

## SOME PROPERTIES OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2 AND MOLECULAR ORGANIZATION OF THE 3' REGION OF THE VIRAL GENOME

N. Abou-Ghanem, S. Sabanadzovic, A. Minafra, P. Saldarelli and G.P. Martelli

*Dipartimento di Protezione delle Piante, Università degli Studi,  
and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, I-70126 Bari, Italy*

### SUMMARY

Grapevine leafroll-associated virus 2 (GLRaV-2) was purified from *Nicotiana benthamiana*. The molecular mass of viral coat protein subunits determined by polyacrylamide gel electrophoresis was ca 21.5 kDa. Double-stranded RNA was isolated from infected *N. benthamiana* and used for cloning and sequencing. Molecular probes and primers generated during cloning were successfully used for virus detection in infected grapevines by dot spot hybridization and reverse-transcription polymerase chain reaction. The sequence of the 3'-terminal 8590 nucleotides of the viral genome was determined, encompassing eight open reading frames (ORFs). The first ORF consisted of two parts, ORF1a, which was incompletely sequenced and contained the conserved domains of virus helicases in its 3' region, and ORF1b, whose product (RNA dependent RNA polymerase) is apparently expressed via a +1 ribosomal frameshift. The ORFs that followed in the 5'-3' direction, encoded proteins of 6 kDa (ORF2); 65 kDa (ORF3), identified as a homologue of cell heat shock proteins; 63 kDa (ORF4); 25 kDa (ORF5), identified as a diverged copy of the coat protein (CP); 22 kDa (ORF6) identified as the CP; 19 kDa (ORF7) and 24 kDa (ORF8). The structural organization of the genome was virtually identical to that of beet yellows virus, the type species of the genus *Closterovirus*, and also resembled that of citrus tristeza virus. This similarity was confirmed by comparative analysis of phylogenetically relevant proteins. GLRaV-2 has morphological, physico-chemical, ultrastructural, and molecular properties that qualify it as a species in the genus *Closterovirus*.

### RIASSUNTO

#### ALCUNE PROPRIETÀ DEL VIRUS 2 ASSOCIATO ALL'ACCARTOCCIAMENTO FOGLIARE DELLA VITE ED ORGANIZAZIONE MOLECOLARE DELLA REGIONE 3' DEL GENOMA VIRALE.

Su preparati del virus 2 associato all'accartocciamento fogliare della vite (GLRaV-2) purificati da *Nicotiana benthamiana*, è stato determinato il peso molecolare della proteina capsidica che è risultato di ca 21.500 daltons. Il clonaggio e sequenziamento dell'RNA genomico virale è stato effettuato da dsRNA estratto da piante infette di *N. benthamiana*. Sonde molecolari ed inneschi ottenuti durante il clonaggio sono stati usati con successo per l'identificazione di GLRaV-2 in vite mediante ibridazione su membrana e amplificazione genica (PCR). Un tratto di 8590 nucleotidi nella porzione 3' terminale del genoma virale è stato sequenziato, identificando otto griglie di lettura (ORF). La ORF 1 è costituita da due parti, di cui la prima (ORF1a) è stata sequenziata solo nella zona 3' terminale che contiene i domini conservati delle elicasi virali. La seconda parte della ORF1 (ORF1b) codifica la polimerasi virale (RdRp), che viene espressa attraverso un meccanismo di "frameshift" ribosomale +1. Le sette ORF che seguono codificano rispettivamente proteine di: 6 kDa (ORF2); 65 kDa (ORF3) identificata come un omologo delle proteine cellulari da shock termico; 63 kDa (ORF4); 25 kDa (ORF5), identificata come duplicato della proteina capsidica; 22 kDa (ORF6), identificata come proteina capsidica; 19 kDa (ORF7) e 24 kDa (ORF8). L'organizzazione genomica di GLRaV-2 è praticamente identica a quella del closterovirus del giallume della barbabietola (BYV) ed è simile a quella del closterovirus della tristezza degli agrumi (CTV). La similitudine è stata confermata dall'analisi comparativa delle proteine filogeneticamente rilevanti. Pertanto, GLRaV-2 possiede caratteristiche morfologiche, fisico-chimiche, ultrastrutturali e molecolari che lo qualificano come specie definitiva del genere *Closterovirus*.

*Key words:* grapevine, closterovirus, GLRaV-2, cloning, sequencing, diagnosis, molecular hybridization, PCR.

## INTRODUCTION

Leafroll is a graft-transmissible disorder of grapevines seriously affecting plant vigour and yield (Martelli, 1993). The putative agents of this disease are serologically unrelated closteroviruses, seven of which, denoted grapevine leafroll-associated viruses (GLRaV-1 to -7), have been identified (Boscia *et al.*, 1995; Choueiri *et al.*, 1996). Of these viruses, only GLRaV-2 (Boscia *et al.*, 1995; Goszczynski *et al.*, 1996a, 1996b) and GLRaV-6 (Gugerli *et al.*, 1997) have been transmitted by sap inoculation to *Nicotiana benthamiana*, the only infectible host. This allowed ultrastructural studies (Castellano *et al.*, 1995) and partial physicochemical characterization (Goszczynski *et al.*, 1996a, 1996b) of GLRaV-2, opening the way for molecular studies, as briefly reported by Abou-Ghanem *et al.* (1997) and, more extensively, in the present paper.

## MATERIALS AND METHODS

**Virus source and purification.** The GLRaV-2 isolate was the same as used in previous studies (Boscia *et al.*, 1995; Castellano *et al.*, 1995), and was propagated in *N. benthamiana* and purified as described by Namba *et al.* (1991) from systemically infected plants.

