Further complexity of the genus *Crinivirus* revealed by the complete genome sequence of *Lettuce chlorosis virus* (LCV) and the similar temporal accumulation of LCV genomic RNAs 1 and 2

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The sequence of *Lettuce chlorosis virus* (LCV) (genus *Crinivirus*) was determined and found to contain unique open reading frames (ORFs) and ORFs similar to those of other criniviruses, as well as 3′ non-coding regions that shared a high degree of identity. Northern blot analysis of RNA extracted from LCV-infected plants identified subgenomic RNAs corresponding to six prominent internal ORFs and detected several novel LCV-single stranded RNA species. Virus replication in tobacco protoplasts was investigated and results indicated that LCV replication proceeded with novel crinivirus RNA accumulation kinetics, wherein viral genomic RNAs exhibited a temporally similar expression pattern early in the infection. This was noticeably distinct from the asynchronous RNA accumulation pattern previously observed for *Lettuce infectious yellows virus* (LIYV), the type member of the genus, suggesting that replication of the two viruses likely operate via dissimilar mechanisms.

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Introduction

The family *Closteroviridae* consists of positive-sense, single stranded (ss)RNA viruses with long, flexuous virions and large genomes of up to 20 kb. Closteroviruses cause serious diseases in a number of economically important fruit, vegetable and ornamental crops worldwide. Taxonomy of the *Closteroviridae*, based on vector transmission and phylogenetic relationships, has defined three genera in the family. These include *Closterovirus*, which consists of aphid-borne viruses with monopartite genomes; *Ampelovirus*, which comprises of mealybug-borne members with monopartite genomes; and *Crinivirus*, which includes tested and proven white flies. Although LCV is endemic to Southwestern U.S.A. and causes frequent disease outbreaks (J. Ng, unpublished data), its genome sequence and molecular biology remain unknown and uncharacterized.

In contrast, *Lettuce infectious yellows virus* (LIYV), the type species of *Crinivirus* has been extensively studied and is the only member in the genus for which cloned infectious complementary (c)DNAs are available (Klaassen et al., 1996). LIYV is also the only crinivirus whose replication has been analyzed. Time course analysis of LIYV progeny RNAs accumulation in LIYV virion (v)RNAs-inoculated tobacco protoplasts showed an asynchronous buildup of LIYV RNAs, in which the initial accumulation of RNA 2 lagged behind that of RNA-dependent RNA polymerase (RdRp) encoded in ORF 1b (Karasev, 2000). They also contain a hallmark five gene array encoding a small hydrophobic protein, a heat shock protein homolog (HSP70h), a protein of 50–60 kDa, depending on the virus, the major coat protein (CP) and the minor CP (CPm) (Karasev, 2000).

*Lettuce chlorosis virus* (LCV) is a member of the rapidly emerging genus *Crinivirus* (Duffus et al., 1996). A limited number of biological and physiochemical studies have revealed features of the virus that are consistent with those described for all other criniviruses (Duffus et al., 1996; Liu et al., 2000; McLain et al., 1998; Wisler et al., 1997). For example, plant infection by LCV is restricted to the phloem, and virions are transmitted in a semi-persistent manner by *Bemisia tabaci* biotypes A and B whiteflies. Although LCV is endemic to Southwestern U.S.A. and causes frequent disease outbreaks (J. Ng, unpublished data), its genome sequence and molecular biology remain unknown and uncharacterized.

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1 (Yeh et al., 2000). This LIYV RNA accumulation pattern is believed to be associated, in part, with the dissimilarity in sequence and/or structure between both genomic 3′ termini (Yeh et al., 2000), which are sites, in positive-sense ssRNA viruses, where interactions with viral replication proteins occur to initiate negative-strand RNA synthesis (Buck, 1996). It is not known if other criniviruses exhibit such a viral RNA accumulation pattern. Analysis of the genomes of extant criniviruses indicated that, other than LIYV, nucleotides at the 3′ non-coding regions (NCRs) of RNAs 1 and 2 display a high degree of similarity within individual criniviruses (Aguilar et al., 2003; Hartono et al., 2003; Kreuze et al., 2002; Tzanetakis et al., 2006; Wintermantel et al., 2005). This suggests that an asynchronous accumulation of RNAs 1 and 2 may not be common among criniviruses, but this suggestion has not been validated experimentally.

Here are presented results of the sequence and phylogenetic analyses of the LCV genomic RNAs, showing that they: 1) contain both unique ORFs and ORFs similar to those of other criniviruses, 2) share the highest homologies with those of the crinivirus Bean yellow disorder virus (BYDV), and 3) have 3′ NCRs that share a high degree of nucleotide sequence identity, a feature that is similar to almost all of the fully characterized members of Crinivirus. Furthermore, our studies have identified the subgenomic (sg)RNAs corresponding to several prominent internal ORFs in the LCV genome, and this is consistent with the hypothesis that sgRNA synthesis is a genome expression strategy employed by LCV. We also provide unprecedented evidence of a crinivirus, LCV, exhibiting a replication pattern that is distinct from the asynchronous accumulation (of RNAs 1 and 2) previously observed in LIYV, suggesting that LCV and LIYV likely do not initiate infection by identical mechanisms.

Results and discussion
dsRNA analysis and cDNA cloning

LCV-specific double stranded RNAs (dsRNAs) were used as templates for cDNA synthesis. Electrophoretic analysis revealed the presence of a prominent, slightly diffused, high molecular weight dsRNA band (approx. 8.6 kb) (data not shown). Several lower molecular weight species were also detected (data not shown). The high molecular weight dsRNA band was excised, purified from agarose gels and used for cDNA synthesis, cloning and sequencing.

Results of 260 reads clustered into two contigs, one with 8442 bp and the other with 3162 bp, corresponding to sequences of RNA 1 and RNA 2 of LCV, respectively. The shotgun sequencing allowed an almost complete coverage (98%) of RNA 1, while the coverage for RNA 2 was lower (40%). Missing genomic sequences were acquired by sequencing individual cDNA clones obtained through RT-PCR amplification, using LCV-specific oligonucleotide primers flanking the gaps. Additional cDNA clones with insert sequences representing the 5′- and 3′-terminal regions of the genome generated using 5′- and 3′-RACE were also obtained, completing the coverage of the entire genome of 8591 and 8556 nt for RNAs 1 and 2, respectively. The small size difference between RNAs 1 and 2 is consistent with the apparent migration of LCV genomic dsRNAs as a single band during gel electrophoresis. The sequence of LCV has been submitted to the GenBank Nucleotide Sequence Database and assigned the accession numbers FJ380118 (RNA 1) and FJ380119 (RNA 2).

