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Development of an RT-PCR for the detection of little cherry virus and characterization of some isolates occurring in Europe

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Abstract

A reverse transcription-polymerase chain reaction (RT-PCR) was developed for the detection of little cherry virus (LChV), a closterovirus responsible for heavy yield losses in sweet cherry. Total RNA was extracted from the leaves of sweet cherry trees affected with 21 virus isolates from different locations in Germany, the Netherlands, the UK, and Switzerland, and used as template for RT-PCR. In all the samples tested, 274-277-nt products were amplified with a pair of oligonucleotide primers specific for the 3′-terminal 276-nt genomic region of the German LChV UW1 isolate of which the complete genome sequence has been published. The PCR products derived from 9 isolates were cloned and sequenced. The sequence comparisons revealed high homology between these isolates and UW1 (86.9% to 96.7% nt sequence identity), thus indicating that the RT-PCR assay may be applicable for the detection of a wide spectrum of natural LChV isolates.

Introduction

Little cherry disease (LCD) has a great impact on the production of sweet cherry. LCD was first recorded in 1933 in a sweet cherry orchard in the Kootenay Valley of British Columbia (Welsh and Cheney, 1976) where it spread rapidly due to high populations of the natural vector of the LCD-associated virus, the apple mealybug *Phenacoccus aceris* (Raine et al., 1986). The disease, which is of worldwide distribution, continues to hamper fruit production in Canada (Eastwell and Li, 1994; Jesperson and Carter, 1994). In recent years, LCD has been critical for sweet cherry production in the 'Altes Land' of Northern Germany (Büttner et al., 1993, 1994; Harms et al., 1996). Symptoms appear on fruits and leaves of sensitive sweet cherry cultivars, e.g. Lambert, Bing, Van, Sam, and Canindex. Affected fruits do not fully ripen, are imperfectly coloured, pointed, of reduced sweetness and up to half to two-thirds normal size. In late summer or early fall, the affected leaves show a red coloration or bronzing of the surfaces (Welsh and Cheney, 1976). The cultivars Sam and Canindex are widely used as indicator hosts in certification programs as they develop very pronounced leaf symptoms.

The identification of elongated virus-like particles in phloem cells of infected trees by electron microscopy (Raine et al., 1975, 1979) indicated an association of the LCD with a virus belonging to the closterovirus group. Subsequently a high molecular weight double-stranded replicative form of RNA (dsRNA) was extracted from sweet cherry leaves thus supporting the previous conclusions on the closterovirus nature of the infectious agent (Hamilton et al., 1980; Jelkmann, 1995; Keim-Konrad and Jelkmann, 1996; Eastwell and Bernardy, 1996). Northern blots of the dsRNA isolated from 48 sources from British Columbia demonstrated a positive correlation between the presence of the dsRNA and the occurrence of LCD (Eastwell and Bernardy, 1996; Eastwell et al., 1996). Very recently, the dsRNA of the German UW1 isolate was cloned as cDNA and the complete sequence of 16,934 nucleotides was obtained (Keim-Konrad and Jelkmann, 1996; Jelkmann et al., 1997). The genome analysis revealed that the virus, named little cherry closterovirus (LChV), has a monopartite positive-strand RNA genome encompassing nine open reading

frames whose products are related to those in the other closteroviruses. However, as the electrophoretic size estimations of the 16.9 kbp LCD-associated dsRNA gave a value of ca. 15 kbp (Keim-Konrad and Jelkmann, 1996), which is in contrast to the 12.6 kbp reported from Canada (Eastwell and Bernardy, 1996), the question has remained open as to whether intrinsic differences exist between the LChV isolates from different geographic regions.

The development of an RT-PCR assay for LChV would provide a notable improvement over the currently used woody indexing for virus detection in nuclear stock plants and for retesting of fruit tree propagation material. RT-PCR has been successfully applied for the detection of a number of viruses affecting fruit trees. A primary goal is often to provide a suitable nucleic acid template preparation which is free from substances interfering with enzymatic reactions, such as phenolic compounds or polysaccarides (Korschineck et al., 1991; Rowhani et al., 1993; Kinard et al., 1996; MacKenzie et al., 1997). Provided suitable trapping antibodies are available immunocapture RT-PCR (IC-RT-PCR) is a highly sensitive method to overcome the limitations for PCR detection of fruit viruses (Wetzel et al., 1992; Kaden-Kreuziger et al., 1995; Jelkmann and Keim-Konrad, 1997). The development of an RT-PCR-based detection method would be of special value for LChV, as herbaceous hosts are lacking and the virus accumulates to very low amounts in the *Prunus* host plants, thus not allowing preparation of purified particles suitable for antibody production and the development of serological methods.