Coat protein was dissociated by boiling purified virus preparations for 5 min in the presence of Laemmli buffer. It was electrophoresed in 12.5% polyacrylamide slab gels (PAGE) in a discontinuous buffer system, and stained with Coomassie brilliant blue (Laemmli, 1970). Reference molecular weight markers were coat protein subunits of grapevine vitiviruses A (GVA) and B (GVB), and a MW-SDS-70L kit (Sigma Chemical Co., St. Louis).

**Total nucleic acids and double-stranded RNAs.** Total nucleic acids (TNAs) were obtained as described by White and Kaper (1989), from healthy and infected *N. benthamiana* and grapevines.

Double-stranded RNAs (dsRNAs) were recovered from infected *N. benthamiana* by phenol extraction and chromatography through cellulose CF-11 (Dodds, 1993). Preparations were incubated with RNase-free DNase (60 µg ml<sup>-1</sup>) and DNase-free pancreatic RNase (0.5 µg ml<sup>-1</sup>) (Saldarelli *et al.*, 1994), then analysed in 6% PAGE under non-denaturing conditions, in comparison with dsRNAs from citrus infected with tristeza virus (CTV), *Chenopodium quinoa* infected with beet yellows virus (BYV) and grapevines infected with GLRaV-3. The gels were stained with silver nitrate. The relative sizes of the dsRNAs were calculated using as markers the double-stranded replicative forms of genomic and subgenomic RNAs of CTV and BYV

(Serghini *et al.*, 1990; Guerri *et al.*, 1991; Agranovsky *et al.*, 1994; Mawassi *et al.*, 1995).

**cDNA cloning and sequencing.** Because of the exceedingly low yields of purified virus preparations, cDNA was cloned from dsRNAs extracted from infected *N. benthamiana*. dsRNA preparations were denatured by 20mM methyl mercuric hydroxide and primed with random DNA hexanucleotides using "cDNA System Plus" (Amersham). Double-stranded cDNA was inserted into the *Sma*I-cut dephosphorylated plasmid vector pUC18 (Pharmacia) and cloned in *Escherichia coli* DH<sub>5α</sub> cells. Sequencing was performed on both strands of cDNA by the dideoxynucleotide chain termination method, using <sup>35</sup>S-ATP and the Thermo Sequenase cycle sequencing kit (Amersham). Smaller subclones were generated by restriction digestion and cloned in the polylinker of pUC18. An excised fragment (460 bp) from a 3' coterminal clone (p38) was purified to synthesize an oligolabelled DNA probe (Dig DNA Labelling Kit, Boehringer), which was used in hybridization assays.

For 3' end sequence determination, GLRaV-2 RNA was polyadenylated by *E. coli* poly(A) polymerase (Gibco-BRL) and reverse transcribed using oligo(dT)<sub>16</sub> and Moloney murine leukemia virus reverse transcriptase. First-strand cDNA thus obtained was PCR-amplified using an oligo(dT)<sub>32</sub> and the specific (+) sense primers aV-2(38) (5'AGAGAAGCGGTGAAGAGG3'), located close to the 3' end. The PCR product was ligated to a pGEM-T vector (Promega), transformed into *E. coli* DH<sub>5α</sub> competent cells, and sequenced.

**Computer-assisted analysis of nucleotide and protein sequences.** Nucleotide and protein sequences were analysed using the Strider 1.1 programme (Marck, 1988). Protein sequences were aligned with CLUSTAL V (Higgins and Sharp, 1988). Search for homologies of putative proteins from the Protein Information Resources (PIR, release 47.0), was done with the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programmes. Tentative phylogenetic trees were constructed, and bootstrap analysis made with the NEIGHBOR, SEQBOOT, PROTDIST, and CONSENSE programmes of the PHYLIP package (Felsenstein, 1989).

**Molecular hybridization.** TNA and dsRNAs extracts from grapevines infected with GLRaV-1, -2, -3, -5, and -7, as well as TNAs and purified dsRNAs from GLRaV-2-infected *N. benthamiana*, were spotted onto nylon membranes (Hybond N<sup>+</sup>, Amersham) after denaturation with 50% formamide, 6.5% formaldehyde in MAE buffer [2 mM 3(N-Morpholino)-propane-sulfonic acid, 0.5 mM sodium acetate pH 7.0, 1 mM Na<sub>2</sub> EDTA] at 65°C for 5 min. The membranes were hybridized

overnight at 42°C using the Dig-labelled DNA probe p38 according to the manufacturer's instructions (Dig DNA Labeling Kit, Boehringer, Mannheim). Hybridization signals were detected by chemiluminescence according to the manufacturer's instructions (Dig Luminescent Detection Kit, Boehringer, Mannheim).

