The entire genome of grapevine leafroll-associated closterovirus-2 (GLRaV-2), except the exact 5′ terminus, was cloned and sequenced. The sequence encompasses nine open reading frames (ORFs) which include, in the 5′ to 3′ direction, an incomplete ORF1a encoding a putative viral polyprotein and eight ORFs that encode proteins of 52 kDa (ORF1b), 6 kDa (ORF2), 65 kDa (ORF3), 63 kDa (ORF4), 25 kDa (ORF5), 22 kDa (ORF6), 19 kDa (ORF7) and 24 kDa (ORF8) respectively, and 216 nucleotides of the 3′ untranslated region. An incomplete ORF1a potentially encoded a large polyprotein containing the conserved domains characteristic of a papain-like protease, methyltransferase and helicase. ORF1b potentially encoded a putative RNA-dependent RNA polymerase. The expression of ORF1b may be via a 1 ribosomal frameshift mechanism, similar to other closteroviruses. A unique gene array, which is conserved in other closteroviruses, was also identified in GLRaV-2; it includes genes encoding a 6 kDa small hydrophobic protein, 65 kDa heat shock protein 70, 63 kDa protein of function unknown, 25 kDa coat protein duplicate and 22 kDa coat protein. Identification of ORF6 (22 kDa) as the coat protein gene was further confirmed by in vivo expression in E. coli and immunoblotting. Phylogenetic analysis comparing different genes of GLRaV-2 with those of other closteroviruses demonstrated a close relationship with beet yellows virus (BYV), beet yellow stunt virus and citrus tristeza virus. GLRaV-2 is the only closterovirus, so far, that matches the genome organization of the type member of the group, BYV, and thus can be unambiguously classified as a definitive member of the genus Closterovirus.

Introduction

Grapevine leafroll is a worldwide graft-transmitted virus disease which adversely affects grape yields and fruit quality. At least seven serologically distinct closteroviruses have been isolated from leafroll diseased vines and designated as grapevine leafroll-associated closteroviruses-1 to -7 (Gugerli et al., 1984; Rosciglione & Gugerli, 1986; Zee et al., 1987; Hu et al., 1990; Zimmermann et al., 1990; Gugerli & Ramel, 1993; Boscia et al., 1995; Choueiri et al., 1996). Grapevine leafroll-associated closterovirus-2 (GLRaV-2) was first reported by Gugerli et al. (1984). Later, Gugerli & Ramel (1993) showed that the grapevine infected with GLRaV-2 (Gugerli et al., 1984) was infected with a mixture of GLRaVs, which were then designated GLRaV IIa and GLRaV IIb. Boscia et al. (1995) recently showed that GLRaV IIb was the same as GLRaV-2 isolated in France (Zimmermann et al., 1990). This isolate (GLRaV IIb) is now regarded as GLRaV-2, while GLRaV IIa was designated GLRaV-6 (Boscia et al., 1995). GLRaV-2 can be mechanically transmitted to herbaceous plant species (e.g. Nicotiana benthamiana) (Boscia et al., 1995; Goszczynski et al., 1996b).

Virions of GLRaV-2 are flexuous, filamentous particles about 1400–1800 nm in length (Gugerli et al., 1984). A dsRNA of about 15 kb was consistently isolated from GLRaV-2-infected tissues (Goszczynski et al., 1996a). The coat protein (CP) of GLRaV-2 is ca. 22–26 kDa (Zimmermann et al., 1990;
Gugerli & Ramel, 1993; Boscia et al., 1995), which is considerable smaller than other GLRaVs (35–43 kDa) (Zee et al., 1987; Hu et al., 1990; Zimmermann et al., 1990; Ling et al., 1997). Although G. P. Martelli (circular of ICTV Plant Virus Subcommittee Study Group on Closteroviruses, 1996) classified GLRaV-2 as a definitive member of the genus Closterovirus based on particle morphology and cytopathology, its molecular and biochemical properties are not well characterized.

