Arch Virol (2001) 146: 127–133

Archives of Virology

Complete nucleotide sequence of RNA-2 of *Olive latent ringspot virus**

Brief Report

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Accepted July 18, 2000

Summary. The complete nucleotide sequence of *Olive latent ringspot virus* (OLRSV) RNA-2 was determined. This RNA is 3969 nucleotides in length and contains a single open reading frame of 3448 nt, that encodes a polypeptide of 1146 amino acids, with a calculated M_r of 126,044. OLRSV RNA-2 has a structural organization typical of nepoviruses, with the coat protein (CP) cistron located in the C-terminal and the putative movement protein (MP) in the N-terminal regions of the polyprotein. Computer-assisted comparison of coat proteins of OLRSV and other nepoviruses disclosed relationships that tally with subgrouping based on physicochemical properties.

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Olive latent ringspot virus (OLRSV), a definitive nepovirus species isolated from symptomless olive trees in Italy [5, 25], has isometric particles *c.* 28 nm in diameter and a single capsid protein with a molecular mass of 57,600. The bipartite single-stranded RNA genome consists of two molecules with M_r 1.4×10^6 (RNA-2) and 2.65×10^6 (RNA-1), respectively [25]. OLRSV RNA-2 has now been completely sequenced, and the taxonomic relationship of this virus with other members of the *Nepovirus* genus [9] is discussed.

The same OLRSV isolate used in previous studies [5, 25], was propagated in *Chenopodium quinoa* and purified as described [25]. Nucleic acids were extracted from sucrose gradient-fractionated virus preparations [12] and further purified by polyacrylamide gel electrophoresis (PAGE) [24]. The presence of a poly(A) tail

*OLRSV RNA-2 sequence is deposited in the EMBL database under the accession number AJ277435.

was ascertained by chromatography on oligo(dT) cellulose columns. cDNA synthesis and cloning were performed as described [13] and small overlapping clones were produced from the original ones [10]. Plasmid DNA was prepared using a commercial kit (JetQuick, Genomed, Germany) and sequence analysis done with T7 DNA polymerase (Sequenase, Amersham, U.S.A.) or by custom automatic sequencing (MWG, Germany). The 5′-terminal 95 nucleotides (nt) of RNA-2 were cloned by rapid amplification of cDNA ends (RACE) protocol [14], using the primer 1 (5′-CTCGTAAGCCTCTAATGGT-3′, complementary to nt 399 to 417 of RNA-2 sequence) and primer 2 (5′-TGGCCAACCTAGCAAGAAG-3′, complementary to nt 115 to 134 of RNA-2 sequence). The 5′-end was directly sequenced by dideoxy-terminated reverse transcription using primer 2 [11]. Primer-extension experiments to determine the exact length of RNA-2 were as described [1].

For peptide sequencing, virus preparations were analysed by PAGE and electrophoretically blotted onto a polyvinylidene difluoride (PVDF) protein sequencing membrane, in a medium containing 10 mM CAPS [3-(cyclohexylamino)-1-propanesulphonic acid]/10% methanol, pH 11.0. Peptides were sequenced with an automatic peptide sequencer (mod. 473A, Applied Biosystems) [16]. The nucleotide sequence was assembled and analysed with the DNA Strider Program [18] and the assistance of the University of Wisconsin Genetics Computer Group programs [2]. Comparisons with the SWISS-PROT protein database of proteins potentially expressed by OLRSV RNA-2 were made with the automatic electronic mail server FASTA [22].

The PILEUP program [2] was used for alignment of amino acid sequences of OLRSV polypeptide with the corresponding proteins (SWISS-PROT database, release 38.0) of the following nepoviruses: *Artichoke italian latent virus* (AILV), *Arabis mosaic virus* (ArMV), *Blueberry leaf mottle virus* (BBLMV), *Blackcurrant reversion virus* (BRV), *Cherry leaf roll virus* (CLRV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine fanleaf virus* (GFLV), *Raspberry ringspot virus* (RRSV), *Tomato black ring virus* (TBRV), *Tomato ringspot virus* (ToRSV), and *Tobacco ringspot virus* (TRSV). The coat protein of *Artichoke mottle crinkle virus* (AMCV) and the movement protein of *Olive latent virus 2* (OLV-2) were used as outgroups in phylogenetic analysis. Cluster dendrogram analysis was made with the assistance of the SEQBOOT, PROTPARS, CONSENSE and DRAWGRAM programs from the PHYLIP version 3.4 package [6].

