First report of *Cherry leaf roll virus* (CLRV) in *Malus domestica*

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Abstract *Cherry leaf roll virus* (CLRV) was detected in apple (*Malus* sp.), a host not previously reported for CLRV in New Zealand. A total of 72 leaf samples were obtained from two orchards in the Waikato region of the North Island of New Zealand and tested by reverse transcription-polymerase chain reaction (RT-PCR). Virus-specific RT-PCR, sequencing and mechanical inoculation on herbaceous host plants detected the presence of CLRV in three samples. This is the first report of CLRV on apple in New Zealand. Based on a thorough review of literature, results obtained in this study may likely represent the first case of CLRV in apple.

Keywords *Cherry leafroll virus* · *Malus* sp. · RT-PCR · Phylogenetic analysis · Mechanical inoculation · New Zealand

Cherry leaf roll virus (CLRV) is a species within subgroup C of the *Nepovirus* genus, *Secoviridae* family (Sanfaçon et al. 2009). CLRV has a wide natural and experimental host range and causes economically significant diseases in horticultural crops. The virus was first described in 1955 causing a disease of sweet cherry in England (Posnette and Cropley

1955). Subsequently the virus was reported to cause leaf rolling and plant death in cherry (Cropley 1961) and a range of other plant species including elderberry, olive, raspberry, rhubarb, walnut and a number of shrub, tree, weed and ornamental species (Ahmed and Bailiss 1975; Cooper and Atkinson 1975; Ford et al. 1972; Hansen and Stace-Smith 1971; Jones and Wood 1978; Larson et al. 1990; Mircetich and Rowhani 1984; Savino and Gallitelli 1981; Tomlinson and Walkey 1967; Walkey and Cooper 1973). The virus has been detected throughout Europe and North America (Bandte and Büttner 2001), Chile (Herrera and Madariaga 2001), Peru (Crosslin et al. 2010), New Zealand (Jones and Wood 1978; Veerakone et al. 2012), Japan (Yamashita and Fukui 2004), Australia and China (Jones 1985).

CLRV is naturally transmitted through seeds of woody perennial hosts which include birch, cherry, elderberry, elm, and walnut (Cooper et al. 1984; Massalski and Cooper 1984) or pollen (Card et al. 2007). It has also been experimentally graft-transmitted by bark patches of English walnut trees (Mircetich et al. 1980) and mechanically transmitted to a wide range of herbaceous species (Cropley 1961; Jones 1985). Unlike many other nepoviruses, CLRV appears not to be transmitted by soil-inhabiting nematodes (Jones et al. 1981; Wang et al. 2002) despite earlier reports to the contrary (Flegg 1969). More recently, it has been suggested that CLRV particles released from roots of infected *Chenopodium quinoa* plants are able to migrate through nutrient solution and infect healthy *C. quinoa* plants (Bandte et al. 2007).

During a recent survey of apple trees (*Malus × domestica* Borkh), predominantly the ‘Royal Gala’ variety on either Geneva 202 or MM106 rootstocks, some leaves were observed with leaf tip deformation, necrotic spots and mottling lesions (Fig. 1a & b). A total of 72 leaf samples were tested for the presence of CLRV using a 1-tube reverse transcription-

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Fig. 1 Symptoms observed in apple leaves: **a** Leaf tip deformation; **b** Necrotic spots and mottling. Symptom development following inoculation of *Cherry leaf roll virus* in *Nicotiana occidentalis*: **c** Localized symptom of yellow mottling; **d** Systemic symptoms of lamina deformation, necrosis and yellow mottling

polymerase chain reaction (RT-PCR) protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from leaf samples using the Qiagen RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RT-PCR was done using the CLRV-specific primers of Werner et al. (1997). A PCR competency control using the nad5-s and nad5-as primers of Menzel et al. (2002) was included for all the RNA extracts tested. The cycling conditions were optimised during this study and consisted of 1 cycle of complementary DNA synthesis at 55 °C for 30 min, a single cycle of denaturation at 94 °C for 2 min, followed by 40 cycles at 55 °C annealing for 30 s, 68 °C extension for 45 s, and 94 °C denaturation for 15 s, with a final extension at 68 °C for 5 min.

RNA extracts from all the 72 leaf samples were confirmed to be RT-PCR competent using the nad5-s and nad5-as primers of Menzel et al. (2002) as a 180 bp product was obtained from all the samples tested. Three samples produced amplicons of the expected size, these were cloned using a pGEM®-T easy vector systems kit (Promega Corporation, USA) and sequenced. The consensus sequences from four clones of two samples were deposited in the GenBank (Accession numbers JN581001, JN581002) while two clones from the third sample generated sequences that have a pairwise identity of only 96.8 % to each other (Accession numbers JN581000, JQ822107). A BLASTn search of these amplicons in GenBank showed 96.8 % – 99.8 % nucleotide identity to two New Zealand isolates from *Rubus* and *Hydrangea* (Accession numbers AJ877162, JN418885). The primer sequences described by Werner et al. (1997) were removed prior to performing a multiple sequence alignment with 58 CLRV isolates selected from reports by Rebenstorf et al. (2006), von Barga et al. (2011) and Veerakone et al. (2012) using the Geneious software (Drummond et al. 2011). Details of isolates obtained from the NCBI database are shown in Table 1. A Neighbour-Joining tree (Fig. 2) with bootstrap values of 1,000 replicates was constructed using Geneious (Drummond et al. 2011). The phylogenetic tree showed that one of the four CLRV sequences (*Malus*-JQ822107-New Zealand) clustered with phylogenetic