In the closterovirus group, several viruses have recently been sequenced. The partial or complete genome sequences of beet yellows virus (BYV) (Agranovsky et al., 1991, 1994), beet yellow stunt virus (BYSV) (Karasev et al., 1996), citrus tristeza virus (CTV) (Pappu et al., 1994; Karasev et al., 1995), lettuce infectious yellows virus (LIYV) (Klaassen et al., 1994, 1995), little cherry virus (LChV) (Keim & Jelkmann, 1996; Jelkmann et al., 1997) and GLRaV-3 [Ling et al., 1998 (accompanying paper)] revealed several common features of the closteroviruses, including the presence of heat shock protein 70 (HSP70) and a duplicate (CPd) of the coat protein gene (Agranovsky et al., 1996; Dolja et al., 1994; Boyko et al., 1992). Characterization of the genome organization of GLRaVs would provide molecular information on the serologically distinct closteroviruses that cause similar leafroll symptoms in grapevine. The accompanying paper reports (Ling et al., 1998) on the 3′-terminal two-thirds of the nucleotide sequence of GLRaV-3. In this work, we report the nucleotide sequence and analysis of the nearly complete (95%) genome of GLRaV-2.

Methods

■ Virus source and dsRNA isolation. Several vines of GLRaV-2-infected Vitis vinifera cv. Pinot Noir that originated from a central New York vineyard served as the source for dsRNA isolation and cDNA cloning. DS RNA was extracted from phloem tissue of infected grapevines according to the method described by Hu et al. (1990). Purification of the high molecular mass dsRNA (ca 15 kb) was carried out by electrophoretic separation of the total dsRNA on a 0.7% low melting point agarose gel and extraction by phenol–chloroform following the method described by Sambrook et al. (1989). The concentration of dsRNA was estimated from the UV fluorescent density of an ethidium bromide-stained dsRNA band in comparison with a known concentration of DNA marker.

■ cDNA synthesis and cloning. cDNA synthesis was performed following the method initially described by Jelkmann et al. (1989) and modified by Ling et al. (1997). About 100 ng of high molecular mass dsRNA purified from low melting agarose gel was denatured in 20 mM methylmercuric hydroxide and incubated at room temperature for 10 min with 350 ng of random primers. First strand cDNA was synthesized by using avian myeloblastosis virus (AMV) reverse transcriptase. Second strand cDNA was obtained by using RNase H and E. coli DNA polymerase I. Double-stranded cDNA was blunt-ended with T4 DNA polymerase and ligated with EcoRI adapters. The cDNA, which had EcoRI adapters at the ends, was activated by kinase reaction and ligated into Lambda ZAPII/EcoRI-prepared arms following the manufacturer’s instructions (Stratagene). The recombinant DNA was then packaged in vitro to Gigapack II packaging extract (Stratagene). The packaged phage particles were amplified and titrated according to the manufacturer’s instruction.

Two kinds of probe were used to identify GLRaV-2-specific clones from the library. One type was prepared from the synthesized cDNA that was amplified by PCR after ligation to the specific EcoRI Uni-Amp adapters (Clontech); the other type was DNA inserts or PCR products from previously sequenced clones. Clones from the cDNA library were selected by colony-lifting hybridization onto the colony/plaque screen membrane (Dupont NEN) with the probe described above. The probe was prepared by labelling with [α-32P]dATP using the Klenow fragment of E. coli DNA polymerase I. Prehybridization, hybridization and washing steps were carried out at 65 °C according to the manufacturer’s instructions (Dupont NEN). Selected plaques were converted to recombinant pBluescript by in vivo excision method according to the manufacturer’s instructions (Stratagene).

