Nucleotide sequence of the coat protein gene of Lettuce big-vein virus

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A sequence of 1425 nt was established that included the complete coat protein (CP) gene of Lettuce big-vein virus (LBVV). The LBVV CP gene encodes a 397 amino acid protein with a predicted Mr of 44486. Antisera raised against synthetic peptides corresponding to N-terminal or C-terminal parts of the LBVV CP reacted in Western blot analysis with a protein with an Mr of about 48000. RNA extracted from purified particles of LBVV by using proteinase K, SDS and phenol migrated in gels as two single-stranded RNA species of approximately 7.3 kb (ss-1) and 6.6 kb (ss-2). After denaturation by heat and annealing at room temperature, the RNA migrated as four species, ss-1, ss-2 and two additional double-stranded RNAs (ds-1 and ds-2). The Northern blot hybridization analysis using riboprobes from a full-length clone of the LBVV CP gene indicated that ss-2 has a negative-sense nature and contains the LBVV CP gene. Moreover, ds-2 is a double-stranded form of ss-2. Database searches showed that the LBVV CP most resembled the nucleocapsid proteins of rhabdoviruses. These results indicate that it would be appropriate to classify LBVV as a negative-sense single-stranded RNA virus rather than as a double-stranded RNA virus.

Introduction

The big-vein disease of lettuce (Lactuca sativa L.) caused by infection with Lettuce big-vein virus (LBVV, genus Varicosavirus) is a serious problem in many lettuce-growing areas (Kuwata, 1996). The main symptoms are vein-banding accompanied by crinkling and distortion of leaves. The economic importance of the disease is a result of the unsightliness of the foliage, which depresses market value, delayed head formation, decreased head size and a reduced proportion of harvestable plants (Zink & Grogan, 1954). LBVV is transmitted by the obligate parasitic soil-inhabiting fungus Olpidium brassicae (Campbell, 1996). LBVV survives in the resting spores of the fungus for many years.

LBVV has labile, rod-shaped particles about 320 to 350 × 18 nm (Kuwata et al., 1983; Vetten et al., 1987; Huijberts et al., 1990). Kuwata (1996) reported that LBVV particles contain a coat protein (CP) with an Mr of 48000 and two double-stranded RNAs (dsRNAs) of approximately 7.0 kbp and 6.5 kbp, but no data have been given to support these figures. LBVV is closely related to Tobacco stunt virus (TStV). It is grouped with TStV, based on morphology, serology and vector relationship (Kuwata & Kubo, 1986). There are several other viruses which have the same vector and virus–vector relationship (Campbell, 1996). Currently these viruses are classified as members of the genus Varicosavirus (Mayo, 2000).

Molecular characterization of LBVV has been slow because of difficulties in obtaining a sufficient amount of purified virions. Attempts to isolate viral dsRNAs directly from infected leaves, as has been successful for other RNA viruses (Jelkmann et al., 1989; Habili et al., 1995; Fazeli et al., 1998), were unsuccessful (Huijberts et al., 1990; data not shown). In this paper, we report the molecular characterization of the LBVV CP gene and demonstrate that the LBVV CP is distantly similar in amino acid sequence to the nucleocapsid (N) proteins of some rhabdoviruses. Moreover, LBVV particles contained not only negative-sense but also positive-sense RNAs, which are separately encapsidated as has been shown for viruses of the genus Tenuivirus (Falk & Tsai, 1998), and negative-sense RNAs were predominant.
Methods

**Virus and virus maintenance.** The source virus originated from the soil of a grower’s field in Kagawa prefecture where crisp-head lettuce plants were showing characteristic symptoms. The virus was maintained in resting spores in dry soil kept in the laboratory. Lettuce cv. Cisco seedlings were transplanted into pots containing the stored soil with the root debris of the diseased plants. Pots were kept under relatively low light intensity in a greenhouse at a constant temperature between 15 and 18 °C (Walsh, 1994).