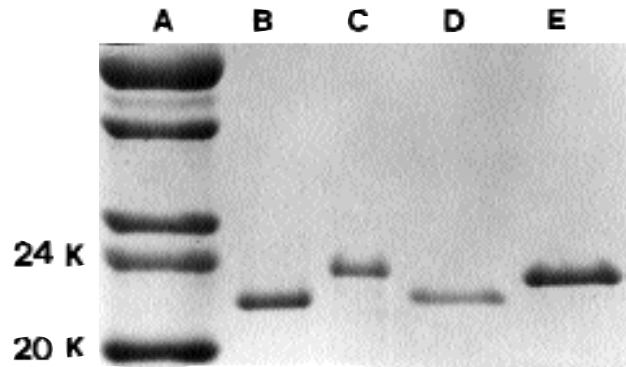
**Reverse transcription-polymerase chain reaction (RT-PCR).** GLRaV-2-specific oligonucleotides LRAV-2(1) (5'AGGCGGATCGACGAATAC3'-sense primer) and LRAV-2(2) (5'ATCCTGTCCGGCGCTGTG3' - antisense primer), were used to amplify an 821 nt fragment of the GLRaV-2 genome. About 250 mg of cortical scrapings from dormant cuttings or from leaf and petiole tissues were ground as described by Wetzal *et al.* (1991) and cDNA synthesized according to Minafra and Hadidi (1994). Five µl (of a total of 50 µl) of cDNA were mixed with 44 µl of the amplification mixture (1x Taq Promega buffer, 1.0 mM MgCl<sub>2</sub>, 200 mM of each dNTP, 120 nM of each primer). After a hot start step at 94 °C for 1 min and 80 °C for 30 sec, 1 unit of Taq DNA polymerase (Promega Corporation, Madison USA) was added. Cycling was as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 52°C, extension for 1 min at 72°C, for 35 cycles, and final extension for 7 min at 72 °C. PCR-amplified products were analyzed by electrophoresis in 6% polyacrylamide gel in 1x TBE buffer and visualized by silver staining (BioRad Laboratories, Hercules, USA).

## RESULTS AND DISCUSSION

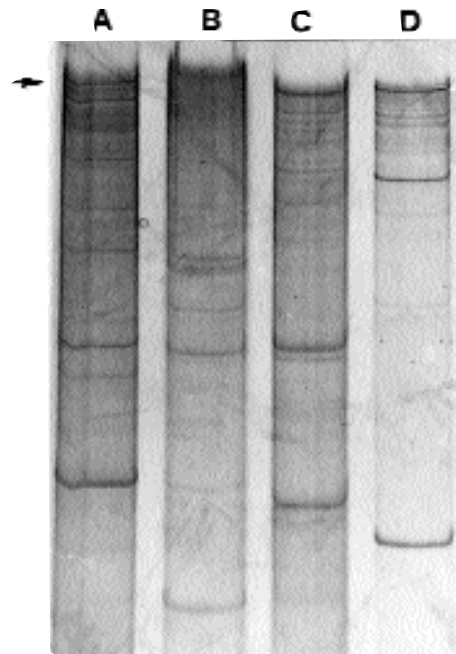
**Physicochemical properties.** Purified virus preparations from cesium sulfate gradients contained a single coat protein (CP) with an estimated  $M_r$  of 21.5 kDa (average of three readings) (Fig.1). This figure tallies with the electrophoretic estimate by Zimmermann *et al.*, (1990) and, as specified below, with the size predicted from sequence analysis of the CP cistron. Thus, it may be closer to the actual CP size than the higher values reported by others (Gugerli and Ramel, 1993; Goszczynski *et al.*, 1996b)

The electrophoretic pattern of dsRNAs extracted from infected *N. benthamiana* was complex like those of CTV, BYV, and GLRaV-3, consisting of a large dsRNA of ca 16 kbp (mean value from five different gels) and several bands of lower  $M_r$  supposed to correspond to subgenomic RNAs (Fig.2).

**Virus detection by molecular hybridization and RT-PCR.** In dot spot assays, probe p38 specifically hybridized with GLRaV-2 dsRNA extracts from *N. benthamiana* and TNA extracts from infected *N. bentha-*

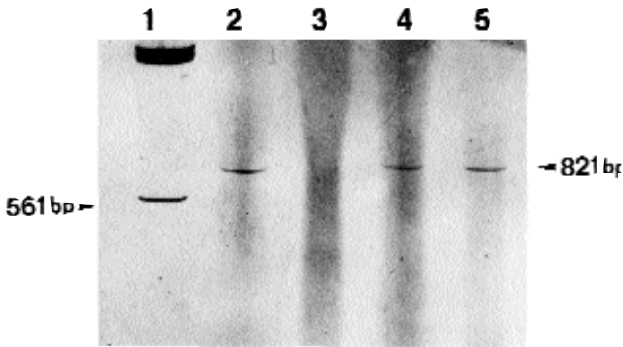


**Fig. 1.** Electropherogram of dissociated coat protein preparations of GLRaV-2 (lanes B and D), GVB (lane C) and GVA (lane E). Mol. wt markers in lane A.



**Fig. 2.** Electrophoretic patterns of dsRNAs extracted from GLRaV-2-infected *Nicotiana benthamiana* (lane A), CTV-infected citrus (lane B), BYV-infected *Chenopodium quinoa* (lane C) and GLRaV-3-infected grapevine (lane D). The arrow indicate the presumed GLRaV-2 full-genome dsRNA.

*miana* and grapevine. No hybridization was obtained with GLRaV-1,-3, and -7 dsRNAs, healthy grapevine and *N. benthamiana* extracts, and with TNA extracts from healthy vines and vines infected by GLRaV- 1,- 3,- 5 , and -7. A DNA fragment of the expected size (821 bp) was amplified from cortical scraping extracts from cv. Semillon vine, from which the virus was originally isolated (positive control), and local vines known to be infected by GLRaV-2, but not from the negative control (Fig. 3). These molecular tools have the potential to be developed into a sensitive and specific GLRaV-2 detection assay useful in field surveys.