Nucleotide sequence analyses and ORFs of LCV RNA 1

LCV RNA 1 contains the replication module (ORFs 1a and 1b), which encodes conserved domains of the viral P-PRO, MTR, HEL and RdRp (Fig. 1A). In LIYV and Beet yellow virus (BYV), this module is sufficient to support the replication of viral RNA (Klaassen et al., 1995; Peremyshlov et al., 1998). ORF 1a encodes a predicted protein of 227 kDa with an AUG codon that is in optimal context for translational initiation (Kozak, 1986; Kozak, 1991). Analysis of the N-terminus of 1a revealed a putative P-PRO domain with significant similarity (62%) to the P-PRO of BrnYDV (Martin et al., 2008). The two catalytic residues, Cys and His, (Peng et al., 2001), are located at positions 406 and 455, respectively. Cys-406 bears the same motif, cCWxx, identified in other criniviruses (Tzanetakis et al., 2005; Tzanetakis et al., 2006), where α represents a hydrophobic residue (A, V, L, I, M, F, W or Y). The presumed cleavage site is between Gly-474 and Val-475; thus, auto-proteolysis would yield a putative leader proteinase of 54.5 kDa. In BYV, P-RPO is a multi-functional protein involved in polyprotein processing, long-distance virus transport, and enhancement of RNA amplification (Peng and Dolja, 2000; Peng et al., 2003). The MTR domain (Rozanov et al., 1992) shares 70% similarity with the BrnYDV orthologous domain. The HEL contains the seven conserved motifs characteristic of RNA helicases (Gorbalenya and Koonin, 1993), and is the most conserved region of 1a, exhibiting 65% aa sequence identity and 81% similarity with the orthologous region of BrnYDV. Overall, the LCV 1a protein shares homology with that of other criniviruses (Aguilar et al., 2003; Coutts and Livieratos, 2003; Klaassen et al., 1995; Kreuze et al., 2002; Livieratos et al., 2004; Martin et al., 2008; Tzanetakis and Martin, 2004; Tzanetakis et al., 2005; Tzanetakis et al., 2006; Wintermantel et al., 2005), but shows minimal homology with the more distantly related CTV (Karasev et al., 1995). As with other criniviruses, a region of more than 800 aa between the MTR and HEL domains shows no significant similarity with any known proteins. ORF 1b encodes the 59 kDa RdRp and contains all eight conserved sequence motifs reported in the RNA polymerases of positive strand RNA viruses (Koonin, 1991; Koonin and Dolja, 1993). The LCV RdRp is most closely related to that of BrnYDV (94% similarity), followed by that of Cucurbit yellow stunting disorder virus (CYSVD; 88%) and Blackberry yellow vein associated virus (BYVaV; 81%). ORF 1b is in a +1 reading frame compared to ORF 1a and may be expressed via a +1 ribosomal frameshift (Karasev, 2000). This process is predicted to yield a fusion protein of 286.5 kDa, identical in size to that predicted for the orthologous protein of CYSVD (Coutts and Livieratos, 2003) and Sweet potato chlorotic stunt virus (SPCSV) (Kreuze et al., 2002). Analysis of the nucleotide sequence in the overlapping region of ORFs 1a and 1b of LCV and several other closteroviruses did not identify any slippery heptanucleotide sequences or structural elements that favor frameshifting (Agranovsky et al., 1994; Klaassen et al., 1995; Klaassen et al., 1996). Nevertheless, +1 frameshifting in the expression of the closterovirus ORF 1b is also believed to be influenced by the context of the nucleotide sequence before the ORF 1a stop codon. In LCV, BrnYDV and CYSVD, the termination signal for ORF 1a is UUAUG. In Beet pseudo-yellows virus (BPYV), BYVaV, LIYV, Potato yellow vein virus (PYVV), Strawberry pallidosis associated virus (SpAV), SPCSV and Tomato chlorosis virus (ToCV), it is UUAUG. In all cases, it has been postulated that the three uridines upstream of the stop codon of ORF 1a may be involved in assisting the +1 ribosomal frameshift (Karasev, 2000).

ORF 2 encodes a putative protein with a predicted molecular mass of 8 kDa (P8) (Fig. 1A) and a potential transmembrane helix between amino acids 29 and 51. When examined for putative signal peptides (SignalP V2.0.b2 at, http://www.cbs.dtu.dk/services/SignalP-2.0/), a potential cleavage site between amino acids 48 and 49 was identified. No sequence homology has been found between P8 and any other proteins in the databases, nor has any conserved motifs been identified in its sequence. Small hydrophobic proteins (SHP) of similar sizes are encoded by most members of the Closteroviridae and are thought to possess membrane-binding properties. However, the number, size and genomic location of these small proteins vary in all of the sequenced criniviruses. A SHP, P6, is essential for virus-cell-to-cell movement in BYV (Alzhanova et al., 2000).

ORF 3 encodes a 22.9 kDa protein (P23) of unknown function. LCV P23 shares 45% sequence similarity with BrnYDV RNA 1-encoded P26 and 41% sequence similarity with ToCV RNA 1-encoded
P22. Coincidentally, a similarly sized protein, which bears no sequence homology with LCV P23 is encoded by an ORF situated at the 3′-terminal region of the CYSDV, LIYV and SPCSV RNA 1. In LIYV this protein is known to function as an enhancer of RNA replication (Yeh et al., 2000).

Nucleotide sequence analyses and ORFs of LCV RNA 2

RNA 2 is consists of 10 predicted ORFs (Fig. 1A). This contrasts with that of most criniviruses, which encodes between 7 and 9 ORFs. LCV RNA 2 is colinear with BnYDV RNA 2, but has an additional ORF (ORF 10; P4.8) at the 3′-distal end. Two small ORFs (ORFs 1 and 2) are situated near the 5′ terminus of the RNA molecule, encoding products of 5.6 (P5.6) and 6 kDa (P6), respectively. However, whereas small protein encoding ORFs situated in a similar genomic location of other criniviruses contain transmembrane helices, P5.6 and P6 do not appear to have this feature (SignalP V2.0,b at, http://www.cbs.dtu.dk/services/SignalP-2.0/). P6 contains a Cys at position 3, the same position the Cys involved in dimerization of the BYV P6 is located (Peremyslov et al., 2004b). As with P8 in RNA 1, P5.6 and P6 do not show any significant sequence homologies with any other proteins in the database.

