Arch Virol (2005) 150: 107–123
DOI 10.1007/s00705-004-0382-z

Archives of Virology
Printed in Austria

Little cherry virus-2: Sequence and genomic organization of an unusual member of the *Closteroviridae*

M. E. Rott[1,2] and **W. Jelkmann**[1]

[1]Federal Biological Research Center for Agriculture and Forestry, Institute for Plant Protection in Fruit Crops, Dossenheim, Germany
[2]Centre for Plant Health, Canadian Food Inspection Agency, Sidney, B.C., Canada

Received April 16, 2004; accepted June 10, 2004
Published online September 21, 2004

Summary. The complete genomic sequence of variant USA6b of *Little cherry virus-2* (LChV-2), has been determined and is 15045 nucleotides in length, coding for 11 open reading frames (ORFs). The sequence shares 77.2% identity with a previously published, *ca.* 6 kb partial replicase sequence of LChV-2 (variant USA6a). Both LChV-2/USA6a and LChV-2/USA6b were obtained from the same tree infected with little cherry disease, and would suggest a mixed infection. LChV-2/USA6b is more closely related to the partially determined genomic sequence of a Canadian isolate of LChV-2, strain LC5 (92.9% identity). LChV-2/USA6b has an unusual genomic organization compared to other members of the *Closteroviridae*. The LChV-2/USA6b genome is potentially ambi-sense, with a negative sense ORF0 at the 5′ terminus, from which an 18.1 kDa protein of unknown function can be expressed *in vitro*. The N-terminal region of the LChV-2/USA6 ORF1a translation product does not code for a papain-like protease motif. ORF1 codes for a novel motif, of unknown function, also present in isolates of the *Grapevine leafroll associated virus-3*, (genus *Ampelovirus*) as well as viruses of the family *Flexiviridae*. ORF3 lacks an AUG start codon, but could potentially be expressed via read-through of the ORF2 stop codon. At the 3′ end, there is a re-organization of encoded genes compared with other members of the *Closteroviridae* including separation of the coat protein and coat protein duplicate genes by 4 other genes as found for LChV-2/LC5.

Introduction

The family *Closteroviridae* comprises a large and diverse group of flexuous, filamentous, single-strand RNA viruses infecting plants (for review see [16]),

comprised of the 3 genera of which several members have been fully or partially sequenced. Members of the *Closteroviridae* with monopartite genomes that are transmitted by aphids include *Beet yellows virus* (BYV) [2], *Citrus tristeza virus* (CTV) [17], *Beet yellow stunt virus* (BYSV) [18] and *Grapevine leafroll-associated virus-2* (GLRaV-2) [48]. The genus *Crinivirus* (essentially bipartite genomes, transmitted by whiteflies) includes *Lettuce infectious yellows virus* (LIYV) [19], *Sweet potato chlorotic stunt virus* (SPCSV) [25], *Cucurbit yellow stunting disorder virus* (CYSDV) [8, 29], and *Cucumber yellows virus* (CuYV) [13]. The genus *Ampelovirus* (monopartite, mealybug-transmitted species) [30], includes Little cherry virus – LC5 (LChV-2/LC5) [47], *Pineapple mealybug wilt-associated virus-2* (PMWaV-2) [35], GLRaV-3 [27], and GLRaV-1 [28]. *Little cherry virus*-1 (LChV-1) [15] is an unassigned species in the family *Closteroviridae*. Conserved among members of the *Closteroviridae* are a common core set of encoded proteins involved in replication [16], a papain-like protease (P-PRO), methyltransferase (MET), helicase (HEL), and RNA dependent RNA polymerase (RdRp). Also conserved are proteins for virus movement/encapsidation [3, 36, 16], consisting of a small hydrophobic protein, coat protein (CP), duplicate coat protein (CPd) and a heat shock 70 homolog (HSP70).

Members of the *Closteroviridae* show a high degree of sequence heterogeneity between virus isolates, perhaps more so than most other plant virus families. Sequence comparisons between two isolates of CTV showed an identical genome organization with 89% identity throughout the ten 3′ ORFs, but only 60–70% identity throughout ORF1, dropping to 35% in some less conserved regions [32]. These findings were confirmed by comparisons of 5 complete CTV and several partial genomic sequences [44]. Comparisons of 75 clones corresponding to GLRaV-1 open reading frames (ORFs) 3, 6 and 7, coding for the heat shock 70 homolog, coat protein and coat protein duplicate, were found to be particularly variable with 1916 nucleotide changes recorded over *ca.* 4450 nt of genomic sequence [28]. The *Closteroviridae* are also associated with defective RNAs. A number of LIYV defective RNAs have been characterized, consisting of 3′ deletions of RNA1 or RNA2, and RNA1/RNA2 molecules with extensive internal deletions [45]. Several classes of defective RNAs are associated with CTV. Small (2–5 kb) defective RNAs consisting mainly of the 5′ and 3′ termini with extensive internal deletions [4], and large (12 kb) infectious defective RNAs which correspond to the 5′ portion of the viral RNA consisting of the intact replicase genes with varying deletions in the 3′ termini [34]. Replication of CTV defective RNAs, requires minimal sequences corresponding to *ca.* 1000 and 270 nt of the 5′ and 3′ termini, respectively [33].

Two distinct viruses associated with little cherry disease have been identified [9, 15, 42] for which the species names *Little cherry virus*-1 (LChV-1) and *Little cherry virus-2* (LChV-2) have been proposed [42], and accepted by the ICTV *Closteroviridae* study group [31]. The partial sequence of a Canadian isolate of LChV, strain LC5 [47], which was given the name Little cherry virus 3, shares 77% identity with a previously published partial sequence of LChV-2 [42] (referred to here as variant USA6a). The amount of genomic sequence heterogeneity is

similar to that observed between isolates of CTV, and would indicate that both are strains of the same virus. LChV-1 is an unassigned species in the family *Closteroviridae*, is monopartite, and has no known vector. LChV-2 can be vectored by the apple mealybug (*Phenacoccus aceris*) [39] and has been classified as a member of the genus *Ampelovirus*. Both LChV-1 and LChV-2 have been detected in Europe and North America [9, 42]. Limited comparisons between the conserved helicase and RNA-dependent RNA polymerase domains have shown that LChV-2/USA6a and LC5 are closely related to another mealybug transmitted virus, GLRaV-3 [39, 42, 47]. We present here the complete genomic sequence of LChV-2, variant USA6b, which shares 77.2% identity to LChV-2/USA6a, and 92.9% identity to LChV/LC5. These comparisons indicate that all 3 viruses are variants of LChV-2.