To obtain clones representing the extreme 3′ terminus of GLRaV-2, dsRNA was polyadenylated by yeast poly(A) polymerase. Using poly(A)-tailed dsRNA as template, cDNA was amplified by RT–PCR with oligo(dT)16 and a specific primer, CP-1/T7R (5′ TGCTGGAGCGTTGAGTCTCGC 3′), derived from the clone CP-1. The resulting PCR product (3′ PCR) was cloned into a TA vector (Invitrogen) and sequenced.

■ Northern hybridization. Specificity of the selected clones was confirmed by Northern hybridization. Northern hybridization was performed after electrophoresis of the dsRNA of GLRaV-2 in a 1% agarose non-denaturing condition gel. The agarose gel was denatured by soaking in 50 mM NaOH containing 0.4 M NaCl for 30 min, and then neutralized with 0.1 M Tris–HCl (pH 7.5) containing 0.5 M NaCl for another 30 min. RNA was sandwich blotted overnight onto Genescreen plus membrane (Dupont NEN) in 10× SSC buffer and hybridized as described above.

■ Sequencing and computer-assisted nucleotide and amino acid sequence analysis. DNA inserts were sequenced in pBluescript SK(+) by using T3 and T7 universal primers for the terminal region sequence and additional oligonucleotide primers designed according to the known sequence for the internal region sequence. Purification of plasmid DNA was performed by a modified mini alkaline-lysis/PEG precipitation procedure described by the manufacturer (Applied Biosystems). Nucleotide sequencing was performed on both strands of cDNA by using an ABI Tag DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Automatic sequencing was performed on an ABI373 Automated Sequencer at Cornell University, Geneva, NY, USA.

The nucleotide sequences of GLRaV-2 were assembled and analysed with the programs EditSeq and SeqMan, respectively, from the DNASTAR package. Amino acid sequences deduced from nucleotide sequences and their encoding open reading frames were depicted using the MapDraw program. Multiple alignments of amino acid sequences, identification of consensus amino acid sequences and generation of phylogenetic trees were performed using the Clustal method in the MegAlign program. The nucleotide and amino acid sequences at various closteroviruses were obtained with the Entrez program; sequence comparisons with nonredundant databases were searched for with the Blast program from the National Center for Biotechnology Information.

■ Expression of the CP in E. coli and immunoblotting. To demonstrate that ORF6 was the CP gene of GLRaV-2, the complete ORF6 was subcloned from a PCR product and inserted into the fusion protein expression vector pMAL-C2 (New England Biolabs). The specific primers used for the PCR reaction were CP-96F (5′ egaqitaacctagg-aggtalgtgctgcag 3′) and CP-96R (5′ accgggtaccttgtagttgctgagattga 3′), in which an EcoRI or BamHII site was included to facilitate
cloning. CP-96F was designed to include the start codon for the CP, and CP-96R was 60 nucleotides (nt) downstream of the stop codon for the CP. The CP was expressed as a fusion protein with maltose-binding protein (MBP) of E. coli under the control of a tac promoter and suppressed by the lac repressor. The MBP–CP fusion protein was induced by adding 0.3 mM IPTG and purified on a one-step affinity column according to the manufacturer’s instructions (New England Biolabs). The MBP–CP fusion protein or the CP cleaved from the fusion protein were tested for reaction with specific antiserum to GLRaV-2 (kindly provided by Charles Greif of INRA, Colmar, France) on Western blot according to the method described by Hu et al. (1990).

Results and Discussion

dsRNA isolation and cDNA cloning

A high molecular mass dsRNA of ca. 15 kb was consistently identified in extracts from GLRaV-2-infected grapevines, but not from healthy vines. In addition, several low molecular mass dsRNAs were also detected from infected tissue (Fig. 1a). The yield of GLRaV-2 dsRNA was estimated to be 5–10 ng/15 g phloem tissue, which was much lower than that for GLRaV-3 (Hu et al., 1990; data not shown). Only the high molecular mass dsRNA that was purified from low melting point agarose gel was used for cDNA synthesis, cloning and establishment of the Lambda ZAPII cDNA library.

