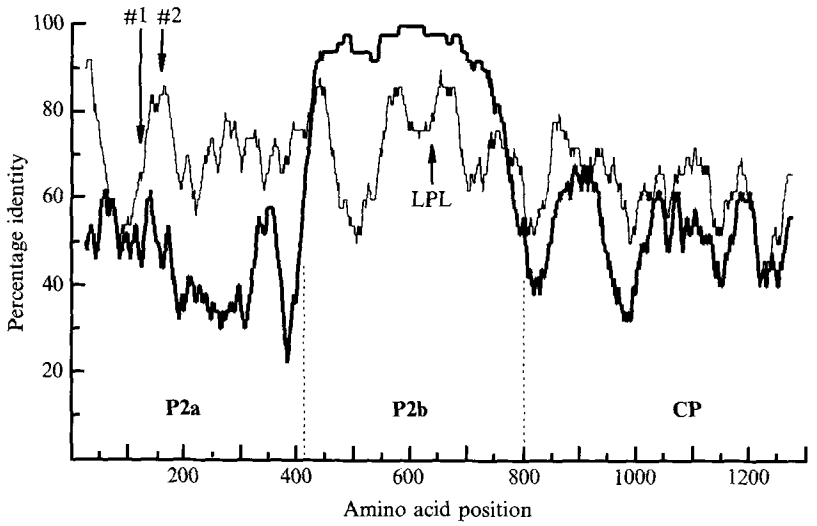

Fig. 2. Percentage amino acid sequence identity along the P2 polyproteins of TBRV-ED and of TBRV-S or GCMV. The percentage identity (according to CLUSTAL V; Higgins *et al.*, 1992), measured in a window of 50 amino acids, was plotted at each position. Thin curve, identity to TBRV-S; thick curve, identity to GCMV. The regions corresponding to P2a, P2b and CP are delineated. The positions of the two 'DnaJ-like repeats' (#1 and #2) and of the 'PL motif' (LPL) are indicated (see text). The amino acid positions indicated only partially reflect the actual positions along the individual sequences since the positions with a gap in any of the sequences were omitted.

GCMV. The level of identity with TBRV-S is steady along the whole sequence but, as seen in Fig. 2, the central region is much more homologous between TBRV-ED and GCMV than the two flanking ones (92·0% identity between positions 447 and 833 of TBRV-ED, including a 181 amino acid block bearing only four conservative changes).

The carboxy-terminal domain of the polyprotein (positions 834–1344 of TBRV-ED) is homologous to the coat protein (CP) regions of TBRV-S (Meyer *et al.*, 1986) and GCMV (Brault *et al.*, 1989), and is likely to also encode the CP in TBRV-ED. The cleavage site allowing its release from P2 would then be at a K/A dipeptide, identical to that present in TBRV-S (Demangeat *et al.*, 1991) and similar to the R/A of GCMV (Brault *et al.*, 1989). Two motifs also found in the CP of other nepoviruses (Serghini *et al.*, 1990; Steinkellner *et al.*, 1992; Blok *et al.*, 1992), $FxGx_6FDAYx(R/K)$ and FxFYGR(S/T), occur at positions 897–911 and 1330–1336 respectively.

The two other domains probably represent the two amino-terminal proteins (denoted P2a and P2b in Fig. 2) described in TBRV-S and GCMV RNA-2 (Demangeat *et al.*, 1991; Hibrand *et al.*, 1992). No significant homology between these two regions and any (non-nepoviral) protein sequence present in data banks was detected using BLAST (Altschul *et al.*, 1990).

In domain P2a, an imperfect duplication of a motif resembling the 'DnaJ repeat' (Ohki *et al.*, 1986) was found in TBRV-ED at positions 132–139 (CvrHhGpG, upper-case letters indicate the characteristic amino acids) and 154–161 (CphCqGtG) and at equivalent positions in TBRV-S and GCMV, but not in other nepoviruses (not shown). In all three viruses, the second cysteine of the first repeat is replaced by an histidine. None of the other typical features of DnaJ proteins, the DnaJ-like NH_2-region and the glycine-rich domain, was found in the P2a domain of TBRV-ED, TBRV-S or GCMV. The biological role of the cysteine-rich repeats in DnaJ proteins is unclear and indeed they are even absent in some DnaJ-like proteins (Cyr *et al.*, 1994), but the presumed binding of metal ions may stabilize the proteins that carry them (Glaser *et al.*, 1992). The cysteine-to-histidine change in the first repeat is a conservative one as far as such binding of metal ions is considered; thus P2a might be a metal-binding protein.