ORF 3 encodes a putative protein of 62 kDa (P62) that contains the signature motifs of the HSP70 protein family. Heat shock proteins are molecular chaperones that possess an N-terminal ATPase domain and a C-terminal substrate identification domain, and play a role in mediating the correct folding of cellular proteins (Alzhanova et al., 2000). The ATPase domain of HSP70 proteins contains five signature motifs (Bork et al., 1992), all of which are present in the HSP70 of LCV. Our analyses revealed that the LCV HSP70 is most closely related to that of BnYDV, CYSDV and SPaV. Studies have demonstrated that the HSP70 is a multi-functional protein whose basic mode of action most likely involves ATPase activity and protein-protein interactions (Agranovsky et al., 1991; Agranovsky et al., 1997; Peremyslov et al., 1999). For example, the HSP70 of BYV and LIYV are structural components of the virion (Napuli et al., 2003; Napuli et al., 2000; Tian et al., 1999) and may be involved in the encapsidation process (Peremyslov et al., 2004a). Further, HSP70 has been identified in the plasmodesmata of BYV-infected hosts (Medina et al., 1999), and is also required for cell-to-cell movement for both BYV (Alzhanova et al., 2001; Alzhanova et al., 2007) and CTV (Satyanarayana et al., 2000).

ORF 4 encodes a putative protein of 6.4 kDa (P6.4) with unknown function and shows a high degree of similarity with proteins present in a similar position in BnYDV, SPaV and CYSDV RNA 2.

ORF 5 encodes a 59.8 kDa (P60) putative protein, the ortholog of the coat protein homologs (CP) of clostero- and criniviruses (Karasev, 2000). LCV P60 is most closely related to the BnYDV CP, with 70% and 84% amino acid sequence identity and similarity, respectively. P60 shares similarity at its C-terminal domain with regions in the CP and CPm of other closteroviruses (Napuli et al., 2000). In particular, two charged amino acid residues, Arg-416 and Asp-455, are invariant within this region of similarity. The two charged amino acids are also found at positions 434 (Arg) and 471 (Asp) for LCV P60. These conserved residues could be involved in virion assembly (Dolja et al., 1991) and virus movement (Napuli et al., 2003), while the protein itself has been shown to be an integral component of the BYV virion (Napuli et al., 2003; Peremyslov et al., 2004a).

The sixth predicted RNA 2 ORF (ORF 6) encodes a putative protein of 9.4 kDa (P9). This putative 79 amino acid peptide is of unknown function and appears to be unique among members of the genus Crinivirus since it does not share similarity with proteins encoded by viruses in the other genera of the Closteroviridae or other proteins in the databases (Karasev, 2000).

ORFs 7 and 8 encode the CP (28.4 kDa) and CPm (54.7 kDa), respectively. Studies of BYV, CTV and LIYV have shown that the CP encapsidates nearly the entirety length of the virion, while the CPm is confined at the tip (Agranovsky et al., 1995; Febres et al., 1996; Tian et al., 1999; Zinovkin et al., 1999), where it also associates with HSP70 and Cph, and encapsidates the 5′ end (approx. 600 nt) of the RNA genome to form a characteristic ‘rattlesnake’ structure (Peremyslov et al., 2004a; Satyanarayana et al., 2004). Sequence alignments have identified three conserved residues involved in protein-RNA interactions in the CP (Ser-118, Arg-165, and Asp-202), and the Cpm, (Ser-348, Arg-392, and Asp-433) (data not shown). In addition to having a structural role in the virion architecture, both proteins may be involved in cell-to-cell movement in BYV (Alzhanova et al., 2000; Alzhanova et al., 2001), and Cpm is a potential determinant of vector transmission for LIYV (Tian et al., 1999).

The predicted ORF 9 encodes a putative protein of 26.9 kDa (P27). Overall, LCV P27 shows significant amino acid similarity with its counterpart proteins expressed in most criniviruses; the most significant of which being BnYDV P27, wherein 54% amino acid sequence identity and more than 77% similarity were observed. The ORF 2 encodes a putative 4.8 kDa protein (P4.8) with a transmembrane domain. This ORF is analogous, in terms of size and genomic location, to ORF 10 of ToCV. However, its deduced amino acid sequence of P4.8 shows no significant similarity with this or any other protein sequences available in the databases.

Phylogenetic analyses

Phylogenetic analyses were performed on the amino acid sequences of the RdRps, HSP70hs, CPs and CPm proteins of criniviruses, using CTV as an outgroup. The data indicated that LCV consistently clustered with other criniviruses and were distinct from those of CTV (Fig. 2). The analyses further grouped LCV into the same lineage with BnYDV and CYSDV; these three viruses formed a distinct cluster apart from all other members of the genus. The closest relative of LCV, as predicted by pairwise amino acid comparisons and confirmed by phylogenetic analyses, is BnYDV (Martin et al., 2008).