**Virion purification.** Leaves showing typical symptoms were harvested 4 to 7 weeks after transplanting and subjected to virion purification immediately. Virus was purified following a procedure modified from that of Kuvata et al. (1983). The first step of sedimenting virions by low speed centrifugation was omitted. The Triton-X treatment was carried out at room temperature. In the second cycle of differential centrifugation, 1% Brij-35 and a 20% caesium sulfate (CsSO₄) cushion were used instead of 1% Triton-X and a 30% sucrose cushion as in the first cycle. In the final CsSO₄ equilibrium centrifugation, 1/10 vol. of 5 M urea solution was added to the virus suspension

**Isolation of RNA.** Approximately 10 μg of purified virions was dissolved in TE (10 mM Tris–HCl pH 8.0 and 1 mM EDTA) containing 1% SDS and 25 μg/ml proteinase K in a total volume of 250 µl and incubated for 2 h at room temperature. Viral genomic RNAs were extracted with an equal volume of phenol/chloroform and subsequently precipitated from 70% ethanol. After further ethanol precipitation, LBVV RNAs were resuspended in 20 µl of TE and were used for electrophoresis or immediately for cDNA synthesis. Total RNAs and poly(A)+ RNAs from healthy or LBVV-infected lettuce were isolated by using an RNeasy Plant Mini kit (Qiagen) and a Dynabeads mRNA Direct kit (Dynal), respectively, following the instructions provided by the suppliers.

**Direct amino acid sequencing of the LBVV CP-derived peptides.** N-terminal amino acid sequences of internal peptide fragments of the LBVV CP were determined according to Hirano et al. (1991). Purified LBVV was dissociated by SDS and electrophoresed on 10% polyacrylamide–SDS gels (SDS–PAGE) and electro-botted onto a PVDF membrane. The protein corresponding to the LBVV CP on the PVDF membrane was excised, carbonyxymethylated and digested by a lysylendopeptidase (Wako). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II). The peptide fragments obtained were isolated by using HPLC with a reverse sphere column (Cosmosil 5C18 AR-II).

**Cloning and sequencing of the LBVV CP gene.** cDNA to the viral genomic RNAs or the poly(A)+ RNAs isolated from LBVV-infected lettuce was synthesized by a random or a Bam-dT13 primer using a cDNA Synthesis kit (Amersham Pharmacia). The oligonucleotide primers used for PCR and the cloning strategy for the LBVV CP gene are shown in Table 1 and Fig. 1, respectively.

The initial cloning of the LBVV genomic RNAs was achieved using degenerate primers D171p and D171y, which were derived from two amino acid sequences of the internal peptide fragments in the LBVV CP. The cDNA was amplified by PCR in 50 μl of 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μM each of the four dNTPs, 1 μM each of the primers and 2.5 U Taq DNA polymerase (Takara). The PCR protocol consisted of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, and finally an extension time of 10 min at 72 °C.

The 5’ end of the LBVV CP gene (positive sense) was determined by using the 5’RACE system for rapid amplification of cDNA ends, version 2.0 (GibcoBRL) according to the protocol provided by the suppliers. The region was amplified from viral genomic RNAs and poly(A)+ RNAs from LBVV-infected lettuce as templates by using three different virus-specific primer sets (3R11p + 3R12p, 3R21p + 3R22p, and m3R21p + m3R22p).

The 3’ end of the LBVV CP gene (positive sense) was determined from viral genomic RNAs and poly(A)+ RNAs from LBVV-infected lettuce as templates by RT–PCR. The primer Bam-dT13 and a virus-specific positive-sense primer (m5R1p, m5R2p or m5R2p) were used, assuming that there might be a polyadenylated stretch in the region. Amplified PCR fragments were separated by electrophoresis in a 1% agarose gel and isolated from the gel using a QIAquick Gel Extraction kit (Qiagen). The purified PCR fragments were directly ligated into plasmid pGEM-T Easy Vector (Promega). At least three clones of each PCR fragment were sequenced in both directions using an automated ABI Prism 377 DNA sequencer with an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). Nucleotide sequence data were compiled and analysed with GENETYX-WIN version 4.3 (Software Development). The amino acid sequence of the LBVV CP was compared with the DDBJ database using the TFASTA program (Pearson & Lipman, 1988).

**Electrophoresis and Northern blot analysis.** Ribonuclease A treatment utilizing high and low salt buffers was used to differentiate ssRNAs and dsRNAs (Zelcer et al., 1981). LBVV RNAs in 0.1 × SSC (ss is 15 mM Na-citrate pH 7.0, 0.15 M NaCl) or 2 × SSC were treated with 2 μg/ml of ribonuclease A for 30 min at 30 °C, followed by 5 μg/ml of proteinase K in 2 × SSC containing 0.05% SDS for 30 min at room temperature. The RNAs were then electrophoresed through a 1% non-denaturing agarose gel in TBE (89 mM Tris–HCl, 89 mM borate, 2 mM EDTA). The Perfect RNA Marker (Novagen) and dsRNAs of Rice dwarf virus (Uyeda et al., 1995) were used as ssRNA and dsRNA molecular markers, respectively. For Northern blot hybridization, LBVV RNAs were transferred onto a Hybond-N+ membrane (Amersham Pharmacia) using 20 × SSC as the transfer buffer. To denature the dsRNAs, the membrane was treated with 50 mM NaOH and 10 mM NaCl according to Habili et al. (1995). A 1-2 kb fragment of the complete LBVV CP gene, which was amplified by RT–PCR using virus-specific primers CPp and CPp' (Table 1), was directly ligated into plasmid pGEM-T Easy Vector. The riboprobes of the LBVV CP gene were prepared from the plasmid harbouring the complete LBVV CP gene using RNA Labelling kit (Amersham Pharmacia). Prehybridization, hybridization, washings and membrane exposures were carried out according to standard protocols (Sambrook & Russell, 2000).