OLRSV RNA-2 was 3969 nt in length excluding the poly(A) tail. Except for the 110 5′-terminal nucleotides the sequence was determined on at least two independent cDNA clones in both directions. The 3′-terminal sequence was determined by sequencing 9 independent cDNA clones containing the natural poly(A) tail. When RNA-2 was directly sequenced using primer 2, the sequence showed two terminal strong stops, thus resembling that reported for ToRSV [23]. However, when cloning and sequencing of the 5′-terminal 95 nt was by RACE, eight independent RACE cDNA clones yielded an identical sequence, with CA in the first and second position, respectively. Thus, the first nucleotide of the sequence was designed N, because it could not be determined if the C residue was part of the viral sequence or of the artificial poly(C) tail added during RACE protocol.

Table 1. Percentage amino acid sequence similarity between putative movement protein (MP) and coat protein (CP) of OLRSV and the corresponding genomic regions of sequenced nepoviruses

Virus	CP	MP
AILV	49	–
ArMV	50	61
BBLMV	47	–
BRV	47	42
CLRV	50	–
GCMV	45	44
GFLV	49	59
RRSV	49	45
TBRV	46	43
ToRSV	47	69
TRSV	50	–

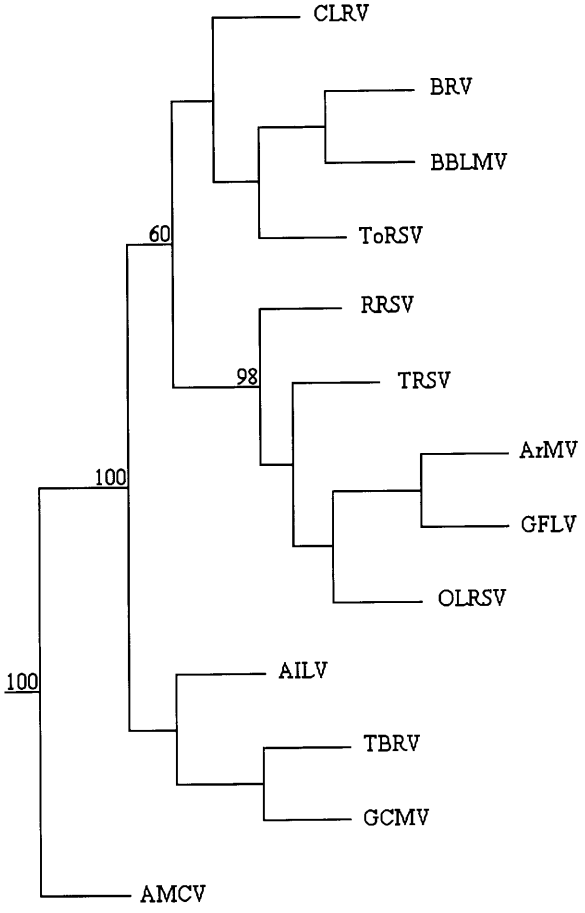

Fig. 1. Relationship dendrogram of deduced amino acids sequences of coat proteins of OLRSV and members of *Nepovirus* genus. AMCV CP, an unrelated protein, was selected as outgroup. Bootstrap scores are on relevant horizontal branches. Database accession numbers: AILV, Q64959; ArMV, Q65029; BBLMV, Q65624; BRV, Q9YK98; CLRV, Q86631; GCMV, P13026; GFLV, P18474; RRSV, P36324; TBRV, P14547; ToRSV, P25247; TRSV, Q88894; AMCV, P14836

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ArMV      LEILYNPVSDEEMEDYRDRGMSAVVIDALEIAINPFGMPGNPTDLTVVATYGHERNMERAFI   (353-414)
GFLV      LEILYNPVSEEEMEEYRDRGMSAVVIDALEIAINPFGMPGNPTDLTVVATYGHERDMTRAFI   (452-513)
OLRSV     MVYYFNPVSQQEIQDAQGQGNDTVVIDAIEISILPFGMPGDATDITGVVMWAQNSDPERAFI   (391-452)
ToRSV     FSFYFNPVSEQEMAEQERGGNTMLSLDAVEVVIDPVGMPGDDTDLTVMVLWCQNSDDQRALI   (1079-1140)
GCMV      TFTVLNRPQFKELNRLAEVGWKEAKSVCLNLHIRSYLPVHLPVYAFCVIMWGHSSNAEQASL   (549-610)
TBRV      VFTVLNRPLYNELNKLAESGWKEAKSVCLNLHIRSYVPVHTPLYAFCVIMWGHSSDAETASL   (576-637)
BRV       VFTPLPRMSDEMLRKFLEKGLGSTSTVALDIGIQSHIPQGMPTVAFVNVMDTRIEDPLYSSL   (839-900)
RRSV      EWINLPRMSENSARKLAEVGWNNADICGVDLAVKSHIAVGTPVRVIISLVDGACSDMPTATM   (344-405)
             §§n     e       G   §    § §  i       p   §   §          a §   consensus

