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Comparison of diagnostic techniques for the detection and differentiation of *Cherry leaf roll virus* strains for quarantine purposes

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A B S T R A C T

Some strains of *Cherry leaf roll virus* (CLRV) are considered as quarantine pests in New Zealand. CLRV was detected in seven plant host species: *Actinidia chinensis*, *Hydrangea macrophylla*, *Malus domestica*, *Plantago major*, *Ribes rubrum*, *Rubus idaeus* and *Rumex* sp. collected from New Zealand between 2005 and 2012. Biological, serological and molecular techniques were compared for the detection and differentiation of CLRV isolates. The biological analysis revealed differences in symptomatology and disease severity among the isolates. The five isolates tested by ELISA were serologically related to each other using polyclonal antisera with only one out of four commercially-available antisera successfully detecting all of them. The phylogenetic analysis of sequences obtained from parts of the coat protein, polymerase and 3′-untranslated regions revealed that the New Zealand CLRV isolates clustered into two closely related but distinct phylogenetic groups with some isolates grouping differently depending on the gene studied. The New Zealand CLRV isolates were clearly distinct to overseas isolates found in phylogenetic groups A, D and E. The conventional RT-PCR using primers targeting the CLRV coat protein coding region is recommended for determining sequence differences between strains. These findings will be useful in making regulatory decisions with regard to the testing requirements and the CLRV strains to be regulated in New Zealand.

1. Introduction

Cherry leaf roll virus (CLRV) belongs to the genus *Nepovirus* within the family *Secoviridae* (Sanfaçon et al., 2012). The genome of CLRV consists of two single-stranded RNAs, RNA-1 and RNA-2 which are packaged into separate isometric particles of about 28 nm in diameter (Jones, 1985). Unlike other nepoviruses, CLRV does not appear to be transmitted by nematode vectors (Jones, 1985; Wang et al., 2002); however, it can be transmitted by pollen, seeds and by mechanical means (Büttner et al., 2011). Some reports also suggested that CLRV particles may enter wounded plant roots through uptake of nutrient solution or water (Büttner et al., 2011; von Bargen et al., 2009). CLRV is distributed worldwide and infects many deciduous trees and shrubs (e.g. *Betula* spp., *Juglans regia*, *Prunus avium*), small fruits (e.g. *Rubus* spp.) and herbaceous plants (e.g. *Rumex* spp.) (Jones, 1985).

CLRV was first described from cherry in 1955 in England (Cropley, 1961). Since then, many isolates of CLRV have been detected using natural and/or experimental hosts, serology, hybridization, restriction fragment length polymorphism or sequencing (Buchhop et al., 2009; Jones, 1985; Massalski and Cooper, 1986; Rebenstorf et al., 2006; Werner et al., 1997). To date, CLRV isolates are divided into six major phylogenetic clusters based on a 375 bp nucleotide sequence of the 3′-end of the untranslated region (3′-UTR) (Rebenstorf et al., 2006).

Due to the occurrence of very divergent strains of CLRV and the potential economic impact of CLRV on New Zealand crops such as grapevine (*Vitis* spp.), peach and plum (*Prunus* spp.), CLRV strains infecting *Prunus* and walnut (groups A, E and D1/D2) are considered as quarantine pests in New Zealand. The current testing requirements for nursery stock of *Vitis* are herbaceous indexing and PCR while for *Prunus*, it requires woody and herbaceous indexing and ELISA or PCR. The exportation of *Prunus* fruit and wine was worth NZ$28.5 million and NZ$1202.4 million, respectively for the year ending 30 June 2013 (Aitken and Hewett, 2013).

In New Zealand, CLRV was first reported in red raspberry (*Rubus idaeus*) in 1978 (Jones and Wood, 1978) and has been recently reported in local orchards of apple (*Malus domestica*) (Woo et al., 2012). CLRV has also been detected in mopheads (*Hydrangea macrophylla*), imported from Australia, in post entry quarantine in New Zealand (Veerakone et al., 2012). In this study, the virus was also detected for the first time in New Zealand in dock (*Rumex* sp.),

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kiwifruit (*Actinidia chinensis*), plantain (*Plantago major*) and redcurrant (*Ribes rubrum*).

The aim of this study was to compare the biological, serological and molecular techniques for the detection and differentiation of strains and/or isolates of CLRV present in New Zealand including the one detected in hydrangea in post entry quarantine. We determined their experimental host range on six herbaceous plant species and their serological relationship by testing the isolates with four commercially available antisera. We also studied the phylogenetic relationship with overseas CLRV isolates for three different regions (coat protein, polymerase and 3′-UTR regions) of the genome with recommendation on the best region to be used for determining sequence differences between strains.

2. Materials and methods

2.1. Source of materials

CLRV isolates were obtained from ten samples belonging to seven plant genera as follows: *Actinidia* (isolates *Actinidia*-1, -2 and -3), *Hydrangea*, *Malus*, *Plantago*, *Ribes*, *Rubus* and *Rumex* (isolates *Rumex*-1 and -2) (Table 1). New Zealand isolates were collected from various regions of the North and South Islands between 2005 and 2012 (Table 1). The CLRV-*Actinidia*-1, CLRV-*Plantago* and CLRV-*Rumex*-2 isolates were collected from the same field. CLRV-*Hydrangea* which originated from Australia was obtained from plants held in post entry quarantine in New Zealand. The CLRV-*Ribes* and CLRV-*Rubus* had been collected in 2007 and 1978, respectively; both isolates were received on *Chenopodium quinoa* while CLRV-*Actinidia*-2 and -3 were received on *Nicotiana tabacum* 'White Burley'.