**Western blot analysis.** Two 19 amino acid long synthetic peptides (LBVV-pe1 and LBVV-pe2), which encompassed amino acid 14 to amino acid 32 and amino acid 37 to amino acid 393 of the LBVV CP, respectively, were synthesized by the solid-phase method (Barany & Merrifield, 1980). The synthetic peptides were coupled to keyhole limpet haemocyanin and used to raise antiserum (anti-LBVV-pe1 and anti-LBVV-pe2) in a New Zealand White rabbit. Approximately 300 μg of conjugated peptides were emulsified in Freund’s complete adjuvant and injected subcutaneously at four sites on the back. Three injections were given at 2-week intervals and thereafter antiserum was obtained from bleeds taken 10 days after the final injection. Total proteins were extracted from LBVV-infected or healthy lettuce leaves and separated by electrophoresis in 10% gels following the method of Laemmli (1970). The gels were transferred to nitrocellulose membranes at 100 mA for 1 h (Altto AE-6675). After blocking with Tris-buffered saline (50 mM Tris–HCl pH 7.4, 200 mM NaCl) containing 50 g/l of gelatin for 1 h, the
Table 1. Synthetic primers used for cloning the LBVV CP gene

Lower-case letters indicate extra nucleotides containing restriction sites introduced for cloning purposes.

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Nucleotide position</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>D171p</td>
<td>283–306</td>
<td>GARWSITGGGAYGAYGARWSIAC</td>
</tr>
<tr>
<td>D171n</td>
<td>537–559</td>
<td>CGCRTCDATRTARTCIAICICCCIG</td>
</tr>
<tr>
<td>CPp</td>
<td>209–229</td>
<td>cgccgatccATGGCAACCCCAATTGAAG</td>
</tr>
<tr>
<td>CPn</td>
<td>1385–1405</td>
<td>gcgcgcgacTCATCCTTCACCTGTTG</td>
</tr>
<tr>
<td>5R1p</td>
<td>490–510</td>
<td>AAGGGGAAAGACACACACCGGA</td>
</tr>
<tr>
<td>5R2p</td>
<td>932–951</td>
<td>CAACCTCGATCGGTCCACAC</td>
</tr>
<tr>
<td>m5R3p</td>
<td>819–838</td>
<td>GCGAATCTTCTATGCTGCTG</td>
</tr>
<tr>
<td>m5R21p</td>
<td>247–266</td>
<td>CTGTGATTTCACAGACGTCA</td>
</tr>
<tr>
<td>mR22p</td>
<td>195–212</td>
<td>gcgcgcgacTCATCCTTCACCTGTTG</td>
</tr>
<tr>
<td>3R11p</td>
<td>612–632</td>
<td>TGGAGGCAGCGATGAATGCT</td>
</tr>
<tr>
<td>3R12n</td>
<td>561–579</td>
<td>caaacgtcgcgcgcgacAGGTTGATGC</td>
</tr>
<tr>
<td>3R21n</td>
<td>976–991</td>
<td>GTTCTGCTCCGTAGTTG</td>
</tr>
<tr>
<td>3R22n</td>
<td>680–696</td>
<td>caaacgtcgcgcgcgacAGGTTGATGC</td>
</tr>
<tr>
<td>Bam-dT35</td>
<td>–</td>
<td>gcgcgcgacAGGTTG</td>
</tr>
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* p. Positive sense; n. negative sense.

Fig. 1. Cloning and sequencing strategy of the LBVV CP gene (positive-sense RNA). Solid lines, cDNA clones synthesized by using viral genomic RNAs as templates; shaded lines, cDNA clones synthesized by using poly(A) + RNAs from LBVV-infected lettuce as templates. Parentheses show the number of independent cDNA clones used for sequencing.

membranes were probed for 1 h on a shaker at room temperature with anti-LBVV-pe1 or anti-LBVV-pe2. Antiserum against TSV was used as control.