ArMV      GSSSTFLGNGLARAIFFPGLQ--YSQEEPRRESLIRLYVASTNATVDADSVLAAISVGTLRQ   (415-475)
GFLV      GSASTFLGNGLARAIFFPGLQ--YSQEEPRRESIIRLYVASTNATVDTDSVLAAISVGTLRQ   (514-573)
OLRSV     GSLSTFVGNGLARAIFFPQLHLSYEHCQVPDNRVLKVILSSTNSTMVGDLVQARVSLGTLRQ   (453-514)
ToRSV     GAMSTFVGNGLARAVFYPGLKLLYANCRVRDGRVLKVIVSSTNSTLTHGLPQAQVSIGTLRQ   (1141-2002)
GCMV      SGAYVYLGDQEASVLQLPLLCGYIGNALEDMEAYKRSLVLSTCFFGTSG-----LSPGQNMF   (611-667)
TBRV      CGAGVYLGDQEAAVLELPLVCSYLGNSLEDFDAYKRSLVLSTVFFGKSG-----LFAGQNVF   (638-694)
BRV       CGSYIDLGRDRAKTLCLPLVNFPMSKLAEDVDDVLNGLMLCTHFQDSTK-----FGVGKPAF   (901-957)
RRSV      CAFEVNLASQNNRSLNLPLLSLPFSRLLADLHDFQNRVKIACQFRDPEG-----FNVGTPML   (406-462)
             §g   a  § §P     §         §   §§§st           § §G      consensus
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Fig. 2. Alignment of the conserved domain of nepovirus putative movement proteins. The conserved proline (P) residue is boxed. In the consensus line: § = hydrophobic amino acids flanking the P motif; lower case = amino acids conserved in six or seven sequences; upper case = amino acids conserved in all sequences. Gaps are indicated by dashes. The position of aligned blocks in the viral polyprotein is shown in parentheses. Database accession numbers are the same as in Fig. 1

The 5′ non coding region (NCR) was 138 nt long with a U+A content (63.5%), typical of leader sequences of plant viruses [8]. The sequence of the first 10 nt (NUGAAAAUAA) was consistent with the consensus motif proposed for nepovirus RNAs [7]. A putative ribosome binding site [15] was found 11 nt upstream of the first AUG codon, which could potentially form 12 pairings (out of 18 residues) with the 18S ribosomal RNA 3′ terminus.

The 3′ NCR (393nt long) had a poly(A) tail, as determined by chromatography on oligo(dT)-cellulose column. Comparison of this sequence with the 3′ NCR of other nepoviruses showed 67% identity in the last 150 nt of the comparable region of BBLMV, the presence of the nucleotide block GUCCACAAAAAUAUUUG similar to a residue stretch conserved in nepoviral 3′ NCR [26], and the octamer UUUCUUUU, found in the comparable genomic region of GFLV, TBRV and GCMV [26].

OLRSV RNA-2 contained a single open reading frame (ORF) of 3448 nt, encoding a protein of 1146 amino acids with M_r 126,044. This ORF was comprised between a AUG codon in position 139 and a UAG stop codon in position 3574. The initiation codon was in a favourable translational context with A and G in position −1 and +4, respectively [17].

The location of the coat protein (CP) gene and the putative cleavage site were ascertained by sequencing the N-terminus of purified CP. The sequence of the N-terminal 10 amino acids of OLRSV CP proved to be AGSTQQESER, which is identical to the sequence between residues 623 to 632 of the polyprotein.