Expression of LCV subgenomic RNAs and detection of novel single stranded RNAs

The genomic organization of LCV suggests that its gene expression likely involves strategies common among closteroviruses, including translational frameshifting, polyprotein processing, and the production of 3′-coterminal sgRNAs (Karasev, 2000). To verify the expression of the sgRNAs corresponding to six prominent LCV internal ORFs, Northern blot analyses were performed using total RNAs, virion (v) RNAs and dsRNA-enriched extracts. Riboprobes complementary to eight different regions of the genome were used for the analyses (Fig. 1A). Probe I hybridized to a RNA species that matched the size (approx. 8.6 kb) of genomic RNA 1 (G1; Fig. 1B), but not of healthy plants or vRNAs. This RNA species also was not detected by probe II. The significant expression of this molecule in the context of the LCV genome expression strategy is currently unknown. Molecules of 0.7–0.8 kb have been detected in both total RNA and dsRNA preparations of CTV (Mawassi et al., 1995), and have been shown to be positive stranded 5′-coterminal sgRNAs whose synthesis are terminated by a sgRNA controller element present in the genomic negative-sense RNA (Che et al., 2001; Gowda et al., 2001). Probe II hybridized with the 8.6 kb genomic LCV RNA 1 and a lower molecular weight (approx. 0.95 kb) RNA species (Fig. 1C). The size of this RNA suggested that it was likely the sgRNA of LCV P8 and/or P23 (sg1). The absence of a detectable sgRNA for ORF 1b is consistent with the hypothesis that this ORF is likely expressed by translation via a +1 ribosomal frameshift rather than by sgRNA production.
Probe III detected a RNA species that matched the size (approx. 8.6 kb) of genomic RNA 2 (G2). Probe III also hybridized to a series of lower molecular weight (ca. 0.4–1.5 kb) species in the total RNA of LCV-infected plants (indicated as ♦ in Fig. 1D), but not of healthy plants or vRNAs. Because these species were not detected by probes (IV–VIII) corresponding to the remaining regions of RNA 2 (Figs. 1E–I), they resembled the positive stranded low molecular weight 5′-coterminal sgRNAs reported for CTV (Che et al., 2001; Mawassi et al., 1995) and the 0.3 kb 5′-coterminal sg-like RNA of LCV RNA 1 (Fig. 1B). Probe IV also detected genomic RNA 2 (G2) like it was with probe III (Fig. 1E). In addition, it detected a smaller sized (approx. 7.0 kb) species, which was observed following an extended exposure of the X-
ray film (Fig. 1E inset). A similarly sized component was also detected by an equivalent riboprobe in the Northern blot analysis of dsRNA-enriched extracts of LCV-infected plants (Fig. 1Ei). The size of this RNA suggested that it was likely the sgRNA of P6 and/or HSP70h (sg2). Probe V was designed to identify the sgRNA of P6.4 and/or P60 (sg3). However, attempts at using it to detect sg3 in both the total RNA and dsRNA-enriched extracts did not result in any definitive hybridization signal (Fig. 1F and data not shown). Nevertheless, it did hybridize to G2 and a ssRNA species that corresponded to sg2 (Fig. 1F). Probe VI also hybridized with the same two RNA species, G2 and sg2, that were detected by probes IV and V (Fig. 1G). The hybridization pattern of this virion-RNA was obscured by similarly sized artifacts (see explanation below).

Evidence of sg4 expression was to be found in the Northern analysis of dsRNA-enriched extracts, in which an equivalent riboprobe was used for hybridization (Fig. 1Gi). The same hybridization also resulted in the detection of an approx. 5.2 kb dsRNA species (Fig. 1Gi), the size of which suggested that it was the sgRNA of P6.4 and/or P60 (sg3). Probes VII and VIII detected an approx. 2.6 kb and 1.1 kb RNA, respectively, in both the total RNA and dsRNA-enriched extracts (Figs. 1H and Hi; and I and Ii). Based on size estimations, these RNAs were likely the sgRNAs of the CPm and P27, respectively.

In Northern analyses (Figs. 1B–I), vRNAs were included as a positive control for the identification of genomic RNAs 1 and 2. Analyses of these vRNAs using probes III, VI and VIII clearly detected the presence of an approx. 2.4 kb RNA species in addition to genomic RNA 2 (Figs. 1D, G and I). The hybridization pattern of this virion-

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**Fig. 1.** Lettuce chlorosis virus (LCV) genome organization and identification of genomic and subgenomic (sg)RNAs. (A) Schematic representation of the LCV genome organization. Rectangles represent the open reading frames (ORFs) in RNAs 1 and 2 encoding the putative viral proteins: P-Pro, papain-like protease; MTR, methyltransferase; HEI, RNA helicase; RdRp, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homolog; CP, major coat protein; CPm, minor coat protein; and proteins that are named after their relative masses (indicated by numbers preceded by “P”): P8, P23, P5.6, P6, P6.4, P60, P9, P27, and P48. ORFs that show similarity with homologous proteins expressed in other crinino- and/or closteroviruses are colored. Those that do not exhibit similarity with any proteins in the databases are uncolored. ORF numbers (1a–3 in RNA 1, and 1–10 in RNA 2) are indicated within each ORF. Black bars below the genome map represent the RNA probes (I–VIII) complementary to the corresponding locations in the genomic RNAs. Fine lines of different lengths underneath the genomic map depict the predicted sgRNAs corresponding to: P8 and/or P23 (sg1), P6 and/or HSP70h (sg2), P6.4 and/or P60 (sg3), P9 and/or CP (sg4), CPm (sg5), and P27 (sg6); with the estimated sizes (kb) being shown above each line. (B–I) Northern blot hybridization analyses using probes I–VIII (as indicated above each blot) to detect the genomic and sgRNAs in total RNA extracts prepared from LCV-infected plants (lanes L; 5 μg/lane). LCV virion (v)RNAs (lanes V; 73 ng/lane) and total RNA extracts prepared from healthy plants (lanes H; 5 μg/lane) were included as hybridization controls. Inset in blot (E) shows the extended exposure of the X-ray film for lane L. Methylene blue-stained 25S rRNAs on blots (B–I) are included to indicate the equal loadings of total RNAs in the respective lanes. In some cases, Northern blot hybridization using RNA probes equivalent to probes IV (Ei), VI (Gi), VII (Hi), and VIII (Ii) were conducted to examine the expression of sgRNAs in dsRNA-enriched extracts prepared from LCV-infected plants. Black triangles to the right of the blots indicate the hybridization signals for genomic RNAs 1 (G1) and 2 (G2), and the sgRNAs (sg1–sg6). The positions of the 5′-coterminal sg-like RNAs detected in blots (B) and (D) are marked by an * and †, respectively. The positions of a potential virion-associated LCV defective RNA in blots (D), (G) and (I) are circled. Sizes of the RNAs were estimated from methylene blue-stained RNA standards included in the same gels used for the Northern hybridizations and are shown on the left of the blots (B) and (D). Estimated sizes of the sgRNAs detected in blots (EI–Ii) are shown on the left of blot (Ei).