Two kinds of probe were used for screening the cDNA library. The initial clones were identified by hybridization with Uni-Amp PCR-amplified cDNA as probes. The specificity of these clones (e.g. TC-1), ranging from 200 to 1800 bp in size, was confirmed by Northern hybridization to dsRNA of GLRaV-2 (Fig. 1b). Additionally, over 40 different clones ranging from 800 to 7500 bp in size were identified following hybridization with the probes generated from GLRaV-2-specific cDNA clones or from PCR products. Over 40 clones were then sequenced on both strands (Fig. 2).

Sequence analysis and genome organization of GLRaV-2 and its relationship with other closteroviruses

A total of 15 000 bp of the RNA genome of GLRaV-2 was sequenced and deposited in GenBank (accession no. AF039204). About 85% of the total RNA genome was revealed from at least two different clones. The sequence in the CP gene region was determined and confirmed from several different overlapping clones. The genome organization of GLRaV-2 is shown in Fig. 2.

ORF1a and ORF1b. Analysis of the amino acid sequence of the N-terminal portion of the GLRaV-2 ORF1a-encoded product revealed a putative papain-like protease (P-PRO) domain, which showed significant similarity to the papain-like leader protease of BYV (Agranovsky et al., 1994). This allowed us to predict the catalytic cysteine and histidine residues for the putative GLRaV-2 protease. Upon alignment of the sequence of the P-PRO of BYV with that of GLRaV-2, the cleavage site at residues Gly–Gly (amino acid 588–589) of BYV aligned with the corresponding Ala–Gly dipeptide of GLRaV-2 (Fig. 3a). Cleavage at this site would result in a leader protein, and a 234 kDa (2090 amino acid) C-terminal fragment consisting of methyltransferase (MT) and helicase (HEL) domains. However, the region upstream of the P-PRO domain in GLRaV-2 did not show similarity to the corresponding region of BYV. In addition, there was variability in the residues located at the scissile bond (Gly in BYV and Ala in the GLRaV-2). Similar variability of the cleavage site residue in the P-PRO domain has been described in LChV (Jelkmann et al., 1997).

Database searching with the deduced amino acid sequence of the ORF1a/1b-encoded protein revealed a significant similarity to the MT, HEL and RNA-dependent RNA polymerase (RdRP) domains of the other closteroviruses. The region downstream of the P-PRO cleavage site showed a significant similarity (57±4% identity in a 266 residue alignment) to the putative MT domain of BYV and contained all the conserved motifs typical of positive-strand RNA viral type I MTs (Fig. 3b). The C-terminal portion of the ORF1a was identified as a HEL domain, the sequence of which showed a high similarity (57±1% identity in a 315 residue alignment) to the helicase domain of BYV and contained the seven conserved motifs characteristic of the Superfamily I helicase of positive-strand RNA viruses (Fig. 3c) (Hodgman, 1988; Koonin & Dolja, 1993).

ORF1b potentially encodes a 460 amino acid polypeptide with a molecular mass of 52 486 Da, counting from the
HSP70 is highly conserved among closteroviruses and is probably involved in ATPase activity and the protein–protein interaction for chaperone activity (Agranovsky et al., 1995, 1997; Karasev et al., 1992). Alignment of the complete ORF3 of GLRaV-2 with the HSP70 homologue of BYV revealed the presence of eight conserved motifs (Fig. 5). The percentage similarity between the GLRaV-2 HSP70 and that of BYV, BYSV, CTV, LIYV and LChV is 47.8%, 47.2%, 38.6%, 20.9% and 17.7%, respectively.

ORF4. This potentially encodes a 551 amino acid polypeptide with a molecular mass of 63,349 Da. Database searching with the ORF4-encoded protein did not identify similar proteins except those of its counterparts in closteroviruses, BYV (p64), BYSV (p61), CTV (p61), LIYV (p59) and LChV (p61). Two conserved motifs which were present in BYV (Agranovsky et al., 1991) and CTV (Pappu et al., 1994) were also identified in the ORF4 of GLRaV-2 (data not shown).