Materials and methods

Cloning, sequencing and sequence analysis

Little cherry disease isolate USA6 is the same as described in [42]. DsRNA and silica capture RNA preparations are as described [14, 41]. Isolates WH, AL4, AL1 and AL5 originated from different sweet cherry varieties from Germany, while UK1/3 originated from the United Kingdom. All were maintained at Dossenheim research field plots. Isolates H2 (*P. serrulata* 'Tai-Haku'), H9 (*P. serrulata* 'Kanzan'), MZ2 (*P. serrulata* 'Hatasakura') were from a botanical garden and a nursery in Germany. Isolates 340-02, 1044-01, 1275-01, 1312-03 and 2030-01 were maintained at the Center for Plant Health in Sidney. 2030-01 and 1312-03 are sweet cherry isolates from Canada and the United Kingdom, respectively. 1275-01 (*P. serrulata* 'Tai-Haku'). 1044-01 (*P. serrulata* 'Kiku Shidara-Sakura') and 340-02 (*P. serrulata* 'Shidara Zakura Perdula') were from a botanical garden and nursery in Canada. Complementary DNA clones spanning ca. 5900 nt of sequence obtained during the initial characterization of LChV-2/USA6a but corresponding to a different LChV-2 variant (USA6b), were sequenced and analyzed in this study. A *ca.* 500 nt cDNA fragment corresponding to the coat protein gene was obtained by DOP-PCR using the DOP7 primer [41]. Sequences between this clone and 5′ sequences were amplified by RT-PCR using specific primers and cDNA primed with random hexamers from viral RNA template prepared by silica capture (clone LCVPCP10). Sequences 3′ of the coat protein gene, were obtained by Uneven PCR [7]. The 5′ and 3′ end terminal sequences were obtained by RACE using viral specific primers and poly (A) tailed viral dsRNA or silica capture RNA as template, essentially as described previously. Additional clones used for sequencing were generated by RT-PCR using virus specific primers and either purified dsRNA or viral RNA prepared by silica capture. PCR fragments were amplified using either TaKaRa LA Taq^{TM} (TaKaRa Biomedicals) or ProofSprinter (AGS-Hybaid) enzyme mixes according to the manufacture's protocol. PCR products were cloned into pBluescript SK (Stratagene) modified into a T-overhang vector [12].

LCV26212, used for *in vitro* translation assays was amplified and cloned as described above by RT-PCR using the viral specific primers LCV261U/LCV262L (Table 1). The 5′ terminal 607 bp of 9 additional LChV-2 isolates (MZ2, H2, H9, UK1/3, 340-02, 1044-01, 1275-01, 1312-03 and 2030-01) were sequenced from amplified fragments using primers LCV01U/LCV607L.

Automated cycle sequencing was performed at ZMBH, Ruprecht-Karls University in Heidelberg, Germany. Sequences were assembled and analyzed using the Lasergene

Table 1. List of primers

Primer	Sequence (5′–3′)
LCV25R3	TATCTACCTCCCCATTGTCCTCTATTA
LCVUSA5RAC	YGTAACTCTGGGAAAGCCGAATCTAAG
LCV224U	TTCTTTTAAATAATCTTCTCATCT
LCV225L	TGTCCATCCCATCAACTAA
LCV261U	TTTTGTATTTCTGTCCTTTTTCCCCCACTT
LCV262L	CTGGCGTCCTGATCTATGTTAGCAGAA
O1M1U	ATATTATATATTTTTAAGTAGGCACAGACCTTTCAAAAGAACG
O1M1L	CGTTCTTTTGAAAGGTCTGTGCCTACTTAAAAATATATAATAT
ONEG1U	AAAGAAAGAGAACAAAAAGTAAAGAAGATTATTTAAAACAAA ACT
ONEG1L	AGTTTTGTTTTAAATAATCTTCTTTACTTTTTGTTCTCTTTCTTT
LCV01U	CCTTTTCAAACTTTCTCTTTATTG
LCV607L	AATTCTCCATACATACATAGTCTCAAGGTTA

Nucleotides altered by *in vitro* mutagenesis are indicated by underlines

Bio-computing Software (DNASTAR Inc.). Sequence comparisons were made with the following viruses; BYSV (accession # U51931), BYV (accession # X73476), CTV (accession # U16304), GLRaV-1 (accession # AF195822), GLRaV-2 (accession # AF039204), GLRaV-3 (accession # AF037268), LChV-1 (accession # Y10237), LChV(LC5) (accession # AF416335), LIYV (accession # U15440, U15441), PMWaV-2 (accession # AF283103), *Grapevine virus A* (GVA) (accession # X75433), *Grapevine virus B* (GVB) (accession # X75448), *Blueberry scorch virus* (BBSV) (accession # L25658), *Papaya mosaic virus* (PMV) (accession # D13975), *Shallot virus X* (ShVX) (accession # M97264), *Grapevine rupestris*

Table 2. Percent amino acid sequence similarities between the conserved LChV-2 putative translation products and sequences encoded by the closteroviruses GLRaV-1, -2, -3, PMWaV-2, BYV, CTV, BYSV, LChV-1, LChV-3 and LIYV. *MET*, methyltransferase domain, *HEL*, helicase domain, *POL*, RNA-dependant RNA polymerase. *HSP70*, heat shock 70-like protein, *CP*, coat protein, *CPd*, coat protein duplicate and *NA*, not available. Note that GLRaV-1 has 2 coat protein duplicates. For the coat protein sequence alignments, the first 120 amino acids of the LChV-1 CPd sequence were not included

	MET	HEL	POL	HSP70	P54	CP	CPd
GLRaV-3	32.0	42.9	39.2	40.3	19.4	26.3	24.4
PMWaV-2	36.5	36.6	33.5	38.8	19.4	26.6	20.5
GLRaV-1	NA	40.1	35.4	39.2	13.5	26.9	19.8/19.0
BYV	25.5	33.1	30.1	27.6	20.2	19.0	19.7
CTV	27.0	30.5	30.7	29.9	18.3	18.1	18.5
GLRaV-2	24.1	32.6	29.4	28.5	17.9	20.8	18.7
BYSV	NA	29.7	30.1	28.7	16.9	18.1	21.4
LChV-1	21.0	31.0	27.2	31.5	15.6	9.5	8.3
LIYV	20.6	32.2	25.1	31.6	13.5	20.5	21.6
LChV-2 LC5	NA	98.0	97.7	97.1	95.4	96.3	93.6

stem pitting associated virus (GRSPaV) (accession # AF026278), *Apple stem pitting virus* (ASPV) (accession # D21829), *Apple chlorotic leaf spot virus* (ACLSV) (accession # M58152), Cherry green ring mottle virus (CGRMV) (accession # AF017780), and Cherry necrotic rusty mottle virus (CNRMV) (accession # AF237816). DNAStar was used for sequence analysis including the coding prediction method of Borodovsky [5]. Percent values listed in Table 2 are "Similarity Index" values as determined by DNAStar running CLUSTALW.

Site-directed mutagenesis and in vitro translations

The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) and the TnT® Coupled Wheat Germ Extract System (Promega) were used to obtain the *in vitro* translation results. Clones O1M103 and ONEG103 were synthesized by mutating clone LCV26212 with the primers O1M1U/O1M1L and ONEG1U/ONEG1L, respectively. *In vitro* translations were conducted according to the manufacture's suggestions with ^{35}S-methionine (Amersham) used as label. Translation products were analyzed by SDS-PAGE, and molecular weight values determined using the Broad Range pre-stained SDS-PAGE standard (BioRad).