**Fig. 2.** Phylogenetic relationships between Lettuce chlorosis virus (LCV) and selected viruses from the family Closteroviridae. Analyses were performed using the amino acid sequence alignments for the RdRp (A), HSP70h (B), CP (C) and CPm (D). Trees were constructed by using the Minimum Evolution Algorithm provided in the MEGA2 software package (Kumar et al., 2001) and assessed by bootstrapping using 1000 pseudoreplicates. The numbers at each node represent the bootstrap values. The scale bars represent the number of residue substitutions per site. Bean yellow disorder virus (ByNVDV), Blackberry yellow vein associated virus (BnYAV), Beet pseudo-yellows virus (BPYV), Cucurbit yellow stunting disorder virus (CYSDV), Lettuce infectious yellows virus (LiYV), Potato yellow vein virus (PyVV), Strawberry pallidosis associated virus (SPaV), Sweet potato chlorosis stunt virus (SPCSV), Tomato chlorosis virus (ToCV) and Citrus trifallax virus (CTV).
Fig. 3. Nucleotide sequence alignment of the 5'- and 3'-terminal regions of the two genomic segments of *Lettuce chlorosis virus* (LCV). (A) Identical nucleotides between LCV RNAs 1 and 2 located at the 5'- non-coding region (NCR) (with respect to nt positions 1–73 on LCV RNA 1) are highlighted (■). A stretch of 14 identical nucleotides from positions 207 to 220 on RNA 1 and positions 189 to 202 located within the 5'-NCR of RNA 2 is also highlighted (■) and underlined. The stop of ORF 1a on LCV RNA 1 is as indicated. (B) Identical nucleotides between LCV RNAs 1 and 2 located at the 3'-NCR (with respect to the 3'-terminal 98 nt of LCV RNA 2) are highlighted (■). The stop codon of the last ORF (ORF 10) in LCV RNA 2 is as indicated.

associated 2.4 kb RNA implied that it contained nucleotide sequences corresponding to the 5' and 3' termini as well as a region within ORF 7 (CP) of RNA 2. It should be noted that the same vRNA preparation was used for all of the above Northern analyses, yet neither was a similarly sized (2.4 kb) RNA species nor any other distinct RNA species detected by the rest of the RNA 1 and 2 probes. Based on these initial data, the 2.4 kb RNA appears to possess features of defective (D)RNAs (Mawassi et al., 1995; Rubio et al., 2000), and seems to contain more than one deleted region of RNA 2 (Figs. 1D–I). However, until it has been cloned, sequenced and analyzed, its identity can only be speculated upon at this time. In addition to all of the above viral-derived RNAs, probes used for the hybridization analyses of total RNAs from LCV-infected plants frequently detected an approx. 3.3 kb and 1.7 kb RNA species in most instances (Fig. 1). These are likely artifacts due to trapping by the traps. Inoculated protoplasts were harvested after various incubation periods (0 to 96 h) following inoculation. Equal amounts (5 μg) of the extracted total RNAs were analyzed by Northern blot hybridization using DIG-labeled single stranded riboprobes complementary to the corresponding to the 5'-termini of RNAs 1 and 2 are identical (Klaassen et al., 1995). Notably, a stretch of 14 identical nucleotides located within 220 nt from the 5'-termini of LCV RNAs 1 and 2 also has been identified in our study (Fig. 3A).

The 3' NCR of RNAs 1 and 2 are highly conserved (Fig. 3B), sharing an 81% nucleotide sequence identity; but no pseudoknot structures such as those identified in PYVV (Livieratos et al., 2004) or Strawberry chlorotic fleck associated virus (Tzanetakis and Martin, 2007) were identified (Zuker, 2003). Conservation of the 3' NCR is a feature common among RNA viruses with multipartite genomes (Dreher, 1999), and is consistent with the notion that identical nucleotide sequences and/or structural elements at the 3' NCR are essential for replication protein binding and initiation of minus-strand synthesis during RNA replication (Boccard and Baulcombe, 1993; Chen et al., 1999; Wintermantel et al., 2005), with the exception of LIYV, which shows ~31% similarity (Klaassen et al., 1995). This anomaly has been postulated to be one reason for the asynchronous temporal replication pattern of the two LIYV RNA species (Yeh et al., 2000) (see below).

5' and 3' non-coding regions

Nucleotide sequences at the 5' and 3' NCRs of RNA viruses play an important role in mediating viral replication. Therefore, an examination of these sequences may provide useful information on the replication strategies adopted by these viruses. The first five nucleotides, GAAT, are identical in both LCV RNAs 1 and 2 (Fig. 3A). The same five nucleotides are also found in the 5'-termini of RNAs 1 and 2 in CYSVD, ToCV and SPCV (not shown). No other obvious sequence homology exists between these regions of the LCV genome and other closteroviruses. In LIYV, a 23-nt stretch situated within 150 nt from the 5'-termini of RNAs 1 and 2 are identical (Klaassen et al., 1995). Notably, a stretch of 14 identical nucleotides located within 220 nt from the 5'-termini of LCV RNAs 1 and 2 also has been identified in our study (Fig. 3A).

The 3' NCR of RNAs 1 and 2 are highly conserved (Fig. 3B), sharing an 81% nucleotide sequence identity; but no pseudoknot structures such as those identified in PYVV (Livieratos et al., 2004) or Strawberry chlorotic fleck associated virus (Tzanetakis and Martin, 2007) were identified (Zuker, 2003). Conservation of the 3' NCR is a feature common among RNA viruses with multipartite genomes (Dreher, 1999), and is consistent with the notion that identical nucleotide sequences and/or structural elements at the 3' NCR are essential for replication protein binding and initiation of minus-strand synthesis during RNA replication (Boccard and Baulcombe, 1993; Chen et al., 1999; Wintermantel et al., 2005), with the exception of LIYV, which shows ~31% similarity (Klaassen et al., 1995). This anomaly has been postulated to be one reason for the asynchronous temporal replication pattern of the two LIYV RNA species (Yeh et al., 2000) (see below).