**Fig.3.** Polyacrylamide gel electrophoresis of RT-PCR products amplified from grapevine sap infected by GLRaV-2 (lanes 4 and 5). Positive control (grapevine cv. Semillon) (lane 2), healthy plant extract (lane 3). Marker (*Hind III*-cut  $\lambda$  phage DNA) (lane 1).

**Nucleotide sequence of the 3' end of GLRaV-2 RNA.** Of 400 recombinant plasmids analyzed, 15 were sequenced, and yielded the sequence of 8590 nt in the 3' terminal region of the genome (Fig. 4). Three cDNA clones coterminal with the 3' region showed identical ends, suggesting that they represented the 3' terminus of the genome. A single nucleotide substitution was found between the sequences of individual clones, *i.e.* that of G for A at nt 5380, which led to an Arg for Gln substitution in ORF4 encoding the 63K protein.

The 8590 nt sequence has been deposited in the EMBL database under the accession number Y14131.

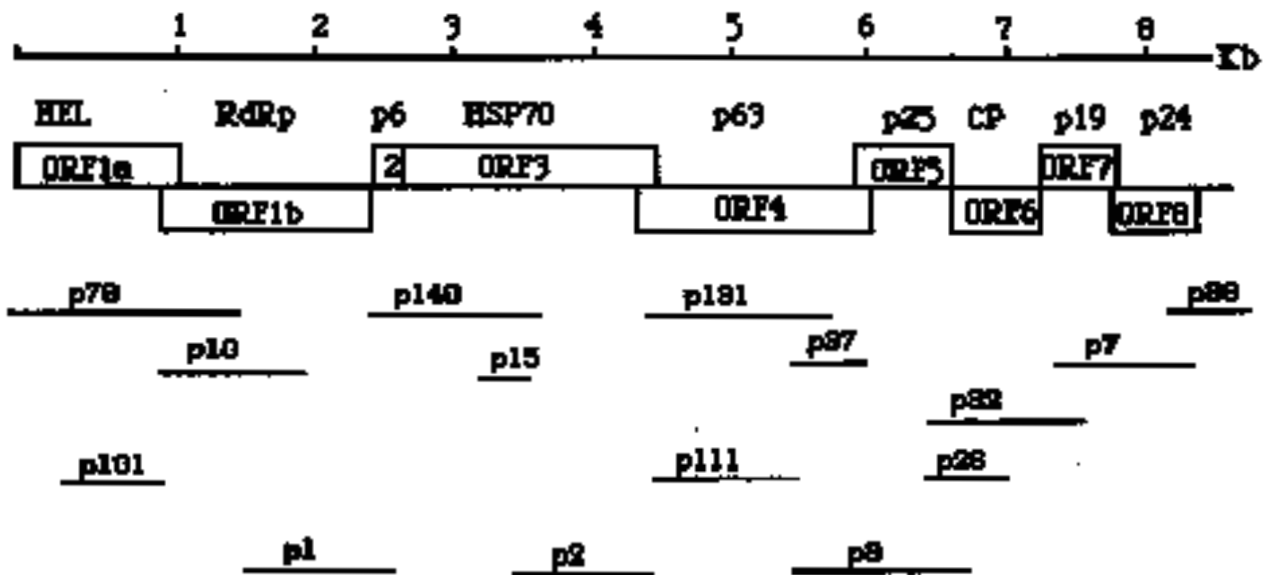
**Structure and organization of the 3'-terminal region**  
In the 5' to 3' direction, the 8590 nt-long sequence contained 8 open reading frames (ORFs) and a 3' non-coding region (NCR) of 180 nt (Fig.4).

The sequenced portion started with the incomplete ORF1a with a UAG termination codon (amber), which encoded a 314 amino acid (aa) protein containing motifs characteristic of virus helicases (Hel) (Gorbalenya and Koonin, 1993).

ORF1b initiated at AUG in position 1165, terminating with an amber stop (UAG) at position 2427. This ORF encoded a 421 aa protein with a  $M_r$  of 47.8 kDa (48K), containing the eight conserved motifs characteristic of viral replicases (RdRps) (Koonin, 1991; Koonin and Dolja, 1993) (Fig. 5). The alignment with RdRp sequences of other closteroviruses showed a degree of similarity and identity extending far beyond the conserved motifs identified by Koonin and Dolja (1993) (Table 1).

Phylogenetic analysis of GLRaV-2 RdRp with those of other positive-strand RNA viruses revealed the closest relationship with high bootstrap values with RdRps of other closteroviruses (Fig. 6). In particular the percentage similarity and identity with RdRps of BYV, Beet yellow stunt virus (BYSV), and CTV varied from 73 to 80% (similarity) and 56 to 61% (identity) (Table 1).

Closteroviruses are thought to express their RdRps via a +1 ribosomal frameshift (Agranovsky *et al.*, 1991; Karasev *et al.*, 1995; 1996; Klaassen *et al.*, 1995; Jelkmann *et al.*, 1997). For BYV, a model of the +1



**Fig.4.** Schematic representation of the sequenced fragments of the GLRaV-2 genome and of the sequencing strategy used. The relative positions of the ORFs and their expression products is shown. The lines below the genome represent the relative size and position of random primed (p) cDNA clones used to determine the nucleotide sequence.