In this paper we report the development of an RT-PCR test, based on total nucleic acid extraction, for the detection of European sources of LChV in sweet cherry. Several LChV isolates were investigated for possible heterogeneity by partial sequence analysis and dsRNA gel electrophoresis.

Materials and methods

Virus sources. Buds from North German sweet cherry trees that had displayed LCD symptoms (Altes Land; AL1/92, 2/92, 3/92 4/92), and from Southern German trees (UW2 to 9, as well as OF300I and LfP-S6/92) were collected and indexed for the presence of LCD by the woody indicators Sam and Canindex. Isolates from Switzerland (CH1 and CH2), the Netherlands (NL1 and NL2), and the UK (UK1) were kindly provided by T. Hasler, G. Jongedijk, and A.N. Adams, respectively. The sources WH70/88, HA119/86, both originating from *Prunus serrulata* cv. Shirofugen, were taken from the virus collection in the field at Dossenheim. Isolate 3/7Roth was a symptom-displaying field isolate that was not maintained. The German beet yellows virus isolate (BYV-D) (Agranovsky et al., 1994) was maintained in the greenhouse on *Tetragonia expansa* and propagated by transmission with *Myzus persicae*.

Sample preparation. Extraction of the dsRNA from symptom-displaying leaf tissue was as described in Jelkmann et al. (1992). Leaf material was taken from the woody indicators Sam or Canindex used to propagate and index the isolates in the field. Total RNA was extracted from fresh sweet cherry leaves by using the RNeasy Total RNA Purification kit (QIAGEN) according to the manufacturer's protocol, with minor modifications. Briefly, 0.1 g of tissue was ground in liquid nitrogen followed by adding 900 μl of the lysis buffer RLT containing guanidinium isothiocyanate (QIAGEN). The lysate was clarified by centrifugation through QIAshredder spin columns, 225 μl of ethanol was added to 450 μl of the flow-through fraction, and the mixture was loaded onto an RNeasy spin column. After washing with the QIAGEN buffers RW1 and RPE, RNA was eluted with 50 μl of water. The samples were stored at –20 °C until use.

Oligodeoxynucleotide primers for RT-PCR. Two LChV-specific oligonucleotides were chosen as primers to amplify a 276 bp fragment corresponding to the 3′-terminal portion of the LChV genome. Oligonucleotide LCV3EC (5′-*GCTCTAGA*GGCACCTTTTATTTTTTATATATGC-3′), complementary to positions 16910 to 16934 (accession number Y10237) (with the addition of 8 non-viral nucleotides to introduce an *Xba*I site), was used as a negative-sense primer in RT reactions and PCR. Oligonucleotide LCV16659 (5′-GTTATAGAATTCACTGCAAGTG-3′) was used as a positive-sense primer for PCR amplification.

Reverse transcriptase – PCR. Total RNA (5 μl) was denaturated for 10 min at 75 °C. The reverse transcription cocktail (20 μl) contained 0.5 μM primer LCV3EC, 10 mM DTT, four dNTPs (0.5 mM each), 20 U RNase inhibitor (MBI, 40 U/μl), 200 U M-MLV reverse transcriptase and the first strand buffer (BRL). The mixture was incubated for 1 h at 42 °C. PCR reactions were performed in 50 μl reactions containing 2 μl of the RT mixture, 1.5 mM $MgCl_2$, 0.4 mM each

dNTP, 0.4 μM each primer, Eurogentec reaction buffer and 2.5 U Taq-polymerase (Goldstar 5 U/μl; Eurogentec). Incubation was carried out in a RoboCycler Gradient 40 (Stratagene) programmed for 35–50 cycles of 45 sec at 95 °C, 45 sec at 54 °C, and 60 sec at 72 °C. The amplification products were analyzed by electrophoresis (90 volts for 1hr) through 1% agarose gels submerged in 1 × TBE.