Results

Nucleotide sequence and analysis of LChV-2

Multiple overlapping cDNA clones were generated spanning the entire length of the LChV-2 genome with most areas covered by more than one clone. Sequencing revealed very high heterogeneity (ca. 20%) between some clones. At least two distinct sequences were observed, one corresponding to LChV-2/USA6a, and the second corresponding to the LChV-2/USA6b. However, other sequence variants were at times observed including what appeared to be recombinant USA6a/USA6b sequences (data not shown). Clones used for sequence determination are depicted in Fig. 1A. Two different primers (LCV25R3 and LCVUSA5RAC, Table 1), were used in separate 5′ RACE experiments to amplify and clone, sequences 5′ of the methyltransferase domain. From the sequence analysis, clone RE519 has an in-frame UAA stop codon at position 3129 (UAA_{3129}) resulting in premature truncation of ORF1a. Four other clones (RE505, RE508, LCV2M1 and USA4, data not shown) contained the sequence CAA at this position resulting in a Q residue in place of the stop codon, therefore, in the final sequence CAA was used instead of UAA. Clones RE505 and RE508 contained an extra A residue at position 2958 resulting in a shift in readings frame and premature truncation of ORF1a. The unusual organization of the LChV-2 5′ open reading frames (ORFs) 0 and 1a (see next section), was confirmed by sequencing the 5′ most 607 nucleotides of 9 additional LChV-2 isolates. Nucleotide sequence identity between USA6 and the other LChV-2 isolates ranged from 95.7–97.0%. Isolate MZ2 was the most divergent and with an extra codon in the 3′ end of ORF0 compared to the other isolates (data not shown).

Comparison of 5944 nucleotides of LChV-2/USA6a genomic sequence (accession number AF333237) [41] with the corresponding sequence from LChV-2/USA6b (nucleotides 875–6818) indicate only 77.2% similarity. Within this region, sequence heterogeneity was not uniformly distributed. The 3′ ca. 3000 nt

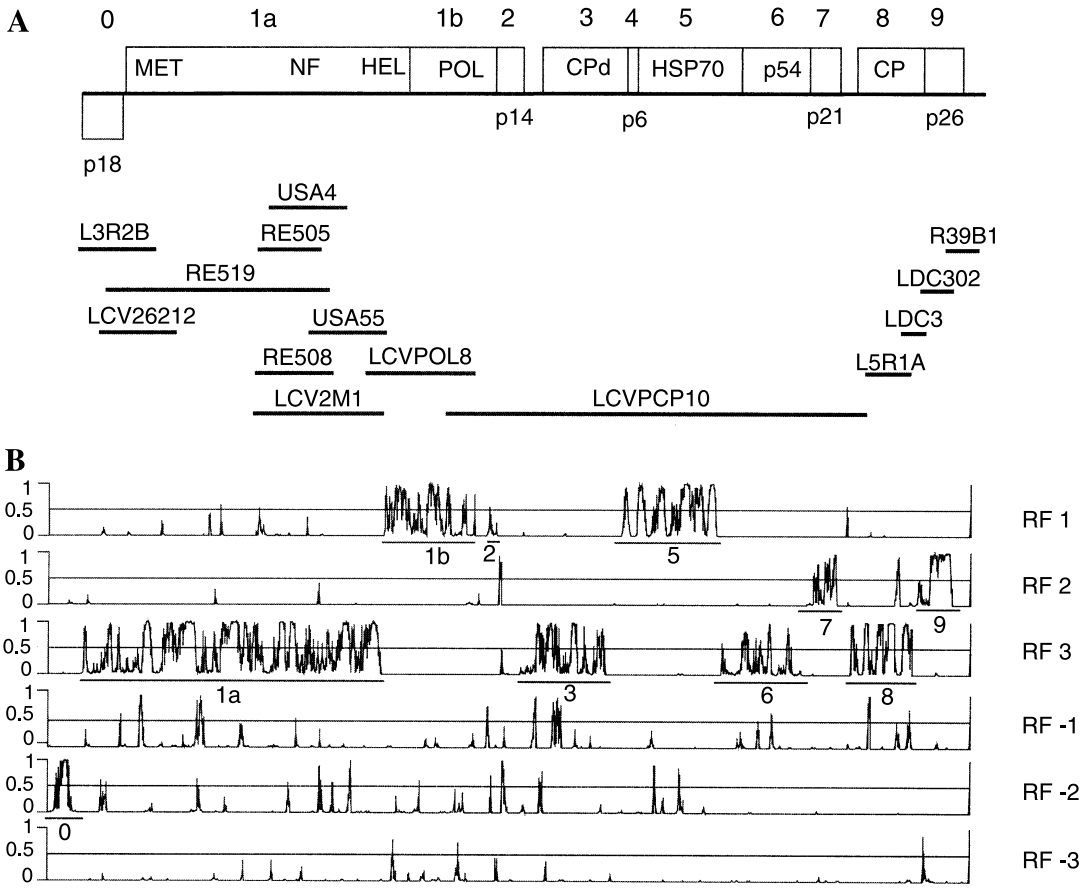

Fig. 1. A Schematic representation of the genomic organization of LChV-2 and selected overlapping cDNA clones. Boxes represent the LChV-2 ORFs with the one negative sense ORF positioned below the other positive sense ORFs. ORFs are consecutively numbered from 0 to 9. Horizontal lines below the genomic organization represent clones used for sequence determination. *MET*, methyltransferase domain; *HEL*, helicase domain; *POL*, RNA-dependent RNA polymerase domain; *CPd*, coat protein duplicate; *CP*, coat protein; *HSP70*, cellular heat shock protein 70 homologue; *NF*, novel motif. **B** Borodovsky coding sequence probability analysis for all 6 reading frames (RF). The threshold value for calling a region coding, is 0.5 while regions likely to be coding with have a value near 1. Method of Borodovsky based on a context of 96 bases and the data file at_lo_4.mat which corresponds to Arabidopsis thaliana, low GC content, 4th order

(corresponding to the conserved helicase and RNA-dependant RNA polymerase domains of ORF1a and ORF1b) were more highly conserved (82.8%) then the 5′ *ca.* 2900 nt (71.4%).

From 2 separate 5′ RACE experiments 6 clones were sequenced 3 of which contained an additional C as the penultimate nucleotide. It is likely that this C is due to an extra unpaired G at the 3′ terminus of the RNA minus strand as reported for CTV [17] and LChV-1 [15]. The 5′ end sequence would then be CUUUU. From 2 separate 3′ RACE experiments, 4 clones were sequenced, 2 of which ended with a C residue and the other 2 with a T residue.

The LChV-2/USA6b RNA genome is 15045 nt (accession number AF531505) in length and codes for 11 ORFs (Fig. 1A). The nucleotide sequence of LChV-2 USA6b is very similar to the partially determined sequence of LChV-2/LC5 sharing 92.9% similarity. LChV-2 has a 5′ non-coding region of only 5 nt in length and a 254 nt 3′ non-coding region.

Analysis of LChV-2 ORFs

The first ORF from the 5′ end, ORF0, is in the negative sense and potentially codes for a 150 amino acid polypeptide with a calculated M_r of 18.1 kDa (p18). No similarity match was detected between this polypeptide and protein sequences available through EMBL/Genbank. To confirm the presence of ORF0 at the 5′ end, this region was cloned and sequenced from 9 additional LChV-2 isolates. ORF0 was conserved in all the other LChV-2 isolates. Amino acid sequence identity of the ORF0 translation product of LChV-2 USA6b and the other isolates ranged from 91.4 to 96.0%. The entire LChV-2/USAb sequence, in all 6 reading frames were analyzed using the coding prediction method of Borodovsky (Fig. 1B). The threshold for calling a region a coding region is 0.5 while regions likely to be coding will have a value near 1. Values of over 0.5 and up to 1 were recorded for ORF0 indicating that this ORF likely contains coding sequence. All other ORFs, except for ORF4, which encodes a very small product, could also be identified as coding using this method.