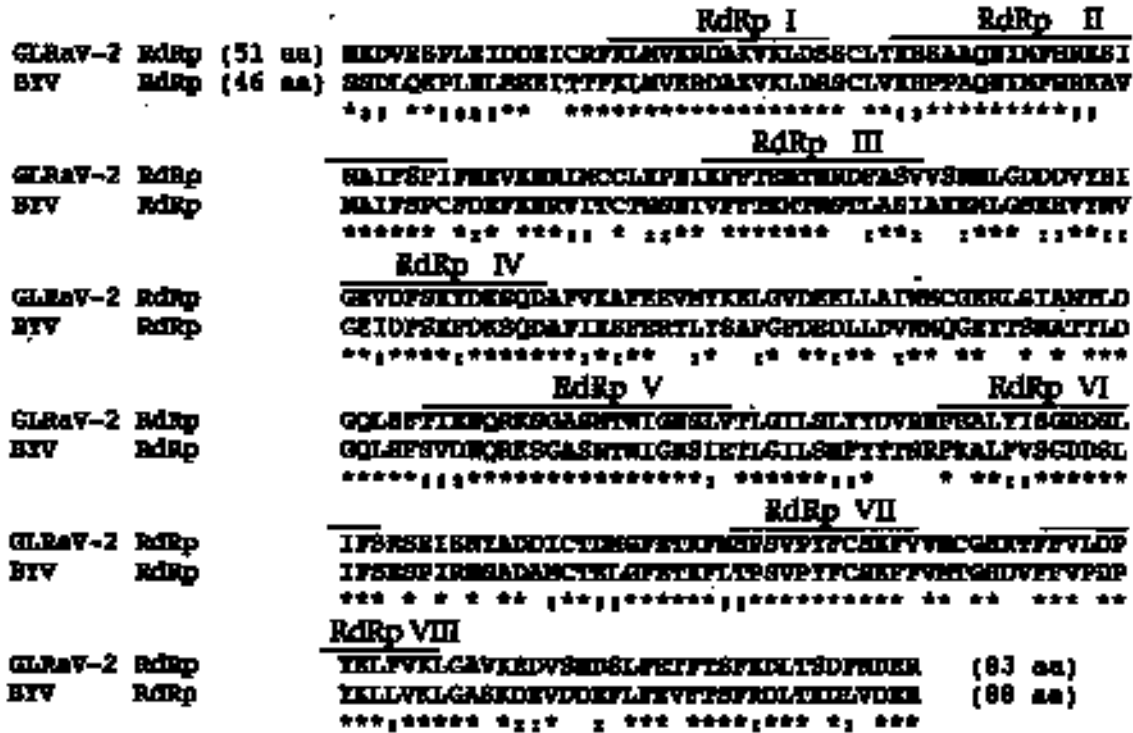


Fig.5. Alignment of the predicted amino acid sequences of the conserved motifs of RNA dependent RNA polymerase domains of GLRaV-2 and BYV. Asterisks indicate identical residues and colons indicate similar ones. The conserved motifs are from Koonin and Dolja (1993).

Table 1. Percentage of similarity (S) and identity (I) of GLRaV-2 RdRp, HSP70, and CP with related proteins of monopartite closteroviruses.

	GLRaV-2		HSP70		CP	
	RdRp		S	I	S	I
BYV	77.0	58.8	70.6	47.7	59.6	35.8
CTV	73.3	56.0	64.2	41.5	54.0	25.2
BYSV	80.7	61.4	69.6	47.4	57.0	34.3
LChV	58.8	32.8	55.0	28.4	58.0	22.2

frameshift has been proposed, implicating the involvement of a GGGUUU slippery sequence immediately upstream of the UAG terminator codon (Agranovsky *et al.*, 1994). Analysis of the GLRaV-2 sequence in the ORF 1a/1b overlapping region (125nt) suggested a +1 ribosomal frameshift for expression of the ORF1b-encoded RdRp. In fact, the alignment of GLRaV-2 and BYV sequences within ORF 1a/1b overlap, indicated that the frameshift site of GLRaV-2 is located in the same position as in BYV. The nucleotide sequence around the proposed frameshift site at position 1049, showed that GLRaV-2 has a UAG terminator at the end of the helicase domain which aligns with the UAG terminator of BYV, and that the suggested slippery sequence is well conserved in GLRaV-2 (Fig.7).

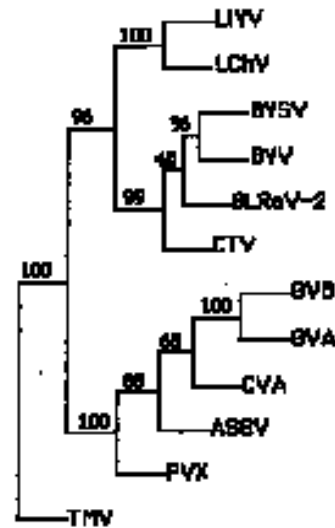


Fig.6. Phylogenetic analysis of GLRaV-2 RNA dependent RNA polymerase (RdRp) with those of members of *Closterovirus*, *Vitivirus*, *Capillovirus* and *Potexvirus* genera. The RdRp tree was generated from an alignment of the amino acid sequences bordered according to Koonin (1991). The GLRaV-2 RdRp domain spans from aa 70 to 323. EMBL accession numbers of the sequences used are: beet yellows virus (BYV) X73476, citrus tristeza virus (CTV) U02547, beet yellow stunt virus (BYSV) U51931, lettuce infectious yellows virus (LIYV) U05242, little cherry virus (LChV) Y10237, grapevine virus A (GVA) X75433, grapevine virus B (GVB) X75448, potato virus X (PVX) M31541, apple stem grooving (ASGV) D14995, cherry virus A (CVA) X82547, tobacco mosaic virus (TMV, outgroup) D13438.