Cloning and sequencing of RT-PCR fragments. Nucleotide sequence reactions were performed with double-stranded DNA and a T7 DNA polymerase sequencing kit (Pharmacia). The PCR products were sequenced after subcloning into the *Eco*RV site of Bluescript M13+ where a single dTTP nucleotide at the 3′ ends had been added according to the method of Marchuk et al. (1990). Standard molecular biological techniques were according to Sambrook et al. (1989). The multiple alignments of amino acid sequences were generated using CLUSTALV (Higgins and Sharp, 1988).

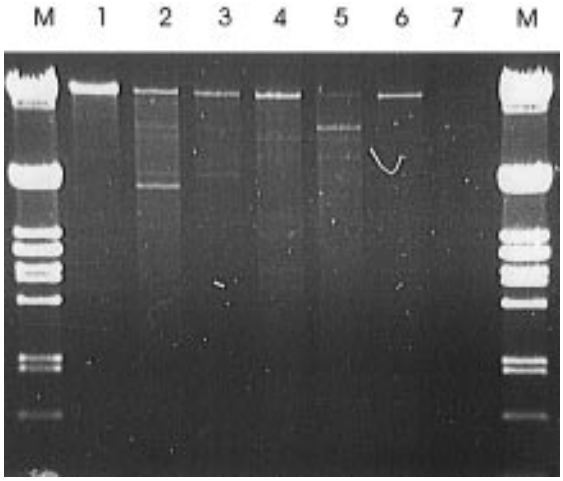

Figure 1. Agarose gel electrophoresis of the dsRNA extracted from little cherry disease symptom-displaying leaf tissue and from beet yellows virus (BYV) infected *Tetragonia expansa* plants. Lanes M on left and right, lambda *Pst*I cut DNA marker; lane 1, BYV; lane 2, UW2; lane 3, 3/7Roth; lane 4, WH70/88; lane 5, LfP-S 6/92; lane 6, AL1/92; lane 7, OF300I.

Results

dsRNA analysis. In agarose gel electrophoresis, the high molecular mass dsRNAs from the LCD-affected plant samples which had been collected from six different locations in Germany, comigrated with the 15,480 bp genomic-size dsRNA of BYV used as a size marker (Figure 1). Similar results have been obtained with the dsRNA of isolates AL2 to 4/92 and UW3 to 9 (data not shown). The nature of faster migrating bands of different intensity (lanes 2 to 7) was not analyzed. The genome size of LChV dsRNA was previously estimated to be ca. 15 kbp (Keim-Konrad and Jelkmann, 1996), whereas the LChV genome consists of 16,934 nucleotides as determined by sequencing (Jelkmann et al., 1997). The dsRNA yield varied in repeated experiments performed with some isolates. Healthy controls remained free of any visible dsRNA. All isolates were indexed in the field and developed typical LCD leaf symptoms on Sam or Canindex indicator plants.

RT-PCR. By using total RNA from leaf tissue purified between late September and middle of October as template for RT-PCR, a PCR product of the expected size (276 bp) was amplified. For all the 21 isolates originating from England, the Netherlands, Switzerland and different locations in Germany, the resulting PCR products were indistinguishable in agarose gels (Figure 2). In the majority of assays 40 PCR cycles were used; under these conditions, no unspecific bands were visible. Furthermore, no PCR products were detected with the healthy leaf tissue controls of the Sam and Canindex indicators and water blanks, independent from the number of amplification cycles. All the isolates that were indexed as LCD-positive by woody indicators, were also positively identified by RT-PCR. For the isolates WH70/88 (Figure 2, lanes 5, 6) and HA119/86 (Figure 2, lanes 15, 16) that had been maintained in the virus collection since the late 1960s, the PCR products were identified using total RNA extracted from leaves of trees of different ages. A positive reaction was also observed with the total RNA isolated from young symptomless leaves, but not from flowers, of a potted plant affected with the AL 4/92 isolate that had been forced in winter (data not shown).