Three CLRV ELISA positive controls originating from *Fraxinus excelsior* (PV-0278, DSMZ GmbH, Braunschweig, Germany), *Prunus avium* (BRB 150953, BIOREBA, Reinach, Switzerland) and *Sambucus* sp. (BIOREBA BRB 252253) were used in the ELISA assays. Two CLRV isolates from *F. excelsior* (PV-0278) and *S. nigra* (PV-0276) were purchased from DSMZ GmbH and were included in the phylogenetic study. 3′-UTR Sequences of CLRV from a further eleven representative isolates (Table 3) previously published by Rebenstorf et al., (2006) were also included in the phylogenetic study.

2.2. Mechanical inoculation

Original plant host tissue infected with their respective isolates of CLRV-*Hydrangea*, and CLRV-*Rumex*-1, along with freeze-dried *C. quinoa* leaves infected with isolates of CLRV-*Ribes* and CLRV-*Rubus*, and freeze-dried *N. tabacum* 'White Burley' leaves infected with CLRV-*Actinidia*-2 were mechanically inoculated onto herbaceous indicator plants (*C. amaranticolor*, *C. quinoa*, *N. benthamiana*, *N. clevelandii*, *N. occidentalis* '37B' and *N. tabacum* 'White Burley'). CLRV-*Plantago* was not inoculated due to insufficient plant material. Herbaceous indicator plants were mechanically inoculated in duplicate using sap from the CLRV-infected leaves diluted in 0.1 M phosphate buffer (pH 7.2) containing 5% (w/v) PVP-40 and 0.12% (w/v) Na_2SO_3. Positive and negative controls were also included in duplicate; the positive control was *Arabis mosaic virus* inoculated in the buffer described above; the negative control was buffer only. Plants were maintained in a glasshouse at an average temperature of 18 °C and were inspected twice a week for symptom development for four weeks. Four weeks after inoculation symptomatic and asymptomatic plants were tested for CLRV by RT-PCR using the Werner et al., (1997) primers.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Symptomatic *N. clevelandii* or *N. tabacum* 'White Burley' inoculated with each CLRV isolate were tested by double antibody sandwich (DAS)-ELISA according to the manufacturer's instructions. ELISA were performed using a broad spectrum CLRV polyclonal antiserum from DSMZ GmbH, a CLRV-birch strain polyclonal antiserum from LOEWE® Biochemica GmbH (Sauerlach, Germany) and two polyclonal antisera from BIOREBA; a CLRV-elderberry strain and a CLRV-cherry strain. Healthy leaves of each indicator plant species were included as negative controls. Three CLRV positive controls from DSMZ, LOEWE® Biochemica GmbH and BIOREBA were included in each test. Samples and controls were tested in duplicates and the absorbance was measured at 405 nm on a Bio-Rad M-680 microplate spectrophotometer (Bio-Rad Laboratories Inc., Hercules, USA). A sample was considered positive when the mean value of its absorbance was more than three times the mean of the value of the negative control.

2.4. Nucleic acid extraction

Leaf samples from the original host plants and from one or more herbaceous plants were used for nucleic acid extraction. Total nucleic acid was extracted from 0.2 g leaf tissue in a 1/10 (w/v) tissue/buffer ratio using the InviMag Plant DNA Mini Kit (Invitek, Berlin-Buch, Germany) on a KingFisher mL workstation (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions.

2.5. RT-PCR amplification

RT-PCR reactions were performed using either the Invitrogen SuperScript III® One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Life Technologies, Auckland, New Zealand) or by a two-step RT-PCR reaction using Invitrogen Superscript™ III reverse transcriptase and Invitrogen random primers (Life Technologies) for cDNA synthesis, and GoTaq® Green Master Mix (Promega, Madison, USA) for the PCR. cDNA synthesis was performed following the manufacturer's instructions. PCR reactions were done in a 20 μL volume, containing 0.5 mg/mL Bovine serum albumin (BSA; Sigma-Aldrich, Castle Hill, Australia), 250 nM of each primer, 2 μL RNA or cDNA and the appropriate PCR reagents as specified by the manufacturer.

RNA extracts were checked for PCR competency with plant internal control primers targeting the *nad*5 gene (Menzel et al., 2002). The presence of CLRV in the original plant hosts and the herbaceous plants from the experimental host range experiment was checked by RT-PCR using the primers published by Werner et al. (1997) which amplify a 416 bp fragment of the 3′-UTR. RNA extractions from one CLRV-infected herbaceous plant from each CLRV isolate with the exception of CLRV-Malus for which the original plant was used, were selected for the phylogenetic study using three sets of primers targeting the 3′-UTR, the coat protein and the polymerase regions (Table 2). Primers were manually designed from sequences available at the time of the research in 2013 using the alignment software Clustal X2.0.

Positive and no template controls were included in the PCR. PCR products were separated on a 1.5% (w/v) agarose gel stained with Invitrogen SYBR Safe (Life Technologies). Amplicons of the expected sizes were excised from the gel and purified using Freeze 'N Squeeze (Bio-Rad). Alternatively, PCR products were purified using the illustra™ MicroSpin™ S-300HR Columns (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer's instructions for either direct sequencing or cloning.