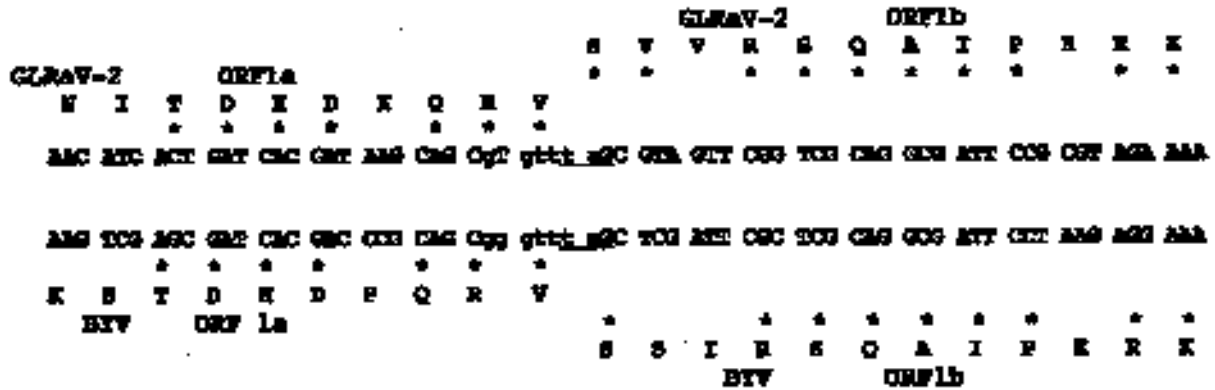


Fig.7. Alignment of GLRaV-2 and BYV nucleotide sequences around the proposed ribosomal frameshift site (nt 1017-1083 in GLRaV-2 and nt 7374-7440 in BYV. Asterisks indicate identical amino acid residues in both sequences. Terminators in GLRaV-2 and BYV sequences are underlined. The slippery sequence is indicated in lowercase letters.

ORF 2 comprises the sequence between nt 2491 and 2661, encoding a small protein of 57 aa ( $M_r$  6.3 kDa). It contains a stretch of nonpolar amino acids terminated by a positively charged residue predicted to form a transmembrane helix in the equivalent hydrophobic proteins of BYV (6K), CTV (6K), BYSV (6K), Lettuce infectious yellows virus (LIYV) (5K) and Little cherry virus (LChV) (4K) (Agranovsky *et al.*, 1991; Karasev *et al.*, 1995; 1996; Klaassen *et al.*, 1995; Jelkmann *et al.*, 1997).

ORF 3 (nt 2677 to 4476) encodes a 600 aa protein with  $M_r$  65 kDa (65K). This was identified as a homologue of the cellular heat shock proteins of the HSP70 family, based on significant similarity with the corresponding HSP70 proteins of other closteroviruses, BYV, BYSV, and CTV in particular (Agranovsky *et al.*, 1991; Pappu *et al.*, 1994; Karasev *et al.*, 1996). The percentage of amino acid similarity and identity with these viruses varied from 55 to 70% (similarity) and from 28 to 48% (identity), respectively (Table 1). ORF 3 contained eight motifs (A-H) (Fig. 8), highly conserved among known HSP70 proteins (Ting and Lee, 1988). Among these, there were the functionally important motifs (A and C) that characterize the HSP70 ATPase domain (Bork *et al.*, 1992) and the C-terminal domain believed to contain a substrate binding site (Milarsky and Morimoto, 1989). These domains may be involved in protein-protein interactions important for chaperone activity (Rothman, 1989). Analysis using amino acid sequences between motifs A and C showed that GLRaV-2 HSP70 is closely related to HSP70 of BYSV, BYV and CTV (Fig. 9).

ORF4 (nt 4403 to 6058) (Fig. 4) encodes a 552 aa protein with a  $M_r$  of 63 kDa (63K). The deduced amino acid sequence of this polypeptide was significantly similar to those of similarly positioned proteins

of BYV (64K), CTV (61K), BYSV, LIYV, and LChV (Agranovsky *et al.*, 1991; Pappu *et al.*, 1994; Klaassen *et al.*, 1995; Karasev *et al.*, 1996; Keim-Konrad and Jelkmann, 1996). Analysis of the amino acid sequences of BYV 64K and CTV 61K proteins had disclosed the presence of domains (I and II) resembling two of the conserved regions in cellular HSP90s (Koonin *et al.*, 1991; Pappu *et al.*, 1994). Direct amino acid sequence comparisons showed that these regions are also conserved in the GLRaV-2 63K protein (not shown).

ORFs 5 (nt 5970 to 6641) and 6 (nt 6710 to 7306) encode two polypeptides of 224 aa and 199 aa with a calculated  $M_r$  of 24.8 kDa (25K) and 21.6 kDa (22K), respectively. The initiation context of AUG codon of ORF 6 fits the consensus of Kozak (1986) because of the presence of C in -2, C in -4 and G in +4. The ORF 6-encoded protein was identified as the viral coat protein (CP) based on its position in the genome, similarity with the CPs of other sequenced closteroviruses (Table 1), and the presence of the conserved residues R/D identifying the salt bridge structure known to occur in CPs of filamentous viruses (Dolja *et al.*, 1991). The value of 21.6 kDa deduced from the amino acid sequence is in excellent agreement with PAGE estimates of CP subunits (Zimmermann *et al.*, 1990 and the present work).

The expression product of ORF 5 also contained the conserved residues R/D and was therefore identified as a duplicated CP gene, present in all sequenced closteroviruses.