In the positive sense, ORF1a begins at AUG_{540} and codes for a putative translation product of 1640 amino acids with a calculated M_r of 182.4 kDa (p182). The start codon is in a good context for translation initiation with a G and a C in the +4 and +5 positions, respectively, and pyrimidines in the −1 and −3 positions [24]. This start codon is conserved in the 9 additional LChV-2 isolates partially sequenced. Upstream of AUG_{540} are 3 in-frame stop codons, the closest at position 449 and it is therefore unlikely that ORF1a is expressed from a non-AUG start codon 5′ of AUG_{540}. The p182 has 94.3% amino acid sequence similarity with the partially determined N terminal 1112 amino acids encoded by the LChV-2/LC5 ORF1a and only 80.6% amino acid sequence similarity with the partially determined N-terminal 1528 amino acids of the ORF1a translation product from LChV-2/USA6a [42]. Alignments between the LChV-2/USA6a and 6b sequences revealed that the N-terminal region 460 amino acids (amino acids 113–570) and C-terminal 540 amino acids (amino acids 1130–1640) are highly conserved (91.3% and 94.2%, respectively), while the middle 560 amino acids are poorly conserved (<60%). The N-termini of p182 contains the 6 motifs comprising the conserved methyltransferase (MET) domain, characteristic of the Sindbis-like supergroup of positive-strand RNA viruses [2, 43]. Similarities between a *ca*. 380 amino acid sequence fragment containing the LChV-2 MET domain, with comparable sequences encoded by PMWaV-2, CTV, BYV, LIYV, LChV-1 and GLRaV-2, are shown in Table 2. Located within the C-termini of the p182 are the 7 motifs comprising the conserved helicase (HEL) domain associated with the Superfamily 1 helicase of positive-strand RNA viruses [10, 11]. Similarities between the

```
                                    * *           *           *    *
LChV-2/USA6b          947    VDCFGDFNTALVQRYTLGGYVSWHADDEPCYSHDDSIVTINFNGQAVFSIQ
LChV-2/USA6a          747    VDCFGDFNTALIQRYTLGGHVSWHADDEPCYSHDDSIVTINFNGPANFPIR
LChV-2/LC5            330    VDCFGDFNTALVQRYTLGGYVSWHADDEPCYSHDDSIVTINFNGPAVFSIQ
GLRaV-3               1596   IKYPSVFDHCLVQKYKMGGGVPFHADDEECYPSDNPILTVNLVGKANFSTK
ACLSV                 742    SKWGIDFNSALIQVYNDGCRLPLHSDNEECY-DDDGILTINVVGDAKFHTT
PMV                   594    KEPLPEFNQCLVQQFKLQAAIPFHRDDEPCYPKGHQVLTINHSGECLTQIA
ASPV                  784    KIPLNFYNQCLVQEYSTGHGLSMHKDDESIYDINHQVLTVNYSGDAIFCIE
GVA                   640    LGLDDSYDHCLIQRYTAGGSIGFHADDEPCYLPGGSVVTVNLHGDATFEVK
GVB                   642    LGLGEDFDHCLAQIYEEDKGINFNADDEPCY-TDPEVVTVNLNGNANFHLK
ShVX                  648    GEKPTTYNQCLVQKYEQGSRIGFHSDEQAIYPKGNKILTVNAAGSGTFGIK
RSPaV                 777    TGGRNYYNSCLAQIYEENSKLALHKDDESCYEIGHKVLTINLIGSATFTIS
CNRMV                 778    ELYGDRFNSCLVQHYTKAATLGLHKDDEDCYEKDHEVMTINLFGKATLFFT
CGRMV                 758    EAYGSRFNSCLVQIYEFGSKLGFHSDDESCYDDDLEVLTVNLFGEACIAFR
BlScV                 745    NGVDETYDCMLAQRYGAQGKIGFHADNEEIFMRGAPVHTVSMDGNADFGTE

Consensus                          fn cLvQ y        h Dde cy  d  vlTvn  G a f
                                   yd                    i         i
```

Fig. 2. Alignment of a conserved block of 40 amino acids located within the putative replicase proteins of the ampeloviruses LChV-2 and GLRaV-3, trichovirus *Apple chlorotic leaf spot virus* (ACLSV), vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB), potexvirus *Papaya mosaic virus* (PMV), carlavirus *Blueberry scorch virus* (BlScV), foveaviruses *Apple stem pitting virus* (ASPV), *Grapevine rupestris stem pitting associated virus* (RSPaV), and unclassified flexiviruses *Cherry green ring mottle virus* (CGRMV) and *Cherry necrotic rusty mottle virus* (CNRMV). Invariant residues are indicated by an*. In the consensus sequence, invariant residues are in capital letters, lower case letters indicate amino acids conserved in at least 7 of the 13 sequences

HEL domains encoded by LChV-2, LChV-1, GLRaV-1, -2, -3, PMWaV-2, LIYV, CTV, BYV and BYSV are shown in Table 2. Unlike for BYV, CTV, LChV-1, LIYV, GLRaV-2 and PMWaV-2, for which the complete or near complete ORF1a sequence is available, the LChV-2/USA6b p182 does not encode a papain-like proteinase (P-PRO). In Entrez searches using BLASTP, the center *ca.* 560 amino acids of p182, gave significant matches with comparable sequences from several viruses of the family *Flexiviridae*, including members of the genera, *Tricho-*, *Fovea-*, *Potex-*, *Carla-* and *Allexiviruses*, in addition to LChV-2/LC5 and GLRaV-3 of the genus *Ampelovirus*. Closer analysis revealed a conserved block of *ca.* 50 amino acids between these viruses (Fig. 2), which has not been previously identified.

ORF1b overlaps the 3′ end of ORF 1a by 47 nucleotides, coding for a polypeptide of 526 amino acids with a predicted M_r of 60.0 kDa (p60). The p60 contains the 8 conserved motifs common to RNA-dependent RNA polymerases (RdRp) of positive strand RNA viruses [21, 22]. The p60 is 93.0% identical to the partial amino acid sequence (468 amino acids) of the ORF1b translation product from LChV-2/USA6a [42] and is 97.7% identical with the LChV-2/LC5 sequence. Similarities to other closterovirus RdRp sequences are shown in Table 2. ORF1b is coded for in a +1 position relative to ORF1a consistent with the ORF 1a/b organization reported for CTV, BYV, BYSV, GLRaV-1, -2, -3, LChV-1, PMWaV-2 and LIYV. Therefore, ORF1b could potentially be expressed by a +1 ribosomal frame-shift mechanism as originally proposed for BYV [2]. Alternatively,

ORF1b could also initiate at AUG_{5446}, which is 14 nt upstream of the ORF1a UAA stop codon. This AUG is conserved in LChV-2 USA6a, USA6b and LC5 sequences.