The central domain (P2b) of the TBRV-ED P2 contains, at positions 651–653 (Fig. 2), the LPL motif present in the movement proteins of many plant viruses belonging to various groups (Koonin *et al.*, 1991). This motif is found at an equivalent position in TBRV-S, GCMV (Koonin *et al.*, 1991) and raspberry ringspot virus (RRV; Blok *et al.*, 1992), and in an altered form in grapevine fanleaf virus (GFLV), arabis mosaic virus (ArMV) and tomato ringspot virus (TomRSV) (Mushegian, 1994). In TomRSV and GFLV, the protein located immediately upstream of the CP has been shown to form tubules possibly allowing cell-to-cell movement of the virus (Wieczorek & Sanfaçon, 1993; Ritzenthaler *et al.*, 1994). Although the level of homology between this region of TomRSV or GFLV on the one hand, and TBRV on the other hand, is low (Rott *et al.*, 1991*a*;

Mushegian, 1994; our observation), it can be assumed that, in TBRV also, P2b is likely to be the movement protein.

The significance of the high level of homology, both at the nucleotide and amino acid levels, between TBRV-ED and GCMV in the P2b region (Fig. 2) can be explained either by a common selection pressure that has maintained it or by a recombination event. In order to discriminate between these two possibilities, we produced a multiple alignment of the nucleotide sequences of the coding regions of the RNA-2 of TBRV-ED, TBRV-S and GCMV with PILEUP (GCG package) and used it to run VTDIST (Sawyer, 1989). This program performs a statistical analysis allowing an evaluation of the probability of recombination (versus random mutations) in the evolution of a set of sequences. VTDIST detected recombination both within the set of three sequences described above and between sequences represented and others not represented in the set, with a probability for the distribution observed being due to random mutations less than 10^{-4} for these two types of event.

In order to confirm this result, we determined the percentage identity at the nucleotide level at the third positions of codons encoding amino acids (*i*) conserved between GCMV and TBRV-ED, and (*ii*) having a four base degeneracy at their third position (codons encoding Ala, Gly, Pro, Thr and Val). Since these nucleotides are supposed to vary independently of the encoded amino acid sequence, similar selection pressure at the protein level and recombination would result in levels of identity respectively similar and higher as compared to the rest of the sequence. The level of identity found at these positions in domain P2b, 55/100, is significantly higher than that in domains P2a and CP, 61/166 ($\chi^2 = 8{\cdot}46$, $P = 3{\cdot}8 \times 10^{-3}$). TBRV-ED RNA-2 is thus the probable result of an interspecific double recombination event, between a yet uncharacterized virus and a virus close to GCMV, that has resulted in the transfer of the whole P2b domain. The actual co-existence of TBRV-English and GCMV in a field isolate has been described for celery yellow vein disease (Hollings *et al.*, 1969), a situation that may have resulted in natural recombination.

In-frame natural RNA recombination events resulting in the exchange of parts of the coding region have been observed between strains of another plant picorna-like virus, plum pox potyvirus (Cervera *et al.*, 1993) as well as in other potyviruses (F. Revers, unpublished) and in luteoviruses (Gibbs & Cooper, 1995). In addition, the terminal regions of the genomic RNAs of some nepoviruses could be the subject of recombination [Rott *et al.*, 1991*b*; Scott *et al.*, 1992; Bacher *et al.*, 1994; Le Gall *et al.*, 1995 (accompanying paper)].

The percentage amino acid sequence identity in the CP domains between TBRV-ED and TBRV-S (62·0%) is lower than between the two virus species GFLV and ArMV (68·5%). However, there are two indications that identity levels in the CP gene are representative of the global level of identity between the two genomes. Firstly, it is in the same range as that observed between the non-structural genes encoded by RNA-2 (Fig. 2). Secondly, an essentially similar figure was observed for a short segment of the RNA polymerase, encoded by RNA-1, fortuitously sequenced for TBRV-ED in the course of this project (not shown). Thus, on the basis of sequence homology, the English and Scottish serotypes of TBRV (Harrison, 1958) could be considered as two distinct, though related, viral species. On the other hand, the close biological relationships between TBRV-ED, TBRV-S and GCMV, between which cross-protection (Harrison, 1958; Doz *et al.*, 1982), pseudorecombination (Harrison & Murant, 1977; Doz *et al.*, 1980) and RNA recombination (this work; Le Gall *et al.*, 1995) can occur, suggest that they behave biologically as a single species.

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