group B according to Rebenstorf et al. (2006) while the other three (*Malus*-JN581000-New Zealand, *Malus*-JN581001-New Zealand and *Malus*-JN581002-New Zealand) clustered with isolates of group C.

Sap from the three positive samples was used to inoculate three *Nicotiana occidentalis* plants. Localized and systemic symptoms of yellow mottling were observed 14 days post inoculation in all three plants (Fig. 1c & d). The presence of CLRV in *N. occidentalis* was confirmed by direct sequencing amplicons with the expected size obtained by 1-tube RT-PCR with CLRV-specific primers (Werner et al. 1997). A BLASTn search of these amplicons showed 92.4 % – 99.4 % nucleotide identity to two New Zealand isolates from *Rubus idaeus* and *Hydrangea macrophylla* (Accession numbers AJ877162, JN418885).

The finding of two sequences within a sample of leaves suggests the presence of two CLRV variants. While the clustering of the four CLRV *Malus* isolates in two phylogenetic groups suggests that it is not likely for the presence of an obvious specific host-virus association. This lack of a distinct phylogenetic grouping according to host has been reported previously (Bousalem et al. 2000; García-Arenal et al. 2001; Tomimura et al. 2004). The clustering in two phylogenetic groups also suggests that there may have been at least two separate introductions of CLRV into New Zealand. Additionally, *Malus*-JN581000-New Zealand, *Malus*-JN581001-New Zealand and *Malus*-JN581002-New Zealand also clustered together with sequences from two other New Zealand CLRV isolates (GenBank accession numbers AJ877162, JN418885) from red raspberry (*Rubus idaeus*) (Jones and Wood 1978) and *Hydrangea macrophylla* (Veerakone et al. 2012) respectively. Although the virulence of CLRV isolated from *Malus* in this study is still to be determined, CLRV isolated from red raspberry was reported to cause stunted growth with small and deformed leaves which had chlorotic mottling and ring spots (Jones and Wood 1978) and severe leaf deformation and chlorosis in *Hydrangea macrophylla* (Veerakone et al. 2012). CLRV has also been reported to cause damaging diseases in cherry (*Prunus avium*) (Jones 1985)

Fig. 2 Neighbour-Joining phylogenetic tree based on a 386 bp nucleotide sequence of the 3' non-coding region of *Cherry leaf roll virus* isolates generated with Geneious (Drummond et al. 2011). The nodal significance was evaluated by 1,000 bootstrap replicates and probability estimate values larger than 70 % are indicated at branch nodes.

CLRV isolates generated from this study are highlighted with * and the CLRV isolates already recorded as present in New Zealand are highlighted with ^. Phylogenetic groups, A to E, according to Rebenstorf et al. (2006) are indicated by bold lines on the right. Phylogenetic tree submitted to TreeBase (2012)

and forest trees including beech (*Fagus sylvatica*) and birch (*Betula* sp.) (Werner et al. 1997). Hence, CLRV may have an economic impact on crops such as *Malus* and *Prunus* in New Zealand.

The virus isolated from *Malus domestica* Borkh was identified as CLRV based on molecular and phylogenetic

analyses and mechanical inoculation of herbaceous test plants. It is understood that the finding of CLRV in apple is a new host record in New Zealand. A thorough review of literature did not reveal any report of an association between CLRV and apple. In view of this, results obtained in this study may likely represent the first case of CLRV in apple.

Table 1 *Cherry leaf roll virus* isolates from the NCBI database included in the phylogenetic analyses