Immediately following the ORF1b UAA stop codon, ORF2 codes for 121 amino acids (p14). This ORF is conserved in the LC5 sequence with the putative translation products sharing 92.6% identity. In both the USA6b and LC5 sequences ORF2 does not contain an AUG start codon. Translation of the ORF could be initiated by a read through of the ORF1b stop codon, or by initiation at a non-AUG start codon. Located within this region a small ORF coding for a putative P5 protein was identified in the LC5 sequence [47] however, this ORF is not conserved in the USA6b sequence.

ORFs 3, 4, 5, 6, 7 and 8 are similar to the corresponding ORFs encoded by LC5. They code for a coat protein duplicate, a p6 membrane bound hydrophobic protein, a HSP70 homolog protein, a p54 movement associated protein, a p22 of unknown function and a coat protein gene. Amino acid sequence similarities between the USA6b and LC5, p6 and p22 products are 94.6% and 96.3%, respectively. Sequence similarities between the other translation products as well as those from other members of the *Closteroviridae* are shown in Table 2.

ORF9 codes for a putative 226 amino acid polypeptide with a calculated M_r of 25.5 kDa (p26), beginning at AUG_{14111}. LC5 also codes for an ORF9, which has only been partially sequenced. The USA6b ORF9 translation product contains an additional 22 N-terminal amino acids. The partial 133 amino acid translation product encoded by LC5 ORF9 shares only 71.4% identity with the USA6b p26 making it the least conserved between USA6b and LC5.

Genomic organization

Figure 3 compares the genomic organization of LChV-2 with those of the ampeloviruses GLRaV-3, PMWaV-2 and GLRaV-1, LChV-1 LIYV (crinivirus) and BYV (closterovirus). LChV-2, as depicted, is an ambisense RNA virus. Typical of the *Closteroviridae*, the ORF1a translation product codes for a conserved P-PRO domain followed by a MET domain at the C-terminus. The LChV-2 genome lacks the P-PRO domain, instead, this region is replaced with the separate, negative sense ORF0. In common with the other fully-sequenced member of the genus *Ampelovirus*, GLRaV-3, the LChV-2 ORF1a contains a conserved NF domain of ca. 50 amino acids of unknown function present in several potex-, allexi-, fovea-, tricho- and carlaviruses. For GLRaV-3, -1, PMWaV-2, LChV-1 and BYV, the CP and CPd genes are clustered together, 3′ of the conserved gene block coding for a p5–p7 (hydrophobic protein), HSP70-like and p46–p64. By comparison, the LChV-2 CPd and CP genes are separated with the CPd located 5′ of the p6 (hydrophobic protein). Furthermore, one of the two small, poorly conserved 3′ encoded genes normally found 3′ of the CP/CPd cluster, would appear to be positioned 5′ of the LChV-2 CP gene. Finally, the p14, located 3′ of the POL gene, lacks an initiating AUG start codon.

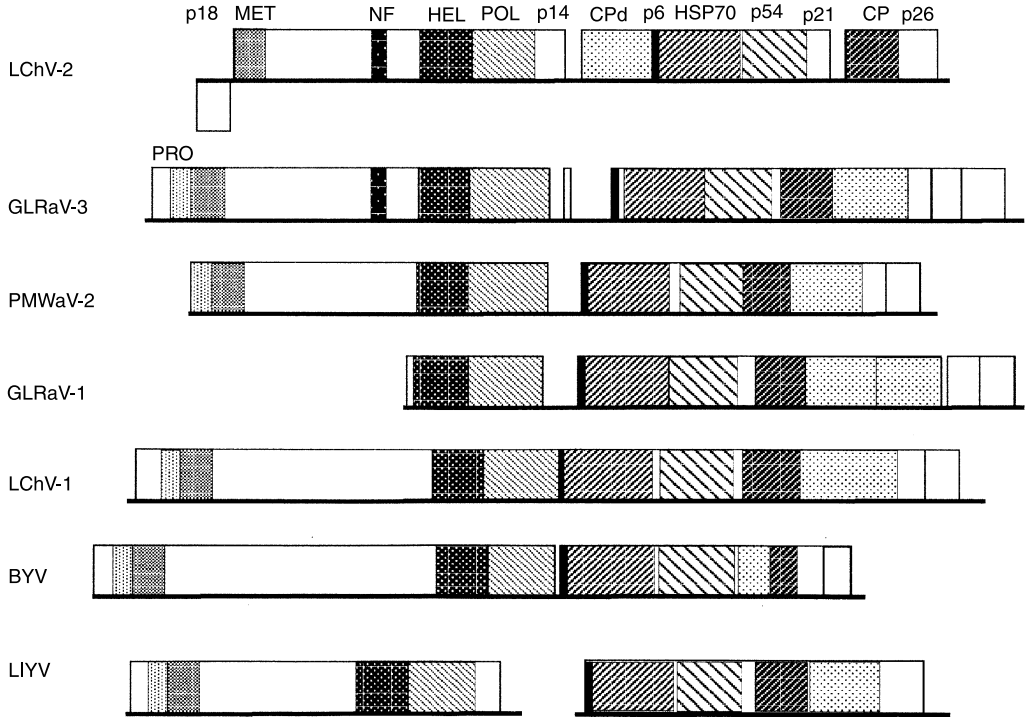

Fig. 3. Genomic organization comparisons between LChV-2 and the other ampeloviruses GLRaV-1, -3 and PMWaV-2, crinivirus LIYV, closterovirus BYV and the unclassified LChV-1. Boxes represent open reading frames (ORFs). The LChV-2 negative sense ORF is depicted below the positive sense ORFs. Conserved domains or homologous genes are indicated by identical patterns. Poorly conserved ORFs are represented by open boxes. *P-PRO*, papain-like protease; *MET*, methyltransferase; *HEL*, helicase; *POL*, polymerase; *p6*, small hydrophobic protein; *HSP70*, cellular heat shock 70 homologue; *CP*, coat protein; *CPd*, coat protein duplicate; *p54*, 54 kDa protein; *NF*, novel motif

Expression analysis of the ORF0 p18 product

To test whether the p18 could be expressed *in vitro*, clone LCV26212, containing a 722 base pair fragment corresponding to LChV-2 nucleotides 42–737, was used as a template for *in vitro* transcription/translation using either the T3 (antisense) or T7 (sense) RNA polymerases. LCV2612 includes both putative AUG start codons for ORF0 and ORF1a. Initiation at the putative ORF1a AUG codon in the positive sense would result in synthesis of a truncated polypeptide of 7.6 kDa. Initiation at the putative ORF0 AUG codon in the negative sense would result in the synthesis of a fusion product with the pBluescript II *lac*Z fragment resulting in synthesis of a 34.1 kDa polypeptide. *In vitro* transcription/translation of the positive sense RNA from LCV26212 resulted in the synthesis of a ca. 7 kDa polypeptide (Fig. 4) in agreement with that expected for translation from the ORF1a AUG codon. *In vitro* transcription/translation of the negative sense RNA from LCV26212 resulted in the synthesis of a ca. 31-kDa polypeptide, in agreement with that expected for translation from the ORF0 AUG codon. When the putative ORF1a