Results

Purification of LBVV virions

Our purification protocol provided sufficient amounts of purified virions for biochemical analyses. Although the yields varied from time to time, LBVV virions were purified with yields of 10–20 µg/kg of leaf tissue. A single band of particle aggregates was always observed in Cs₂SO₄ equilibrium centrifugation. Electron microscopy showed that most particles were in aggregates and were similar in size and structure to those reported by Kuwata et al. (1983), Vetten et al. (1987) and Huijberts et al. (1990). When the single LBVV fraction from the

Fig. 2. Analysis by 10% acrylamide–SDS gel electrophoresis of proteins associated with purified LBVV virions. The protein was stained with Coomassie brilliant blue. Molecular weight markers are indicated on the left.
Sequence of the LBVV CP gene

Attempts to construct an LBVV cDNA library using as template RNA extracted from purified LBVV particles were unsuccessful. Thus we determined the complete nucleotide sequence of the LBVV CP gene by using RT–PCR methods as described below.

N-terminal amino acid sequences of six internal peptide fragments of the LBVV CP were determined after digestion of the LBVV CP with lyslendopeptidase (Fig. 3). Two out of these six sequences obtained were ESWDDESTIAMP and NLEVPGVDYIDA. Four degenerate primers were designed on the basis of these sequences. RT–PCR using the combination of degenerate primers D171p and D171n gave only a single product of approximately 0.3 kb and the sequence of 277 nt was determined. The PCR product was not detected with total RNAs and poly(A)+ RNAs from healthy lettuce as templates.

The nucleotide sequence of the 5′ end of the LBVV CP gene (positive sense) was determined by 5′ RACE. A total of 15 clones was amplified from viral genomic RNAs or poly(A)+ RNAs from LBVV-infected lettuce as templates by PCR and their nucleotide sequences were determined. In eight clones the 5′-terminal sequence was 5′ UGGAAACCCA…3′ in the positive sense. Three clones lacked the first UGG residues and three lacked the first UGGAA residues. One clone started with 5′ CAUUACCA…3′.

The 3′ end of the LBVV CP gene (positive sense) was determined by sequence analysis of cDNA clones synthesized from poly(A)+ RNAs from LBVV-infected lettuce using the

Cs₂SO₄ gradients was analysed for purity by SDS–PAGE (Fig. 2), only a single protein with an Mₚ of 48 000 was detected, which corresponds to the estimated size of the LBVV CP given previously (Kuwata, 1996).
The analysis identified a protein with an amino acid sequence from the LBVV CP gene. Purified LBVV virions existed in the deduced amino acid sequence of the six internal peptide fragments derived from the LBVV CP gene. This open reading frame encoded a 397 amino acid protein with a calculated initiation site. This open reading frame was preceded by a noncoding sequence of 1425 nt (Fig. 3). It contained one open reading frame, beginning with either of the AUG codons at positions 209 or 230. Several trials to directly determine the N-terminal amino acid sequence of the LBVV CP failed, presumably because the N terminus of the LBVV CP was blocked. One amino acid sequence obtained by internal peptide fragments of the LBVV CP was MLDAFSDVVE. Furthermore, the sequence surrounding the first AUG at position 209, UAAAAUGGC, was close to the most favoured AUG initiation sequence context for plants, AACAUGGC (Lütcke et al., 1987). These results indicate that the first AUG was the authentic translation initiation site. This open reading frame encoded a 397 amino acid protein with a calculated $M_r$ of 44,486. All amino acid sequences of the six internal peptide fragments derived from purified LBVV virions existed in the deduced amino acid sequence from the LBVV CP gene.

In order to elucidate the nature of this predicted protein, antisera against the synthetic peptides of its N-terminal and C-terminal parts were prepared and used in Western blot analysis. The analysis identified a protein with an $M_r$ of 48,000 from purified virions and LBVV-infected lettuce leaves, but not from healthy lettuce leaves (Fig. 4). In parallel experiments with the antisera against TSV, a protein of the same size was observed.

The open reading frame was preceded by a noncoding region of 205 nt. The 5' noncoding region (positive sense) contains direct-repeated pyrimidine-rich motifs such as CCCUA(C/U) and UUCC.