Host plant	Accession no.	CLRV isolate	Geographic origin	Phylogenetic group ^a
<i>Aegopodium podagraria</i>	AJ877157	E575	Fellinghausen, Germany	B
<i>Allium tuberosum</i>	AB168098	Chinese chive	Japan	C
<i>Betula nigra</i>	AJ877125	E836	Hannover-Herrenhausen, Germany	A
<i>Betula pendula</i>	AJ877118	E120	Berlin-Spandau, Germany	A
<i>Betula pendula</i>	AJ877119	E499	Berlin-Zehlendorf-Berkaer Str., Germany	A
<i>Betula pendula</i>	AJ877120	E896	Berlin-Zehlendorf-Thielallee, Germany	A
<i>Betula pendula</i>	AJ877121	E696	Klößenstein, Germany	A
<i>Betula pendula</i>	AJ877122	E111	Klößenstein, Germany	A
<i>Betula pendula</i>	AJ877123	E806	UK	A
<i>Betula pendula</i>	S84124	I ₂ -RNA-1	UK	A
<i>Betula pendula</i>	S84125	I ₂ -RNA-2	UK	A
<i>Betula pendula</i>	AJ877124	E1469	UK	A
<i>Carpinus betulus</i>	AJ877156	E141	Niedereimer, Germany	E
<i>Cornus florida</i>	AJ877161	E797	USA	A
<i>Fagus sylvatica</i>	AJ877159	E113	Bonn-Siebengebirge, Germany	A
<i>Fraxinus excelsior</i>	AJ877158	E325	Schwäbische Alb, Germany	A
<i>Fraxinus excelsior</i>	AJ888533	E678	Andechs, Germany	B
<i>Fraxinus excelsior</i>	AJ888534	E698	Idar-Oberstein, Germany	B
<i>Hydrangea macrophylla</i>	JN418885	—	New Zealand	C
<i>Juglans regia</i>	AJ877146	E326	Bonn-Oberkassel, Germany	D1
<i>Juglans regia</i>	AJ877147	E648	France	D1
<i>Juglans regia</i>	AJ877148	4WJUG	UK	D1
<i>Juglans regia</i>	AJ877149	E800	UK	D1
<i>Juglans regia</i>	AJ877126	GAY	Gaydon, UK	D1
<i>Juglans regia</i>	AJ877150	E156	Trans-Danubia, Hungary	D2
<i>Juglans regia</i>	AJ877151	CTIFL	France	D1
<i>Juglans regia</i>	AJ877152	Ludmila	Slovakia	D2
<i>Malus domestica</i>	JN581000	26AYLS	This study	C
<i>Malus domestica</i>	JQ822107	26BYLS	This study	B
<i>Malus domestica</i>	JN581001	54OHS	This study	C
<i>Malus domestica</i>	JN581002	66OHS	This study	C
<i>Prunus avium</i>	AJ877127	E327	Bonn, Germany	A
<i>Prunus avium</i>	AJ877128	E803	UK	A
<i>Prunus avium</i>	AJ877129	E1472	UK	A
<i>Rheum rhaponticum</i>	AJ877165	E395	Bornheim, Germany	B
<i>Rheum rhabarbarum</i>	S84126	R25-RNA-1	UK	B
<i>Rheum sp.</i>	NC015415	E395	Bornheim, Germany	B
<i>Rumex acetosella</i>	AB168099	Rumex AGBC	Japan	C
<i>Rumex acetosella</i>	AB168100	Rumex acetosella-21	Japan	C
<i>Rubus idaeus</i>	AJ877162	E802	New Zealand	C
<i>Rubus procerus</i>	AJ877163	E805	UK	A
<i>Sambucus canadensis</i>	AJ877145	E804	USA	E
<i>Sambucus nigra</i>	AJ877130	E676	Helgoland, Germany	B
<i>Sambucus nigra</i>	AJ877131	E485	Fischland, Germany	E
<i>Sambucus nigra</i>	AJ877132	E603	Werder, Germany	E
<i>Sambucus nigra</i>	AJ877133	E583	Neuruppin, Germany	E
<i>Sambucus nigra</i>	AJ877134	E119	Berlin-Zehlendorf-Lentzeallee I, Germany	E
<i>Sambucus nigra</i>	AJ877135	E622	Berlin-Zehlendorf-Lentzeallee II, Germany	E
<i>Sambucus nigra</i>	AJ877136	E839	Berlin-Zehlendorf-Lentzeallee III	E

Table 1 (continued)

Host plant	Accession no.	CLRV isolate	Geographic origin	Phylogenetic group ^a
<i>Sambucus nigra</i>	AJ877137	E541	Berlin-Zehlendorf-Bitterstr.	E
<i>Sambucus nigra</i>	AJ877138	E443	Berlin-Neukölln	E
<i>Sambucus nigra</i>	AJ877139	E441	Aschersleben	A
<i>Sambucus nigra</i>	AJ877140	E950	Aschersleben	E
<i>Sambucus nigra</i>	AJ877141	E568	Balve	E
<i>Sambucus nigra</i>	AJ877142	E576	Fellinghausen	E
<i>Sambucus nigra</i>	AJ877143	E492	Sümege	E
<i>Sambucus nigra</i>	AJ877144	PV-0276	Bonn-Siebengebirge	E
<i>Sorbus aucuparia</i>	AJ877153	E697	Hamburg-Osdorfer Born	B
<i>Sorbus aucuparia</i>	AJ877154	E695	Pinneberg, Germany	B
<i>Sorbus aucuparia</i>	AJ877155	E693	Titisee-Neustadt, Germany	E
<i>Ulmus americana</i>	AJ877160	E801	USA	A
<i>Vitis vinifera</i>	AJ877164	E1636	Neustadt-W.-Königsbach, Germany	A

^a Phylogenetic grouping according to Rebenstorf et al. (2006)

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