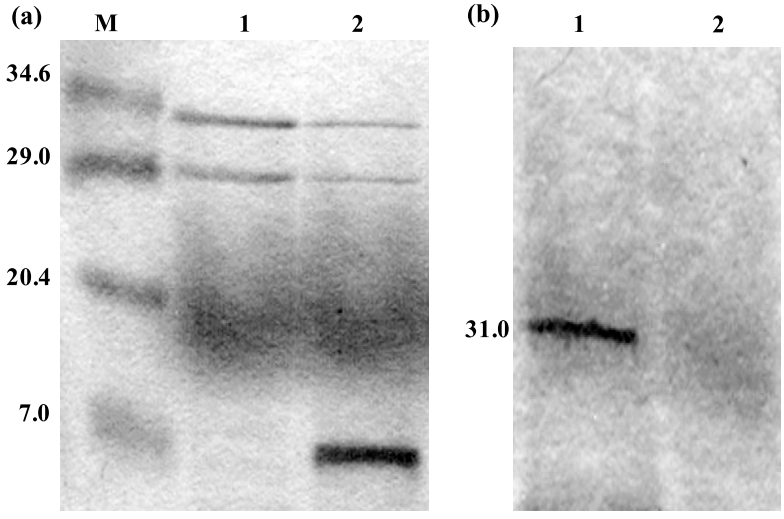

Fig. 4. *In vitro* translation analysis. **a** *In vitro* translation of ORF1a. *M* (marker), *1* clone O1M103 genomic positive sense transcript in which the ORF1a AUG start codon at position 540 has been mutated to the stop codon UAG. *2*, clone LCV26212, wild-type sequence genomic positive sense transcript. The two additional bands of ca. 29 and 34 kDa in both *1* and *2* are non-specific bands observed in the *in vitro* translation experiments using the T3 polymerase. **b** *In vitro* translation of ORF0. *1*, clone LCV26212 genomic negative sense transcript. *2*, clone ONEG103 genomic negative sense transcript in which the ORF0 AUG codon at position 458 has been mutated to the stop codon UAA. Indicated molecular weights are in kDa

AUG start codon was mutagenized to the stop codon TAG (clone O1M103) and the ORF0 AUG codon to the stop codon TAA (clone ONEG103), both the 7 kDa and 31 kDa products were abolished in the *in vitro* transcription/translation assays, respectively.

Discussion

LChV-2 USA6b and USA6a were obtained from the same tree infected with little cherry disease, indicating a mixed infection of at least 2 different LChV-2 strains. A heterogeneous population of sequence variants were observed, some more closely related to either USA6a or USA6b, and some which appeared to be recombinants between USA6a/USA6b. The tree infected with little cherry disease was graft inoculated to Canindex at the Dossenheim research station in 1988. The original innoculum, sent as *Prunus serrulata* 'Shirofugen' to Germany in the early 1950s from Davis California for use as a woody indicator for the detection of *Prune dwarf* (PDV) and *Prunus necrotic ringspot viruses* (PNRSV), probably contained a mixed population of LChV-2 at that time. The possibility that the isolate became mixed infected in Germany is unlikely as the isolate is surrounded by healthy controls which have never displayed symptoms nor tested positive by PCR. Considering the relatively long life span of a typical

cherry tree, the possibility for mixed infections, heterogeneous sequence variants in infected trees may not be uncommon. During the sequencing of LChV-1 [15], we also observed a variant sequence of LChV-1 with only ca. 80% similarity to the published sequence obtained from the same infected tree (unpublished data). Genetic variations in CTV isolates have been well documented in a number of studies, as well as, recombination between variant CTV sequences [20, 32, 43]. In a study of 5 California CTV isolates, one isolate was found to contain 2 predominant sequence variants which could have arisen by mixed infection. Each of these 2 predominant sequences were more closely related to CTV isolates obtained elsewhere, than to each other. This is similar to what is observed with LChV-2. LChV-2/USA6b is more closely related to LChV-LC5 from Canada then to LChV-2/USA6a. The amount of sequence heterogeneity between USA6a, USA6b and LC5 is similar to that observed between isolates of CTV.

Compared to other members of the *Closteroviridae*, the LChV-2 5′ terminus contains a truncated ORF1a lacking a P-PRO domain and presence of a negative sense ORF0 of unknown function. Attempts to clone alternative sequences 5′ of the MET domain were unsuccessful even when using primers designed to conserved P-PRO sequences. Instead, ORF0 and lack of an ORF1a encoded P-PRO, was confirmed by partially sequencing 9 isolates of LChV-2 obtained from Germany, Holland, Canada and the UK. It is unlikely that the LChV-2 5′ sequences are derived from a defective RNA (dRNA). Both the CTV and LIYV have been associated with dRNA molecules. These RNAs are chimeric molecules consisting of sequences derived from the 5′ and 3′ ends of the viral RNA with large internal deletions [6, 33, 45], very different from what is observed in the LChV-2 sequence.

The P-PRO domain is considered an essential and characteristic feature of the *Closteroviridae* [16]. Complete sequences of LChV-1, CTV, BYV, LIYV and GLRaV-1 and the near complete sequence for PMWaV-2, all code for one or more P-PRO domains. The BYV P-PRO has been shown to self cleave from the 1a polyprotein *in vitro* [2, 49] and is associated with BYV induced membranous vesicle aggregates thought to be important for virus replication [2, 49]. Autocatalytic cleavage by the P-PRO domain, releases the P-PRO domain from the rest of the ORF1a translation product and is essential for RNA replication [38]. Mutation studies of the BYV P-PRO showed that in the absence of P-PRO expression, insertion of an artificial start codon immediately upstream of the MET domain (mutant N-ATG-ΔALL), restored RNA replication to the same basal levels produced by functional P-PRO in the absence of the N-terminal sequences [37]. A possible interpretation of these results given by the authors is that, the P-PRO domain has no additional function for RNA replication other then to release the N-terminal sequences required for RNA amplification. The LChV-2 sequence contains a start codon immediately upstream of the MET domain, similar to the BYV N-ATG-ΔALL mutant, and could also be sufficient for basal levels of RNA replication. This is supported by the *in vitro* experiments that demonstrate that the ORF1a AUG can function as a start codon.

LChV-2 is mealybug transmitted virus as are GLRaV-3 and PMWaV-2 and could therefore be classified in the genus *Ampelovirus*. GLRaV-1 might also be transmitted by mealybugs, and is also classified in the genus *Ampelovirus*. Amino acid sequence comparisons between the putative viral encoded MET, HEL, POL, HSP70, CP and CPd products of these 4 viruses reveals the close relationship between LChV-2, GLRaV-1, GLRaV-3 and PMWaV-2, in comparison to other members of the *Closteroviridae* (see Table 2). However, LChV-2 has several unique genomic features that differentiate it from all known members of the *Closteroviridae*: the presence of a negative sense ORF0 at the 5′ end and the lack of an ORF1a encoded protease motif. LChV-2 is a single stranded RNA (ssRNA) virus [9]. All known plant ssRNA viruses are either of positive or negative sense with the exception of the tospoviruses and tenuiviruses which have ambisense genomes [23, 46]. Genomic sequence analysis, computer analysis of the coding potential of ORF0, and *in vitro* expression data suggests that LChV-2 may be the first identified member of the *Closteroviridae* with an ambisense ssRNA genome.