**Properties of LBVV RNA species**

Two species of RNAs (ss-1 and ss-2) were observed in nondenaturing gels following electrophoresis of nucleic acids extracted from purified LBVV using proteinase K, SDS and phenol (Fig. 5a). Ss-1 and ss-2 were shown to be single-stranded by their susceptibility to digestion by RNase A both at high and low salt concentration, and resistance to digestion by DNase I (data not shown). The estimated sizes of ss-1 and ss-2 were approximately 7.3 kb and 6.6 kb, respectively, under nondenaturing conditions when determined using Perfect RNA Markers (Novagen) as size standards. Analysis of the same LBVV RNA preparations after denaturation by heat (94 °C, 3 min) and then annealing at room temperature showed two additional species (ds-1 and ds-2) that were resistant to RNase A in high salt concentration, indicating that the two additional species of RNA were double-stranded. The estimated sizes for ds-1 and ds-2 were approximately twice the ss-1 and the ss-2 values, respectively.

To confirm the location of the LBVV CP gene and the relationships among these four RNA species, the LBVV RNA preparations after denaturation by heat and then annealing at room temperature were analysed by Northern blot hybridization analysis using positive- and negative-sense riboprobes of the LBVV CP gene (Fig. 5b). Not only ss-2 but also ds-2 hybridized to a positive-sense riboprobe. In contrast only ds-2 hybridized to a negative-sense riboprobe. These results indicate that the LBVV CP gene is located in ss-2. Furthermore, the ss-2 is a negative-sense ssRNA and ds-2 is the double-stranded form of ss-2.
only a single protein with an albeit in very low yields. succeeded in obtaining pure LBVV virions from lettuce plants, preparations by host species. After numerous efforts, we form aggregates, and are contaminated in purified virus are present only at low concentration in infected lettuce plants, factor limiting studies of LBVV has been the difficulty of virion

Discussion

LBVV and TStV are serologically related viruses that have been classified in a new genus, Varicosavirus (Mayo, 2000). Specific defining characteristics of these viruses, including nucleotide sequence data, are lacking. The most important factor limiting studies of LBVV has been the difficulty of virion purification, because LBVV particles are extremely unstable, are present only at low concentration in infected lettuce plants, form aggregates, and are contaminated in purified virus preparations by host species. After numerous efforts, we succeeded in obtaining pure LBVV virions from lettuce plants, albeit in very low yields.

SDS–PAGE analysis of purified LBVV virions detected only a single protein with an $M_r$ of 48,000, which corresponds to the estimated size of the LBVV CP given previously (Kuwata, 1996). Only typical rod-shaped particles as virus-like particles were observed in our purified preparations under the electron microscope and any other virus-like particles were not observed. In Western blotting analysis, the antisera against synthesized peptides based on the determined sequence reacted with the purified LBVV virions as well as the preparation from LBVV-infected lettuce plants but did not react with the preparation from healthy lettuce plants. Furthermore, by RT–PCR using virus-specific primers, a single PCR product was detected from total RNAs extracted from LBVV-infected lettuce, but not from healthy plants (data not shown). Therefore, it can be concluded that our purified preparations contained pure and true LBVV virions, and that the sequence we determined derived from a true LBVV CP gene.

The most unusual characteristic found here for LBVV was that although LBVV particles have been reported to contain dsRNA (Kuwata, 1996), we found both ssRNAs and dsRNAs in the LBVV RNA preparations. Interestingly, only two ssRNAs were detected in the LBVV RNA preparations by using proteinase K, SDS and phenol, but some dsRNA was formed by heat-denaturation and then annealing at room temperature or repeatedly freezing and thawing the LBVV RNAs (data not shown). It is unlikely that the dsRNAs came from replicative structures that co-purified with the LBVV particles. It also seems improbable that some LBVV particles encapsidate ssRNAs and others encapsidate dsRNAs. One possible explanation for this phenomenon is that only ssRNAs are encapsidated during assembly, but that positive-sense and negative-sense RNAs are separately encapsidated, and during disruption of the LBVV particles in proteinase K and SDS, the RNAs form a secondary structure without annealing to each other. If so, LBVV RNAs prepared by treatment with proteinase K, SDS and phenol contain only ssRNAs, but the two-stranded RNAs aneal to form dsRNAs after denaturation by heat. A precedent for this behaviour is that ribonucleoprotein particles of some tenuiviruses contain both positive-sense and negative-sense RNAs that are separately encapsidated, and anneal to form dsRNAs under different extraction conditions (Falk & Tsai, 1998). Our Northern hybridization experiments using viral transcripts indicated that negative-sense RNAs are more abundant than positive-sense RNAs. Thus, it would be appropriate to classify LBVV as a negative-sense ssRNA virus rather than as a dsRNA virus.

The results described here appear to be the first report of the negative-sense single-stranded nature of LBVV RNAs and the molecular characterization of the LBVV CP gene. The taxonomic position of Varicosavirus in the virus kingdom will become clearer when more sequence information of other LBVV genes becomes available.

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References


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