Synthesis and accumulation of single stranded LCV RNAs in tobacco protoplasts

To test whether the replication of LCV RNAs is temporally regulated, as is the case with LIYV, we assessed the time course accumulation of viral RNAs in LCV vRNA-inoculated tobacco protoplasts. Inoculated protoplasts were harvested after various incubation periods (0 to 96 h) following inoculation. Equal amounts (5 μg) of the extracted total RNAs were analyzed by Northern blot hybridization using DIG-labeled single stranded riboprobes complementary to the 3'-proximal region of the positive- and negative-sense strand of
genomic RNAs 1 (probe II) and 2 (probe VIII) (Fig. 1A). From the densitometry of the exposed X-ray films shown in Fig. 4, the relative accumulation rates of LCV RNAs were determined. In all cases, the vRNA inocula were never detectable at 0 hpi (Fig. 4, lanes 0); while control LCV vRNAs were detectable only when the blots were hybridized using minus-sense riboprobes (compare Figs. 4A and B, lanes V; and Figs. 4C and D, lanes V). Negative-sense genomic RNA 1 was clearly detectable as early as 12 hpi (Fig. 4B, lane 12). The accumulation of this RNA species increased rapidly (approx. 9 fold) from 12 to 24 hpi, becoming more gradual thereafter (from 24 to 96 hpi), and continued to increase up to the final sampling time of 96 hpi (Fig. 4B, lanes 12 to 96; inset C1). The fold difference in accumulation between the 96 hpi and 12 hpi sampling was approx. 14.6, whereas that between the 96 hpi and 24 hpi sampling was only 1.6. Similar to negative-sense genomic RNA 1, both the positive-sense genomic RNA 1 and the sgRNA of LCV P8 and/or P23 (sg1) were also detected as early as 12 hpi (Fig. 4A, lane 12; insets A1 and A2). The accumulation pattern of positive-sense genomic RNA 1 was very similar to that of negative-sense genomic RNA 1 (Fig. 4A, lanes 12 to 96; inset A1). The P23 sgRNA (sg1) exhibited a similarly rapid increase in accumulation from 12 hpi to 24 hpi, but reached maximum accumulation by 48 hpi (Fig. 4A, lanes 12 to 48; inset A2).

The relative accumulation of full-length positive- and negative-sense genomic RNA 2 and several RNA 2 sgRNAs are shown in Figs. 4C and D. Full-length negative-sense genomic RNA 2 were clearly detectable 24 hpi (Figs. 4C and D, lane 24). Further exposure of the X-ray film also revealed a detectable amount of these RNAs at 12 hpi (Figs. 4C and D, lane 12; insets C1 and D1). These RNA 2 accumulation patterns are significant since they differ markedly from that of LIYV (Yeh et al., 2000). The accumulation of both positive and negative-sense RNA 2 increased by approx. 1.5 fold from 0 to 12 hpi (Figs. 4C and D, lanes 0–12). This appeared to be slightly, but not significantly lower than the approx. 2 to 4 fold increase observed for positive- and negative-sense RNA 1 occurring over the same period (Figs. 4C and D, lanes 0–12). The increase in accumulation of negative-sense RNA 2 occurred rapidly (approx. 4 fold) from 12 to 24 hpi and appeared to have reached a maximum by 48 hpi (Fig. 4D, lanes 12–96; inset D1). In contrast, after its initial rapid accumulation (approx. 4 to

![Fig. 4. Time course of LCV RNAs accumulation in LCV virion (v)RNA-inoculated tobacco protoplasts. To analyze the replication of LCV RNAs in tobacco protoplasts, total RNAs were isolated from LCV vRNA-inoculated protoplasts collected at 0, 12, 24, 48, 72 and 96 hour post-inoculation (hpi), and from water (W)-inoculated protoplasts collected at 96 hpi. Five μg of total ssRNAs was analyzed by Northern blots. The positive and negative-sense genomic RNAs 1 (G1) and 2 (G2), and putative subgenomic RNAs corresponding to: P23 (sg1), HSP70h (sg2), P6.4 and/or P60 (sg3), CPm (sg5), and P27 (sg6) were detected with a DIG-labeled negative- (A) and positive-sense (B) RNA 1 probe (probe II; see Fig. 1A for the genomic location to which probe II hybridizes), and a DIG-labeled negative- (C) and positive-sense (D) RNA 2 probe (probe VIII; see Fig. 1A for the genomic location to which probe VIII hybridizes). The polarity (+ or −) of viral RNAs being probed is indicated under each panel. Hybridization signals from several distinct but unidentified RNA species detected using the negative-sense probes II and VIII in blots (A) and (C), respectively, are marked by a *. LCV vRNAs (V; 80 ng) isolated from LCV-infected Chenopodium murale plants were used as hybridization controls. Insets A1, A2, B1, C1, C2 and D1 show the extended exposure of the respective lanes indicated at the top of the blots. Sizes of the RNAs were estimated from methylene-blue-stained RNA standards included in the same gels used for the Northern hybridizations and are shown on the left of each blot. Methylene blue-stained 25S and 18S rRNAs on the blots (bottom panels) are included to indicate the equal loadings of total RNAs in the respective lanes.](image-url)
5 fold) from 12 to 24 hpi, positive-sense RNA 2 continued to increase gradually from 24 hpi up to the final sampling time of 96 hpi (Fig. 4E, lanes 12–96). The fold difference in accumulation between the 96 hpi and 12 hpi sampling was approx. 9.3, whereas that between the 96 hpi and 24 hpi sampling was 2.1.

As with the RNA 1-derived P23 sgRNA (sg1), the expression and accumulation of most of the RNA 2-derived sgRNAs were readily identifiable at 24 hpi (Fig. 4C, arrows sg2–sg6). However, unlike sg1, no detectable traces of sg2–sg6 could be seen at 12 hpi even after prolonged exposure of the X-ray film, an example of which is shown in Fig. 4C (lane 12; inset C2). Artifacts associated with an approx. 3.3 kb and 1.7 kb RNA species were observed in Fig. 4 similar to those detected in Fig. 1. The 3.3 kb artifact most likely contributed to masking of the 3.3 kb CP sgRNA (sg 4 in Fig. 1). Our Northern blot analyses also detected several unidentified RNA species (indicated as * in Fig. 4) that were presumably of viral origin as they were absent in mock (water)-inoculated samples (Figs. 4A–D), although they were also not seen in the total ssRNA extracts of LCV-infected plants (Fig. 1).