ORF5 and ORF6. These potentially encode polypeptides with molecular masses of 24,803 Da and 21,661 Da, respectively. The start codon for both ORFs is in a favourable context for translation. ORF6 was identified as the CP gene of GLRaV-2 based on sequence comparison with other closteroviruses. The calculated molecular mass of the protein product of ORF6 is in good agreement with the previously estimated 22–26 kDa based on SDS–PAGE (Zimmermann et al., 1990; Boscia et al., 1995).

Database searching with the deduced amino acid sequence encoded by GLRaV-2 ORF6 showed a similarity with the CPs of closteroviruses, BYV, BYSV, CTV, LIYV, LChV and GLRaV-3. At the nucleotide level, the highest percentage similarity was with the CP of BYSV (34.8%); at the amino acid level, the highest percentage similarity was with the CPs of BYV (32.7%) and BYSV (32.7%) (data not shown). Alignment of the amino acid sequence of the CP and CPd of GLRaV-2 with respect to other closteroviruses revealed that the invariant amino acid residues (N, R, G, D) were present in both ORF5 and ORF6 of GLRaV-2 (Fig. 6a). Two of these amino acid residues (R and D) are believed to be involved in stabilization of molecules by salt bridge formation and proper folding in the
GLRaV-2 nucleotide sequence

<table>
<thead>
<tr>
<th>GLRaV-2-P-PRO</th>
<th>BYV-P-PRO</th>
<th>Consensus</th>
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<tr>
<td>TTRYPKFCTAHLKJYVACAPFIGNRTDPR(PF)GTPAATKINMSELQGSGLNYGATYRSHFLGDFXOULRMSAV/A/G</td>
<td>LQREGKELAOAHVACALQCRKRPEEFVMYPKYDFAVKLRAGSALKLVHVRQGQVFRSFLVMDKASFAFSPYS/FLRTG/G</td>
<td>…R...G.CYAH...CA...P...F...G...PT...R...L...L...G...SR...FHD...F...I/I/G</td>
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Fig. 3. Comparisons between ORF1a/ORF1b of GLRaV-2 and BYV. (a), (b), (c) and (d), conserved domains of papain-like protease (P-PRO), methyltransferase (MT/MTR), helicase (HEL) and RNA-dependent RNA polymerase (RdRP), respectively. Exclamation marks indicate the predicted catalytic residues of the leader papain-like protease; slashes indicate the predicted cleavage sites. The conserved motifs of the MT, HEL and RdRP domains are overlined and lettered appropriately. The alignment was constructed with the MegAlign program in DNASTAR.

most conserved core region of CPs of all filamentous plant viruses (Dolja et al., 1991).

Identification of ORF6 as the CP gene was further confirmed by Western blot. The complete ORF6 was cloned into the protein expression vector pMAL-C2 and expressed as an MBP–CP fusion protein after IPTG induction. As predicted, a 64 kDa MBP–CP fusion protein consisting of a 22 kDa ORF6-encoded protein and a 42 kDa maltose-binding protein was produced in E. coli; the 22 kDa GLRaV-2 CP was released from the MBP–CP fusion protein after treatment with factor Xa (New England Biolabs). Specific antiserum to GLRaV-2 reacted with the MBP–CP fusion protein, the cleaved CP and the native CP from GLRaV-2-infected grapevine tissue (data not shown). In contrast, the non-recombinant plasmids or uninduced cells did not react with antiserum to GLRaV-2. The putative phylogenetic tree of the CP and CPd of GLRaV-2 with those of other closteroviruses showed that GLRaV-2 is closely related to aphid-transmissible closteroviruses (BYV, BYSV and CTV) (Candresse, 1994) than to whitefly-(LIYV) or mealybug-transmissible closteroviruses (LChV and BCJD).
Fig. 4. Alignment of the nucleotide (a) and deduced amino acid (b) sequences of the ORF1a/ORF1b overlapping region of GLRaV-2, BYV, BYSV and CTV. Identical nucleotides and amino acids are shown in the consensus. The GLRaV-2 putative +1 frameshift site (TAGC) and corresponding sites in BYV (TAGC) and BYSV (TAGC) and CTV (CGGC) at nucleotide and amino acid sequences are underlined.