The presence of a diverged duplicate of the CP gene located just upstream of the CP cistron was first demonstrated in BYV (Boyko *et al.*, 1992). A comparable situation was then found in CTV (Boyko *et al.*, 1992; Pappu *et al.*, 1994) and BYSV (Karasev *et al.*, 1996). The CP gene and its duplicate have also been

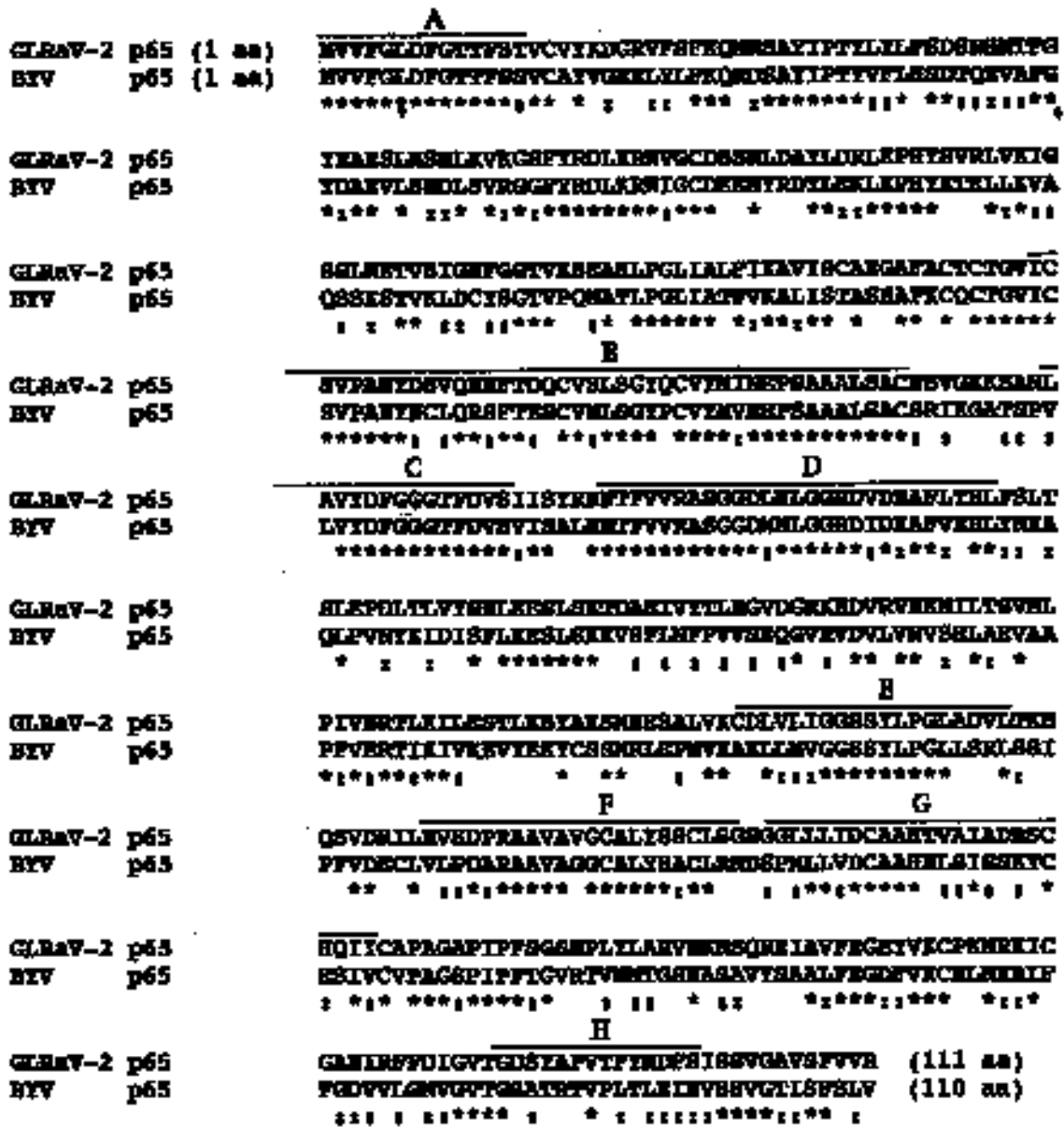


Fig.8. Amino acid sequence alignment of p65 proteins of GLRaV-2 and BYV. Asterisks and colons show identical and similar residues, respectively. The conserved motifs (A through H) typical of cellular HSP70 proteins are overlined.

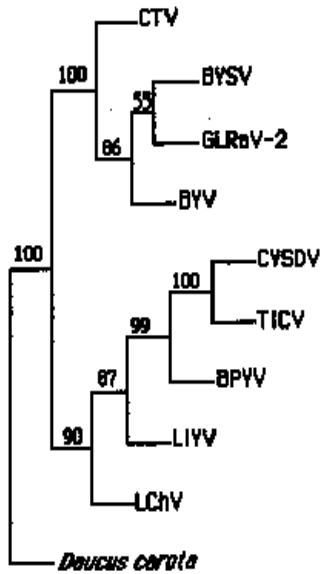
detected in LIYV, Sweet potato sunken vein virus (SPSVV), and LChV, (Klaassen *et al.*, 1995; Keim-Konrad and Jelkmann, 1996) where the two genes are arranged in the reverse order with respect to the homologous genes of BYV, CTV and BYSV. GLRaV-2 therefore has the same type of CP gene duplication as in BYV, CTV and BYSV.