Results from the LCV replication studies presented here clearly demonstrated the presence and accumulation of minus-sense LCV genomic RNAs 1 and 2, as well as most of the RNAs 1- and 2-derived sgRNAs in LCV-inoculated tobacco protoplasts, thus indicating that the detected RNAs were generated by de novo synthesis. More significantly, our data also indicated that LCV genomic RNAs 1 and 2 displayed similar patterns of temporal expression at the early stages of infection. This is supported by the results presented in Fig. 4. Both positive- and negative-sense LCV RNAs 1 and 2, and the RNA 1-derived P23 sgRNA were detected within the same time frame (approx. 12 hpi) after protoplasts were inoculated with LCV vRNAs, and then increased drastically from 12 to 24 hpi. The slightly slower rate of accumulation, from 0 to 12 hpi, of positive- and negative-sense RNA 2 relative to those of RNA 1 is not unusual. ORFs with deduced amino acid sequences bearing the hallmark of viral replication proteins are all located within LCV RNA 1, a finding that is consistent with those reported for all other criniviruses characterized to date (Hartono et al., 2006; Wintermantel et al., 2005). It is conceivable that after being translated from RNA 1, the viral replication proteins need time to identify and associate with RNA 2 in trans. This notion is in agreement with that currently recognized for plant viruses with multipartite positive-sense ssRNA genomes (Buck, 1996; Dreher, 1999; Sztaba-Solinska and Bujarski, 2008). In the case of LCV, the association of RNA 2 with the viral replication proteins probably occurs relatively quickly (within 12 hpi) after the viral replication proteins have been expressed. We believe that the similarity between the sequences at the 3′ NCRs of LCV RNAs 1 and 2 (Fig. 3) is one likely contributor to this observation. Because the majority of criniviruses sequenced to date also exhibit high similarity in the 3′ termini, it is plausible that they too display RNA accumulation kinetics that are comparable to that of LCV. Our analyses of the 3′ NCRs of the LCV genomic RNAs have not identified any significant structural conformations to allow any postulations about their roles in RNA replication (Zuker, 2003).

The accumulation of positive- and negative-sense LCV RNAs 1 and 2 within the same time frame early in the infection (12 hpi) is clearly in contrast with that of LIYV; both positive- and negative-sense LIYV RNAs 1 and 2 have been found to accumulate asynchronously in LIYV vRNA-inoculated tobacco protoplasts, with those belonging to RNA 1 appearing 12 hpi and those belonging to RNA 2 accumulating considerably only between 24 and 36 hpi (Yeh et al., 2000). This unique temporal expression pattern for LIYV RNAs 1 and 2 has been attributed to the dissimilarity in the sequences at their 3′ NCRs and postulated to be associated with the cis-preferential replication of LIYV RNA 1 by the RNA 1 encoded viral replication proteins (Yeh et al., 2000). A recent study in which mutants of LIYV ORF 1a and 1b failed to replicate either alone or in the presence of a WT helper RNA 1 within inoculated tobacco protoplasts has provided further evidence for the cis-preferential replication model of LIYV RNA 1 (Wang et al., 2009). At the 5′ termini of LIYV RNAs 1 and 2, some sequence homology does exist, particularly the first five terminal nucleotides (GGTAA) and a 23-nucleotide stretch situated within 150 nt from the 5′ termini, which are identical (Klaassen et al., 1995). Because the 5′ termini of RNA viruses are also essential for the replication of viral RNAs (Boccard and Baulcombe, 1993; Chen et al., 2001; Ishikawa and Okada, 2004; Yi and Kao, 2008; Zhou and Jackson, 1996), the absence of extensive sequence homology between RNAs 1 and 2 in this region of LIYV also has been previously postulated to have an effect on RNA 2 replication. This unique genomic feature of LIYV is also mirrored in LCV, wherein five terminal nucleotides (GAAAT) and a 14-nucleotide sequence, located within 220 nt from the 5′ termini are identical between LCV RNAs 1 and 2 (Fig. 3).

It has been suggested that the 3′ terminal ORF, ORF 2, of LIYV RNA 1 encodes a trans enhancer, P34, for the efficient accumulation of RNA 2 perhaps as a means for the virus to cope with the lack of homology between the 3′ termini of both RNAs (Wang et al., 2009). The sgRNA of ORF 2 is readily detected in tobacco protoplasts 12 hpi and is the most abundant of all the LIYV RNAs detected in LIYV-infected cells (Yeh et al., 2000). The 3′ terminal ORF of LCV RNA 1, encoding a P23 product, is also expressed early (12 hpi) and abundantly in LCV-infected cells (Fig. 4A). However, P23 does not share any amino acid sequence homology with the LIYV ORF 2 product. Rather, sequence alignment analysis revealed that it is similar to BnYDV P26 and ToCV P22, an RNA silencing suppressor (Canizares et al., 2008). LIVY P34 is a sRNA-binding protein and has not yet been found to exhibit suppressor of RNA silencing activity (B. Falk, personal communication). However, PHI-BLAST analysis revealed that LIVY P34 contains motifs of plant RNase III proteins. In SPSCV, an RNase III-like protein encoded by RNA 1 enhances the RNA silencing suppression activity of SPSCV P22 (Kreuze et al., 2005). Plasticity of the closterovirus genome has been well documented (Dolja et al., 2006) and could play a role in facilitating criniviruses of different lineages to obtain host proteins that evolved to accommodate similar viral functions. In the case of LIYV and LCV, it is possible that differences in the two genomes have driven the evolution of P34 and P23, two apparently different proteins that may share similar functions.

How the identical sequence elements at the 5′ termini of RNAs 1 and 2 affect RNA replication, and whether cis-preferential and/or enhancer-mediated replication indeed exist for LCV are issues that need to be addressed in future studies. However, we do know from the current study that the accumulation kinetics of LCV RNAs 1 and 2 are clearly distinct from that of LIYV. The availability of the cloned infectious cDNAs of LCV RNAs 1 and 2 should allow us to address these and other replication related questions.