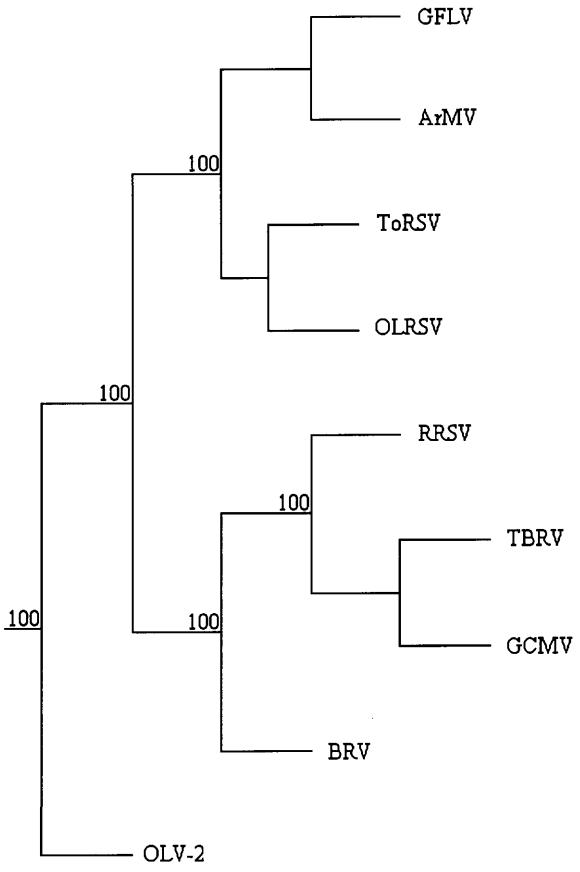

Fig. 3. Relationship dendrogram of deduced amino acid sequences of putative MPs from OLRSV and members of the *Nepovirus* genus. OLV-2 MP (accession Q9YNE3) is an unrelated protein selected as outgroup. Bootstrap scores are on the relevant horizontal branches. Database accession numbers are the same as in Fig. 1

The CP gene started at nt 2005, consisting of 523 amino acids with a calculated M_r 57,916, in good agreement with the M_r 57,600 determined by SDS PAGE [25]. The proteolytic cutting site, identified as K/A between residues 622 and 623 of the polyprotein amino acids sequence, was the same as that reported for TBRV [4].

Comparison of OLRSV CP with proteins from the SWISS-PROT database revealed a good similarity with CPs of other nepoviruses (Table 1). A cluster of six amino acids (FYGRSA, aa 1134 to 1139 of the polypeptide) fitted the C-terminal nepoviral motif FYGRSX, which is supposed to stabilize the CP quaternary structure [3]. Phylogenetic analysis using amino acid sequences of CPs of other nepoviruses (Fig. 1) showed that OLRSV CP clustered with CPs of nepoviruses with a heterogenous B component (particles encapsidating one molecule of RNA1 or two molecules of RNA2).

Pairwise comparison of the polyprotein region N-terminal to OLRSV CP, i.e. the putative cell-to-cell movement protein (MP) [20], with comparable polypetide portions of other nepoviruses showed a high level of similarity with ToRSV (69%), ArMV (61%) and GFLV (59%) (Table 1). In the phylogenetic tree constructed with available nepoviral MPs sequences, OLRSV clustered with the three above viruses, which belong to Mushegian's [21] nepovirus family A (Fig. 3). OLRSV

RNA-2 sequence contained also the proline residue (aa 470) surrounded by a hydrophobic domain (Fig. 2), which occurs in all known nepovirus sequences [21].

No significant sequence similarity was found between the N-terminal 250 amino acids encoded by OLRSV RNA-2 and proteins in the SWISS-PROT database, and pairwise alignment of this region with the similarly located regions of other nepoviruses revealed no consensus sequences.

The results of the present study have shown that OLRSV RNA-2: (i) is monocistronic and polyadenylated at the 3′ end; (ii) its 5′- and 3′ NCR share a number of consensus sequences with other nepoviruses [7, 26]; (iii) it codes for a polyprotein, whose C-terminal region is the capsid protein, whereas the region N-terminal to CP encodes a putative cell-to-cell movement protein. All these features are consistent with alleged molecular properties of nepoviruses, thus adding strength to the assignment of OLRSV as a definitive species in the genus *Nepovirus* [5, 25]. Interestingly, comparison of different nepoviral CPs sequences generated a tree with three distinct clusters, which tallies with a previous tentative grouping of the *Nepovirus* genus based on physicochemical properties of its members [19].

Acknowledgements

The authors thanks Prof. S. Papa and Dr. T. Cocco of the Dipartimento di Biochimica Medica e Biologia Medica (University of Bari) for OLRSV CP sequencing. This work was supported by a grant of the European Union/Italian Ministry of Agricultural Policies “Programma Operativo Multiregionale: attività di sostegno ai servizi di sviluppo per l’agricoltura, misura 2, progetto A32.”

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Received June 5, 2000