GLRaV-3) (Raine et al., 1986; Jelkmann et al., 1997; Rosciglione & Gugerli, 1989; Engelbrecht & Kasdorf, 1990; Cabaleiro & Segura, 1997; Petersen & Charles, 1997) (Fig. 6b).

ORF7 and ORF8. These potentially encode polypeptides of 162 amino acids with a molecular mass of 18,800 Da and of 206 amino acids with a molecular mass of 23,659 Da, respectively. Database searching with ORF7- and ORF8-encoded products showed no significant similarity with any other proteins. Nevertheless, these genes were similar in size and location to those observed in the sequence of other closteroviruses, BYV (P20, P21), BYSV (P18, P22) and LChV (P21, P27) (Fig. 7). However, conserved regions were not observed between ORF7 or ORF8 and its counterparts in BYV, BYSV and LChV.

3' Terminal untranslated region. The 3' UTR consists of 216 nt. Nucleotide sequence analysis revealed a long oligo(A) tract close to the end of the GLRaV-2 genome which is similar to that observed in the genome of BYV and BYSV (Agranovsky et al., 1991; Karasev et al., 1996). The genome of BYV ends in CCC; BYSV and CTV end in CC with an additional G or A in the double-stranded replicative form of BYSV (Karasev et al., 1996) and CTV (Karasev et al., 1995), respectively. GLRaV-2 had CGC at the 3' terminus of the genome. Recently, a conserved 60 nt cis-element was identified in the 3'-UTR of three monopartite closteroviruses which included a prominent conserved stem-loop structure (Karasev et al., 1996). Alignment of the 3'-UTR sequence of GLRaV-2 with the same regions of BYV, BYSV and CTV showed the presence of the same conserved 60 nt stretch (data not shown). Apart from this cis-element, conserved sequences were not found in the 3' UTRs of GLRaV-2, BYV, BYSV and CTV.

The closteroviruses studied so far (e.g. BYV, BYSV, CTV, LIYV, LChV and GLRaV-3) have apparent similarities in genome organization, which include replication-associated genes that consist of MT, HEL and RdRP conserved domains and a five-gene array unique to closteroviruses (Dolja et al., 1994; Agranovsky et al., 1996; Jelkmann et al., 1997; Ling et al., 1998). Our data clearly show that GLRaV-2 is a closterovirus. In the genome of GLRaV-2, a putative P-PRO was identified and an autoproteolytic cleavage process was predicted. The replication-associated proteins, consisting of MT, HEL and RdRP conserved motifs, were also identified and were phylogenetically closely related to the replication-associated proteins of other closteroviruses. A unique gene array including a small hydrophobic transmembrane protein, HSP70 homologue, HSP90 homologue, CPd and CP, was also preserved in GLRaV-2. In addition, the calculated molecular mass (21,661 Da) of the CP (ORF6) of GLRaV-2 is in good agreement with that of the other closteroviruses (22–28 kDa) (Martelli & Bar-Joseph, 1991; Candresse & Martelli, 1995).
Two ORFs downstream of the CP are similar in size and location to those observed in the genome of BYV. Furthermore, lack of a poly(A) tail at the 3' end of GLRaV-2 is also in good agreement with other closteroviruses. Like all other closteroviruses, expression of ORF1b is suspected to occur via a -1 ribosomal frameshift and the 3'-proximal ORFs are probably expressed via formation of a nested set of subgenomic RNAs. Since the slippery sequence, stem–loop and pseudoknot structure involved in the frameshift of BYV were absent in GLRaV-2, the +1 frameshift of GLRaV-2 might be the same as proposed for CTV (Karasev et al., 1995) and BYSV (Karasev et al., 1996).