Comparison of the CP of GLRaV-2 and its diverged copy with the CPs and diverged copies of other closterovirus showed that in both cases GLRaV-2 is more closely related to the aphid-transmitted closteroviruses

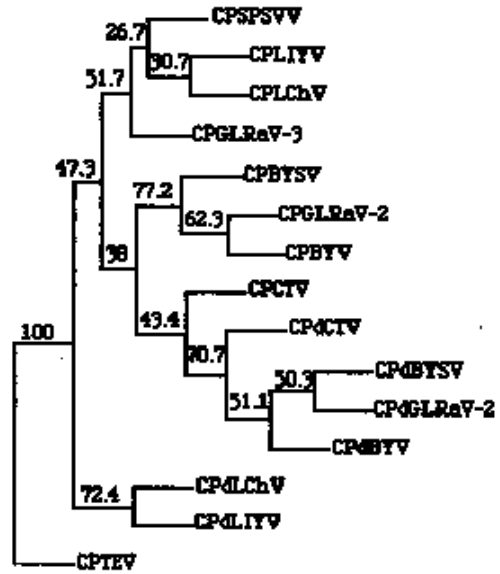
(BYV, BYSV and CTV) than to the those transmitted by whiteflies (LIYV) or mealybugs (LChV) (Fig. 10).

ORFs 2 through 6 constitute the five-gene module conserved in all closteroviruses so far sequenced (Agronovsky, 1996)

ORFs 7 (nt 7301 to 7791) and 8 (nt 7793 to 8410) potentially encode proteins of 162 aa and 206 aa with calculated  $M_r$  of 18.7 kDa (19K) and 23.7 kDa (24K) respectively. Direct comparison with the 3'-proximal ORFs of BYV indicated that the ORF 7-encoded p19 and the ORF 8-encoded p24 show some similarity with



**Fig.9.** Phylogenetic comparison of GLRaV-2 HSP70 protein with those of members of the *Closterovirus* genus. The HSP70 tree was generated from an alignment of the amino acid sequences between motifs A and C. EMBL accession numbers of the sequences used are: beet yellows virus (BYV) X73476, citrus tristeza virus (CTV) U02547, beet yellow stunt virus (BYSV) U51931, lettuce infectious yellows virus (LIYV) U05242, little cherry virus (LChV) Y10237, beet pseudo yellows virus (BPYV) U67447, tomato infectious chlorosis virus (TICV) U67449, cucumber yellow stunt disorder virus (CYSDV) U67448. The HSP70 sequence from carrot (*Daucus carota*) was used as outgroup.



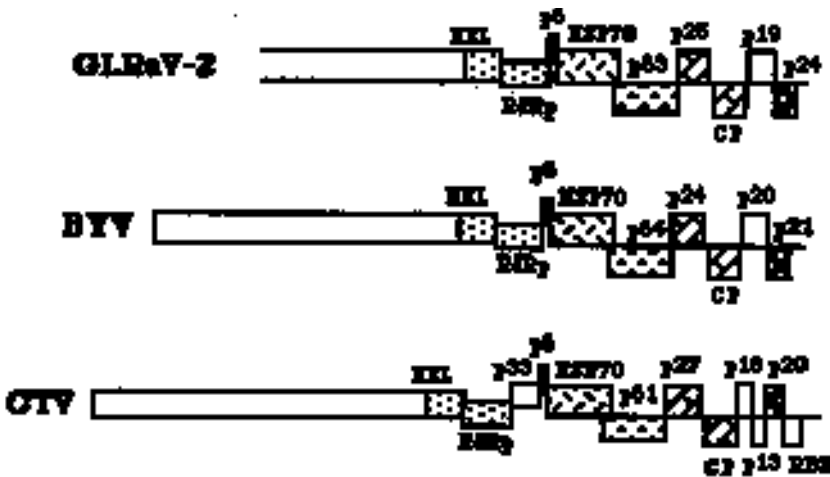
**Fig.10.** Phylogenetic analysis of GLRaV-2 coat protein and its diverged copy with those of other closteroviruses. EMBL accession numbers of the sequences used are: beet yellows virus (BYV) X73476, citrus tristeza virus (CTV) U02547, beet yellow stunt virus (BYSV) U51931, lettuce infectious yellows virus (LIYV) U05242, little cherry virus (LChV) Y10237, grapevine leafroll-associated virus 3 (GLRaV-3) U82937, sweet potato sunken vein virus (SPSVV) X80995, tobacco etch potyvirus (TEV, outgroup) M11458 Domains are bordered according to Dolja *et al.* (1991).

the p20 protein encoded by ORF 7 and the putative p21 product of ORF 8 of BYV, respectively (not shown).

The 3' non coding region (NCR) of GLRaV-2 consisted of 180 nt, showing 25% similarity with the 3' NCR of BYV.

In conclusion, the structural organization of the GLRaV-2 genome is virtually identical to that of BYV and resembles also that of CTV (Fig. 11). This is in line

with recent reports by Zhu *et al.* (1997) in an independent study of another isolate of GLRaV-2. These findings strengthen the case, already supported by morphological, physicochemical and ultrastructural observations (Zimmermann *et al.*, 1990; Boscia *et al.*, 1995; Castellano *et al.*, 1995; Goszczynski *et al.*, 1996b), for placing GLRaV-2 as a definitive species in the genus *Closterovirus*.



**Fig.11.** Structural organization of the 3'-terminal portion of the GLRaV-2 genome and of the complete genome of BYV and CTV. ORFs and their expression products are shown. ORFs encoding proteins with statistically significant similarities are filled similarly. RBP is a 23.5 kDa protein with possible RNA-binding properties (Dolja *et al.*, 1994).

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