A novel, *ca*. 40 amino acid, domain was identified in the LChV-2 p182 which was also located in the GLRaV-3 ORF1a translation product, but is not encoded by PMWaV-2 or any of the other sequenced member of the *Closteroviridae*. Not only is this domain not characteristic of the *Closteroviridae*, but is also not specific of the *Ampeloviruses*. More surprisingly, the domain can be found in translation products of selected members of new plant virus family *Flexiviridae* of which a few have been identified here. The significance of this domain is unknown, as it does not correspond to any known conserved amino acid motif. That it is only conserved among some members of the *Flexiviridae* and *Closteroviridae* suggests that it is not essential for replication, but raises questions as to the origin and function of this domain.

ORF1b contains the RNA dependent RNA polymerase (RdRp) domain and is in a +1 position relative to ORF1a, typical of all other sequenced members of the *Closteroviridae*. It has been postulated that ORF1b is expressed as a read-through product during translation of ORF1a by a +1 ribosomal frame-shift mechanism [1, 9]. Alternatively, the LChV-2 ORF1b could be expressed from the AUG codon at position 5446. Whether or not this AUG can function as a start codon for ORF1b expression is unknown. It is in a relatively poor context for translation initiation [24], which is perhaps not significant, as it is generally assumed that the RdRp is expressed at low levels. What is interesting is that this AUG is conserved in all three LChV-2 sequences, USA6a, USA6b and LC5.

ORF1b is followed directly in-frame by ORF2, separated by a UAA stop codon. Analysis of the ORF2 sequence by the method of Borodovsky indicates that this region had a high probability of containing coding sequence (Fig. 1). Expression via read-through of an UAA stop codon has not been reported. However, conservation of this ORF in both USA6b and LC5 would further indicate that this ORF is expressed. Either via read-though of the UAA stop codon or initiated from a non-AUG start codon. In the published sequence for LC5 [47], the authors identified a small ORF coding for a p5 in place of the ORF2 identified

here. This small ORF is not conserved in the USA6b sequence and is likely not expressed.

Evidence for RNA recombination among the *Closteroviridae* is extensive from defective RNAs produced by CTV [6] and GLRaV-3 [26], and recombination between strains of CTV [32]. The genomic organization of LChV-2 is also suggestive of several recombination events. Loss of the 5′ P-PRO sequences and addition of the negative sense ORF0 could also have arisen by one or more recombination events. For the *closteroviruses* BYV, GLRaV-2, BYSV and CTV, the CPd is coded immediately 5′ of the CP gene. For LIYV (genus *Crinivirus*) GLRaV-3 and PMWaV-2 (genus *Ampelovirus*) and the unclassified LChV-1, the order of these two genes is reversed. The LChV-2 CPd is coded for 5′ of the CP and is separated from the CP (ORF8) by the p6 (ORF4), HSP70-like product (ORF5), p54 (ORF6) and the p21 (ORF7). Furthermore, CTV, GLRaV-1, -2, -3, BYV, BSYV, PMWaV-2, LIYV and LChV-1, code for 2 poorly conserved ORFs 3′ of the CP/CPd with putative translation products of between 18 and 25 kDa, whereas LChV-2 codes only for a p24. It is probable that the p21 corresponds to the second of these 2 products, however, in LChV-2 it is positioned 5′ of the CP. It has been suggested that *Closteroviridae* genomes consist of two, conserved gene blocks [16]. The first, comprising the ORFs1a/1b replicase genes, which are conserved among all the Sindbis-like supergroup viruses. The second, a 5-gene block, consisting of a small p6 hydrophobic protein, an HSP70-like protein, a p54–61 protein, followed by 2 structural proteins in that order. These 5 genes have been shown to be required for the cell-to-cell movement of BYV [3, 36]. All 5 products are conserved in the LChV-2 genome but not their order, with the addition of a sixth gene (p21) inserted within the cluster indicating that this gene block does not need to be absolutely conserved. The BYV p20, which may be analogous to the LChV-2 p21, is not required for cell-to-cell movement [3] further suggesting that genes required for movement do not necessarily need to be grouped together. Only the order of the p6, p60 (HSP70-like) and p54 is maintained, these possibly may represent a core group of dedicated movement proteins.

Acknowledgements

Our thanks goes to Sabine Wetzel for her technical support. The work was supported by the European Union grant FAIR5-CT97-3889.

References

1. Agranovsky AA (1996) Principles of molecular organization, expression and evolution of closteroviruses: over the barriers. Adv Virus Res 47: 119–158
2. Agranovsky AA, Koonin EV, Boyko VP, Maiss E, Frötschl R, Lunina NA, Atabekov JG (1994) Beet yellows closterovirus: complete genome structure and identification of a leader papain-like thiol protease. Virology 198: 311–324
3. Alzhanova DV, Hagiwara Y, Peremyslov VV, Dolja VV (2000) Genetic analysis of the cell-to-cell movement of beet yellows closterovirus. Virology 268: 192–200

4. Ayllon MA, Lopez C, Navas-Castillo J, Mawassi M, Dawson WO, Guerri J, Flores R, Moreno P (1999) New defective RNAs from citrus tristeza virus: evidence for a replicase-driven template switching mechanism in their generation. J Gen Virol 80: 817–821
5. Borodovsky M, McIninch J (1993) Recognition of genes in DNA sequences with ambiguities. Biosystems 30: 161–171
6. Che XB, Dawson WO, Bar-Joseph M (2003) Defective RNAs of Citrus tristeza virus analogous to crinivirus genomic RNAs. Virology 310: 298–309
7. Chen X, Wu R (1997) Direct amplification of unknown genes and fragments by Uneven polymerase chain reaction. Gene 185: 195–199
8. Coutts RHA, Livieratos IC (2003) Nucleotide sequence and genome organization of Cucurbit yellow stunting disorder virus RNA1. Arch Virol 148: 2055–2062
9. Eastwell KC, Bernardy MG (2001) Partial characterization of a closterovirus associated with apple mealybug-transmitted little cherry disease in North America. Phytopathology 91: 268–273
10. Gorbalenya AE, Blinov VM, Donchenko AP, Koonin EV (1989) An NTP-binding motif is the most conserved sequence in a highly diverged monophyletic group of proteins involved in positive strand RNA viral replication. J Mol Evol 28: 256–268
11. Gorbalenya AE, Koonin EV (1993) Helicases: amino acid sequence comparison and structure-function relationship. Curr Opin Struct Biol 3: 419–429
12. Hadjeb N, Berkowitz GA (1996) Preparation of T-over-hang vectors with high PCR product cloning efficiency. BioTechniques 20: 20–22
13. Hartono S, Natsuaki T, Genda Y, Okuda S (2003) Nucleotide sequence and genome organization of Cucumber yellows virus, a member of the genus crinivirus. J Gen Virol 84: 1007–1012
14. Jelkmann W (1995) Cherry virus A: cDNA cloning of dsRNA, nucleotide sequence analysis and serology reveal a new plant capillovirus in sweet cherry. J Gen Virol 76: 2015–2024
15. Jelkmann W, Fechtner B, Agranovsky AA (1997) Complete genome structure and phylogenetic analysis of little cherry virus, a mealybug transmissible closterovirus. J Gen Virol 78: 2067–2071
16. Karasev AV (2000) Genetic diversity and evolution of closteroviruses. Ann Rev Phytopath 38: 293–324
17. Karasev AV, Boyko VP, Gowda S, Nikolaeva OV, Hilf ME, Koonin EV, Niblett CL, Cline K, Gumpf DJ, Lee RF (1995) Complete sequence of the citrus tristeza virus RNA genome. Virology 208: 511–520
18. Karasev AV, Nikolaeva OV, Mushegian AR, Lee RF, Dawson WO (1996) Organization of the 3′ terminal half of beet yellow stunt virus genome and implications for the evolution of closteroviruses. Virology 221: 199–207
19. Klaassen VA, Boeshore ML, Koonin EV, Tian T, Falk BW (1995) Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. Virology 208: 99–110
20. Kong P, Rubio L, Polek M, Falk BW (2000) Population structure and genetic diversity within California Citrus tristeza virus (CTV) isolates. Virus Genes 21: 139–145
21. Koonin EV (1991) The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol 72: 2197–2206
22. Koonin EV, Dolja VV (1993) Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit Rev Biochem Mol Biol 28: 375–430
23. Kormelink R, de Haan P, Meurs C, Peters D, Goldbach R (1992) The nucleotide sequence of the mRNA segment of tomato spotted wilt virus, a bunyavirus with two