Overall, GLRaV-2 is more closely related to monopartite closteroviruses, BYV, BYSV and CTV, than to GLRaV-3 (Ling

Fig. 6. Comparison of the CP and CPd of GLRaV-2 with other closteroviruses. (a) Alignment of the amino acid sequence of CP and CPd of GLRaV-2 with BYV, BYSV and CTV. The conserved amino acid residues are in bold and the consensus sequences are indicated. (b) Tentative phylogenetic tree based on comparison of the CP and CPd of GLRaV-2 with BYV, BYSV, CTV, LIYV, LChV and GLRaV-3. To facilitate the alignment, only the C-terminal 250 amino acids of CP and CPd of LIYV, LChV and GLRaV-3 were used. The scale beneath the tree represents the distance between sequences. Units indicate the number of substitution events. Sequence alignment and phylogenetic tree were constructed by Clustal in the MegAlign program of DNASTAR.

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Two ORFs downstream of the CP are similar in size and location to those observed in the genome of BYV. Furthermore, lack of a poly(A) tail at the 3’ end of GLRaV-2 is also in good agreement with other closteroviruses. Like all other closteroviruses, expression of ORF1b is suspected to occur via a -1 ribosomal frameshift and the 3’-proximal ORFs are probably expressed via formation of a nested set of subgenomic RNAs. Since the slippery sequence, stem–loop and pseudoknot structure involved in the frameshift of BYV were absent in GLRaV-2, the +1 frameshift of GLRaV-2 might be the same as proposed for CTV (Karasev et al., 1995) and BYSV (Karasev et al., 1996).

Overall, GLRaV-2 is more closely related to monopartite closteroviruses, BYV, BYSV and CTV, than to GLRaV-3 (Ling
et al., 1998) (Fig. 7), even though the latter causes similar leafroll symptoms in grapevine (Rosciglione & Gugerli, 1986; Hu et al., 1990). After we finished this work, an abstract (Abou-Ghanem et al., 1997) was published on the sequencing of the 3′ region of GLRaV-2 that had been purified from infected N. benthamiana. Their proposed 3′ end genome organization of GLRaV-2 supports our results.

Closteroviruses are a diverse group with complex and heterogeneous genome organizations. So far, GLRaV-2 is the only closterovirus that matches with the genome organization of BYV, the type member of the genus Closterovirus. In addition, the genomic RNA of GLRaV-2 is about the same size as that of BYV. Although the transmission vector of GLRaV-2 is unknown, the genome organization of GLRaV-2 is more closely related to the aphid-transmissible closteroviruses (BYV and CTV) than to whitefly- (LIYV) or mealybug-transmissible closteroviruses (LChV and GLRaV-3). Is it possible that GLRaV-2 is transmitted by an aphid? Aphid transmission experiments with GLRaV-2 should answer the question and provide information that might help towards the control of GLRaV-2.

Conclusion

A total of 15 000 nt or over 95% of the estimated GLRaV-2 genome has been cloned and sequenced. GLRaV-2 and GLRaV-3 (Ling et al., 1998) are the first grapevine leafroll-associated closteroviruses that have been almost completely sequenced. Our data clearly justify the inclusion of GLRaV-2 in the genus Closterovirus. In addition, the information regarding the genome of GLRaV-2 would provide a better understanding of this and related GLRaVs, and add to our fundamental knowledge concerning closteroviruses.

Note added in proof. Further analysis of the sequence presented in this paper revealed that there were actually two P-PRO domains in the 5′-terminal region.

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