Molecular cloning and sequence analysis. Since several other primer combinations at other parts of the LChV genome (data not described) failed to amplify PCR products from all the samples investigated, thus indicating a possible isolate sequence heterogeneity, the 276 bp RT-PCR fragment of some isolates, representing the different geographic origins, was sequenced. RT-PCR fragments from six German isolates and one each from England, the Netherlands, and Switzerland were included in the study. The nine PCR products, encompassing the 3′-terminal 276

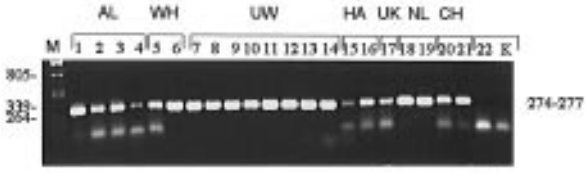

Figure 2. Agarose gel electrophoresis of RT-PCR products from different little cherry virus (LChV)-infected sweet cherry sources. Lane M, size of lambda *Pst*I cut DNA marker in bp; lanes 1 to 4, LChV Altes Land (AL) 1 to 4/92; lanes 5 and 6, LChV WH70/88; lanes 7 to 14, LChV UW2 to 9; lanes 15 and 16, LChV HA119/86; lane 17, LChV UK1; lanes 18 and 19, LChV NL1 and NL2; lanes 20 and 21, LChV CH1 and CH2; lane 22, water control; lane K, healthy control.

nucleotides of the type strain (UW1; EMBL database accession number Y10237) showed a slight length variation from 274 to 277 nucleotides (Figure 3). The nine fragments revealed sequence identities in comparison to the master UW1 sequence ranging from 86.9% to 96.7%.

Discussion

As the previous electrophoretic size estimations of the LCD-associated dsRNA of ca. 15 kbp (Keim-Konrad and Jelkmann, 1996) and 12.6 kbp (Eastwell and Bernardy, 1996) are both in contrast to the complete LChV sequence of 16,934 bp, we analyzed the LChV dsRNA in agarose gel electrophoresis using BYV dsRNA as a size marker. The genomic-size dsRNA of BYV is of 15,480 bp according to the sequencing data (Agranovsky et al., 1994), which is in reasonable agreement with the earlier electrophoretic estimates (MW = 8.4×10^6) (Dodds and Bar-Joseph, 1983).The anomalously fast migration of the LChV dsRNA observed with all the isolates tested might be due to the structural conformation. The discrepancy between the electrophoretic estimates of the dsRNA by our group and by Eastwell and Bernardy (1996) can be explained by the use of different size standards or by intrinsic differences between the LChV isolates from Europe and the isolates sampled in the Kootenay and Okanagan Valleys of British Columbia.

Thus far, indexing on susceptible indicator hosts has been the only method available for the LChV detection in sweet cherry. This approach has drawbacks as it is time-consuming and provides an answer based on the disease symptomatology rather than genotype. The PCR-based detection method will therefore be of special value, in view of the fact that LChV accumulates to very low amounts in its host plants, thus hitherto not allowing isolation of purified particle preparations suitable for antibody production and development of serological methods. The rapid and sensitive RT-PCR

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                Primer LChV16659
UW1             GTTATAGAATTCACTGCAAGTGAAAAAGATGAC-ATGCATATCATGTCAA    49
WH70/88         ................................-.................    49
UW5             .........................G......-...TT...T.......    49
CH1             ................................-...TT...T.......    49
UW4             ................................-...TT...T.......    49
UW3             ................................-...TT...T.......    49
UW6             ................................-...TT...T.......    49
UK1             ................................-...TT...T.......    49
AL1/92          ................................-...TT...T.......    49
NL1             ..............................TT...A....A..T..T.    50
                ************************** *****  ***  *** ** ** *

UW1             CAATATTACACATTAGTTAAATAAGATATAATAATTAATATTTTTTATGT    99
WH70/88         .T.....G............G.TTATA....GTT.CT.....A.AA..A.    99
UW5             .T.....G............G.TTATA....GTT.CT.....A.AA..A.    99
CH1             .T.....G............G.TTATA....GTT.CT.....A.AA..A.    99
UW4             .T.....G............G.TTATA....GTT.CT.....A.AA..A.    99
UW3             .T........T.....C...G.TTATA....GTT.CT.....A.AA..A.    99
UW6             .C.................G.TTATA....GTT.CT.....A.AA..A.    99
UK1             .T.....G............G.TTATA....GTT.CT.....A.AA..A.    99
AL1/92          .T.....G............G.TTATA....GTT.CT.....A.AA..A.    99
NL1             .T.....G..T............TT...A.T.TTACGT.TAAA.AGTAA.   100
                * ***** ** ***** *** *     * *        *    *     *