ambisense RNA segments [published erratum: J Gen Virol 1993 74: 790]. J Gen Virol 73: 2795–2804
24. Kozak M (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15: 8125–8148
25. Kreuze JF, Savenkov EI, Valkonen JPT (2002) Complete genome sequence and analyses of the subgenomic RNAs of Sweet potato chlorotic stunt virus reveal several new features for the genus Crinivirus. J Virol 76: 9260–9270
26. Ling KS, Gonsalves D (1997) Recent information on the molecular characterization of grapevine leafroll virus-3. In: Vesely D (ed), Recent research development in plant pathology – filamentous viruses of woody plants. Research Signpost, Trivandrum, India
27. Ling KS, Zhu HY, Drong RF, Slightom JL, McFerson JR, Gonsalves D (1998) Nucleotide sequence of the 3′-terminal two-thirds of the grapevine leafroll-associated virus-3 genome reveals a typical monopartite closterovirus. J Gen Virol 79: 1299–1307
28. Little A, Fazeli CF, Rezaian MA (2001) Hypervariable genes in grapevine leafroll associated virus 1. Virus Res 80: 109–116
29. Livieratos IC, Coutts RHA (2002) Nucleotide sequence and phylogenetic analysis of Cucurbit yellow stunting disorder virus RNA 2. Virus Genes 24: 225–230
30. Martelli G, Agranovsky A, Bar-Joseph M, Boscia D, Candresse T, Coutts R, Dolja VV, Falk B, Gonsalves D, Jelkmann W, Karasev AV, Minafra A, Namba S, Vetten H, Wisler G, Yoshikawa N (2002) The family *Closteroviridae* revised. Arch Virol 147: 2039–2044
31. Martelli GP, Agranovsky AA, Bar-Joseph M, Boscia D, Candresse T, Coutts RHA, Dolja VV, Duffus JD, Falk BW, Gonsalves D, Jelkmann W, Karasev A, Minafra A, Murant AF, Namba S, Niblett CL, Vetten HJ, Yoshikawa N (2000) Family *Closteroviridae*. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (eds), Virus taxonomy. Classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego
32. Mawassi M, Mietkiewska E, Gofman R, Yang G, Bar-Joseph M (1996) Unusual sequence relationships between two isolates of citrus tristeza virus. J Gen Virol 77: 2359–2364
33. Mawassi M, Satyanarayana T, Albiach-Marti MR, Gowda S, Ayllon MA, Robertson C, Dawson WO (2000) The fitness of citrus tristeza virus defective RNAs is affected by the lengths of their 5′- and 3′-termini and by the coding capacity. Virology 275: 42–56
34. Mawassi M, Satyanarayana T, Gowda S, Albiach-Marti MR, Robertson C, Dawson WO (2000) Replication of heterologous combinations of helper and defective RNA of citrus tristeza virus. Virology 267: 360–369
35. Melzer MJ, Karasev AV, Sether DM, Hu JS (2001) Nucleotide sequence, genome organization and phylogenetic analysis of pineapple mealybug wilt-associated virus-2. J Gen Virol 82: 1–7
36. Napuli AJ, Falk BW, Dolja VV (2000) Interaction between HSP70 homolog and filamentous virions of the beet yellows virus. Virology 274: 232–239
37. Peng CW, Dolja VV (2000) Leader proteinase of the beet yellows closterovirus: mutation analysis of the function in genome amplification. J Virol 74: 9766–9770
38. Peremyslov VV, Hagiwara Y, Dolja VV (1998) Genes required for replication of the 15.5-kilobase RNA genome of a plant closterovirus. J Virol 72: 5870–5876
39. Raine J, McMullen RD, Forbes RD (1986) Transmission of the agent causing little cherry disease by the apple mealybug *Phenacoccus aceris* and the dodder *Cuscuta lupuliformis*. Can J Plant Pathol 8: 6–11
40. Rosciglione B, Gugerli P (1989) Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by the mealybug *Planococcus* ficus. Phytoparasitica 17: 63

41. Rott ME, Jelkmann W (2001) Characterization and detection of several filamentous viruses of cherry: adaptation of an alternative cloning method (DOP-PCR), and modification of an RNA extraction protocol. Eur J Plant Pathol 107: 411–420
42. Rott ME, Jelkmann W (2001) Detection and partial characterization of a second closterovirus associated with little cherry disease, little cherry virus-2. Phytopathology 91: 261–267
43. Rozanov MN, Koonin EV, Gorbalenya AE (1992) Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. J Gen Virol 73: 2129–2134
44. Rubio L, Ayllon MA, Kong P, Fernandez A, Polek M, Guerri J, Moreno P, Falk BW (2001) Genetic variation of Citrus tristeza virus isolates from California and Spain: evidence for mixed infections and recombination. J Virol 75: 8054–8062
45. Rubio L, Yeh HH, Tian T, Falk BW (2000) A heterogeneous population of defective RNAs is associated with lettuce infectious yellows virus. Virology 271: 205–212
46. Takahashi M, Toriyama S, Hamamatsu C, Ishihama A (1993) Nucleotide sequence and possible ambisense coding strategy of rice stripe virus RNA segment 2. J Gen Virol 74: 769–773
47. Theilmann J, Mozafari J, Reade R, Wu Z, Xie W, Jesperson G, Bernardy M, Eastwell KC, Rochon D (2002) Partial nucleotide sequence and genome organization of a Canadian isolate of Little cherry virus and development of an enzyme-linked immunosorbent assay-based diagnostic test. Phytopathology 92: 87–98
48. Zhu HY, Ling KS, Goszczynski DE, McFerson JR, Gonsalves D (1998) Nucleotide sequence and genome organization of grapevine leafroll-associated virus-2 are similar to beet yellows virus, the closterovirus type member. J Gen Virol 79: 1289–1298
49. Zinovkin RA, Erokhina TN, Lesemann DE, Jelkmann W, Agranovsky AA (2003) Processing and subcellular localization of the leader papain-like proteinase of Beet yellows closterovirus. J Gen Virol 84: 2265–2270

Author's address: Dr. Michael Rott, Canadian Food Inspection Agency, Center for Plant Health, 8801 East Saanich Road, Sidney, B.C., Canada, V8L1H3; e-mail: rottm@inspection.gc.ca