Materials and methods

Virus source, dsRNA isolation, cDNA synthesis, cloning and library construction

LCV was obtained from diseased lettuce plants (Lactuca sativa). Leaves were ground in liquid nitrogen, and dsRNAs were obtained using two rounds of CF-11 cellulose (Whatman, Springfield Mill, England) column chromatography (Valverde et al., 1990). DRsRNAs were subjected to electrophoresis in a 1% agarose gel. The highest molecular weight nucleic acids was excised and purified by the Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, CA). The final eluted product was stored at ~80°C until ready for use.

cDNAs were synthesized using purified LCV dsRNAs. Approx. 0.3 μg of LCV dsRNAs were denatured by incubation with 20 mM methyl mercuric hydroxide for 5 min at 94°C, and then subjected to one of the following three cDNA synthesis and cloning strategies: (1) Preparation of a randomly primed cDNA library: the SuperScript™ choice
system (cDNA synthesis kit; Invitrogen Corp., Carlsbad, CA) was used per manufacturer’s instructions. Taq DNA polymerase (1 U; 72 °C, 1 h) was used to incorporate adenylate residues at the ends of synthesized double stranded (ds)cDNAs, and the resulting ‘A-tailed’ products were purified using phenol:chloroform:isoamyl alcohol (25:24:1) (Sambrook and Russell, 2001). These products were ligated into the pGEM-T Easy vector (Promega Corp., Madison, WI) and transformed into Escherichia coli competent cells (E. coli™) using the UltraClone™ ligation and transformation kit (Lucigen Corp., Middleton, WI). 288 bacterial transformants were selected and each was transferred to 200 μl of fresh TB medium (Lucigen Corp), with ampicillin (100 μg/ml) selection, within a well of a 96-well culture plate. A total of three 96-well plates were used for the scale-up of the selected bacterial transformants by shaking them at 280 rpm at 37 °C for 15 h. 

(2) RT-PCR amplification and cDNA cloning of nucleotide sequences located at regions (gaps) of the LCV genome that were not found in the cDNA library: this was achieved by using LCV-specific oligonucleotide primers complimentary to LCV sequences flanking the gaps. 

(3) 5′- and 3′-RACE (Rapid Amplification of cDNA Ends) PCR for the amplification of terminal nucleotide sequences of the LCV genome and cDNA cloning: the 5′/3′-RACE PCR was performed using both the 5′-RACE and the GeneRacer kits (Invitrogen Corp., Carlsbad, CA), and oligonucleotide primers based on the sequences determined from cDNA clones generated from strategies (1) and (2). The resulting amplified DNA fragments were fractionated in a 1% agarose gels and purified using the MiniElute Gel Extraction kit (Qiagen, Valencia, CA). All DNA fragments were ligated into the pGEM-T Easy vector or the pcR2.1 vector (Invitrogen Corp., Carlsbad, CA), and transformed into E. coli (DH5α or DH10β) competent cells.

Nucleotide sequencing and phylogenetic analysis

Double stranded sequence information for all cDNA clones was determined using an ABI3730x1 Automated Sequencer (UCR, CIF) and sequences were analyzed using the Vector NTI Advance™ software. The nucleotide sequences were analyzed using the Vector NTI Advance™ software. They were then analyzed using the Scion Image software (Scion Corp.).

Preparation of riboprobes

Riboprobes (probes I–VIII) were generated from recombinant plasmids containing the cDNAs corresponding to specific regions of the LCV genome (Fig. 1A). RT-PCR products amplified using the oligonucleotide primers shown in Table 1 (supplemental materials) were cloned into the pGEM-T Easy vector to generate the final cloned product for probes I, IV, V, VII, and VIII, and the intermediate cloned product for probes III and VI. The intermediate cloned products for probes III and VI were digested with Sal I and Pst I, respectively, and the intermediate cloned products for probes I, IV, V, VII and VIII, and the intermediate cloned products for probes I, IV, V, VII and VIII, were cloned into the pGEM-T Easy vector or the pcR2.1 vector (Invitrogen Corp., Carlsbad, CA), and transformed into E. coli (DH5α or DH10β) competent cells.

The extraction of LCV virion RNAs was essentially similar to that of LiYV as previously described (Klaassen et al., 1994; Klaassen et al., 1996). Protoplasts prepared from suspension cultures of N. tabacum var. Xanthi (Passmore et al., 1993) were inoculated with LCV virion RNAs (approx. 0.5–1 μg per 0.5 × 10⁶ protoplasts) according to the procedure of Lindbo et al. (1993). For viral RNA accumulation time course analyses, vRNA-inoculated protoplasts were harvested 0, 12, 24, 48, 72 and 96 hours (h) post-inoculation (pi). Mock (water)-inoculated protoplasts were harvested 96 hpi to serve as a negative control for background and non-specific probe binding. The collected cells were subjected to total RNA isolation by the TRizol® method (Invitrogen), and total RNA (approx. 5 μg) from each time point was analyzed by Northern blot hybridization. Total RNA from healthy and LCV-infected C. murale plants was extracted using the LiCl method of Martienssen et al. (1989).

Northern blot analysis

Total RNA was denatured with glyoxal and dimethyl sulfoxide and fractionated in a 1% agarose gel, as previously described (Klaassen et al., 1994), for 5–5.5 h (at a field strength of 3.48 V/cm). RNAs were transferred to a Hybond-N+ membrane (Amersham) using 5× SSC and the VacuGene XL vacuum blotting system (Amersham) at 70 mbar for 3 h. Following the transfer, the membrane was crosslinked by UV irradiation (Fisher BF UV XL-1000), stained with 0.04% methylene blue for 3–5 min and destained with water. Hybridizations and washes were performed as described previously (Rao et al., 1994). Briefly, after overnight prehybridization in hybridization buffer (50% formamide, 5× SSC, 1% SDS, 0.02 M phosphate buffer, pH 7, 5× Denhardt’s solution, 0.15 mg/ml sheared salmon sperm DNA, 0.5 mg/ml yeast tRNA) at 65 °C, the blot was hybridized overnight with 100 ng/ml of DIG-labeled riboprobes at 65 °C. The washes were performed at 65 °C, first with buffer containing 2× SSC and 1% SDS for 30 min, and then with buffer containing 0.2× SSC and 0.2% SDS for 1 h. In some cases, dsRNA-enriched extracts were used for Northern analysis. dsRNAs were treated to similar Northern blot conditions as described for total RNA except that prehybridization and hybridization of blots were performed using the DIG Easy Hyb buffer (Roche Applied Science) supplemented with 0.67 mg/ml yeast tRNA, and the first wash was performed at room temperature with buffer containing 2× SSC and 0.1% SDS for 10 min, followed by two washes at 65 °C with buffer containing 0.1× SSC and 0.1% SDS for 15 min. For densitometry, X-ray film images were digitized using a Canon LiDE scanner and saved as.tif files. They were then analyzed using the Scion Image software (Scion Corp.).
resuspended with sterile water. Labeling efficiency and yield of transcripts were examined according to manufacturer’s instructions (Roche Applied Science). Detection procedures were as previously described (Yeh et al. 2000).

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Appendix A. Supplementary data


References