UW1             ATTA-CTGT--GTGTTTAATTTAAATAAAGAGGAGGGTTTTTATACCGCT   146
WH70/88         ....T.AA.AA......T.A.............................    149
UW5             ....T.AA.AA......T.A.............................    149
CH1             ....T.AA.AA......T.A.............................    149
UW4             ....T.AA.AA......T.A.............................    149
UW3             ....T.AA.AA......T.A.............................    149
UW6             ....T.AA.AA......T.A.............................    149
UK1             ....T.AA.AA......T.A............G................    149
AL1/92          ....T.AA.AA......TTAA.T..AT..AGA.GA..G............    149
NL1             G..-----.ATA.TA..GTA..TTGA.T.A..AGA..............    145
                 **     *   *  **    *      *      ** ************

UW1             TACCTTCTCTAGTCATAAA-CTGGTTTTTTACTGAGTGTTTATTATAAAA   195
WH70/88         ...................-.........T....................    197
UW5             ...................-.........-....................    197
CH1             ...................-.........-....................    197
UW4             ...................-.........-....................    197
UW3             ...................-.........-....................    197
UW6             ...................-.........-.................G..    197
UK1             ...................A.........-.....................-    197
AL1/92          ...................-.........-C...................    197
NL1             ...................-.........-....................    193
                ******************* *********  *****************

UW1             TCTAAATAAAATGCAACTTTTAAATAGTTTATCTGTTAAGATAAACCACC   245
WH70/88         ....................--G.............................    245
UW5             ....................--G.............................    245
CH1             ....................--G.............................    245
UW4             ....................--G.........................-...    244
UW3             .....................-.............................    246
UW6             .....................-.............................    246
UK1             ....................--G.............................    245
AL1/92          .....................-.............................    246
NL1             ...G..............C.T.............................    243
                *** **************    ************************ ***

UW1             TAGGTTGCATATATAAAAAATAAAAGGTGCC   276
WH70/88         ...............................   276
UW5             ...............................   276
CH1             ...............................   276
UW4             .....G.........................   275
UW3             ...............................   277
UW6             ...............................   277
UK1             ...............................   276
AL1/92          ...............................   277
NL1             .GA............................   274
                *  ** ************************
                   3' CGTATATATTTTTTATTTCCACGGAGATCTCGGC 5'
                      Primer LChV3EC
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Figure 3. Comparison of the nucleotide sequence products obtained from nine cloned little cherry virus RT-PCR fragments and the master UW1 sequence. The 276 nucleotides of LChV UW1 represent the 3′ terminus of the complete sequence encompassing 16,934 nucleotides (EMBL database accession number Y10237). Dots indicate identical nucleotides. Residues found to be identical in all isolates are indicated by asterisks. The oligonucleotides used for RT and RT-PCR are underlined.

assay reported here is based upon the primers specific to the 3′-terminal sequence of the LChV genome (Keim-Konrad and Jelkmann, 1996; Jelkmann et al., 1997) and requires, as a preparatory step, only the extraction of total RNA from leaf tissues. The extracted total RNA has been shown to be a suitable template for the RT-PCR detection of cherry virus A (Jelkmann, 1995) (data not shown) and possibly for a number of other viruses occuring in cherry for which nucleotide sequences have been obtained to date. This conclusion corroborates the recently reported data on the extraction of RNA virus template preparations from leaves and budwood of pome and stone fruits, and from grapevine (MacKenzie et al., 1997).

Our data on the PCR amplification and sequencing of the LChV isolates clearly show that they have highly homologous (albeit not identical) sequences in the 3′-terminal region. This indicates that the RT-PCR assay with the given primer combination may be applicable for testing an even wider spectrum of the naturally occuring LChV isolates than was tested in this study. However, it should be kept in mind that comparisons of the Canadian LChV isolates by Northern blot hybridization indicate a significant sequence variation between them (Eastwell et al., 1996). Hence, the PCR primer pair used in this study might not fit some LChV isolates that have diverged 3′-proximal RNA sequences, and further validation of the test is required with non-European isolates. On the other hand, the 3′-terminal sequence may be an optimal target for the PCR, as this genomic region is apparently well conserved among the strains of other closteroviruses. For two isolates of citrus tristeza closterovirus the 3′-untranslated sequences were found to be nearly identical, in contrast to substantial variability in the 5′-proximal part of the genome (Mawassi et al., 1996). Likewise, the Ukrainian and German isolates of BYV possess nearly identical 3′-untranslated regions (Agranovsky et al., 1994). It is expected that the RT-PCR assay will greatly facilitate LChV testing for quarantine and certification purposes.

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