SHORT COMMUNICATION

A NEW HERBACEOUS HOST OF *CITRUS LEAF BLOTCH VIRUS*

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SUMMARY

Citrus leaf blotch virus (CLBV), the type species of the putative new genus *Citrivirus*, causes a bud union disorder of Nagami kumquat and Calamondin scions grafted on trifoliate rootstocks. This virus was successfully transmitted to *Nicotiana cavicola* using leaf extracts of infected Nagami kumquat and Etrog citron, thus widening its herbaceous host range. The infection was latent but confirmed by the positive response of RT-PCR using virus-specific primers and by electron microscopy. The positive transmission of CLBV to *N. cavicola* should in principle facilitate laboratory investigations, as it provides a new source of virus alternative to and more manageable than citrus.

Key words: Electron microscopy, RT-PCR, mechanical transmission, Troyer citrange, Etrog citron.

Citrus leaf blotch virus (CLBV), the type species of the putative new genus *Citrivirus*, family *Flexiviridae* (Mayo and Haenni, 2006), has filamentous particles 960 nm long containing a single-stranded, positive-sense RNA, 8747 nt in size (Galipienso *et al.*, 2001) The viral genome is made up of three open reading frames (ORFs) which encode, in the order, a polyprotein involved in replication, the movement protein (MP) and the coat protein (CP) (Vives *et al.*, 2001)

CLBV, first detected in Nagami kumquat (*Fortunella margarita* Lour. Swingle) from Corsica in 1984 (Navarro *et al.*, 1984), was later associated with bud union crease in Troyer citrange (*Citrus sinensis* x *Poncirus trifoliata*) and citrumelo (*C. paradisi* x *P. trifoliata*) (Galipienso *et al.*, 2001), and reported from different citrus varieties in Japan, Australia, Florida and Spain. Recently, CLBV has been found in Nagami kumquat (Guardo *et al.*, 2007a) and Calamondin (*Citrus mitis* or *madureusis* Lour) (Guardo *et al.*, 2007b) in Italy, and in a number of citrus varieties in New Zealand (Harper *et al.*, 2008).

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CLBV was recently suggested (Vives *et al.*, 2005) to be the same as the agent of Dweet mottle, a disease characterized by a psorosis-like mottling of the leaves of Dweet tangor (Roistacher and Blue, 1968), which was transmitted from citrus to citrus by contaminated knife blades (Roistacher *et al.,* 1980). CLBV was also transmitted to a low percentage (*ca*. 2.5%) through seeds of citrange, kumquat and sour orange (Guerri *et al.*, 2004), but apparently not by inoculation of sap to herbaceous hosts (Navarro *et al.*, 1984; Galipienso *et al.*, 2000) until recently, as reported by Vives *et al.* (2008a, 2008b), when the present study was virtually completed. In fact, in the course of an extensive investigation on the properties of a partially sequenced Italian isolate of CLBV (ISA 10-CT-I) successful transmission to a *Nicotiana* species was obtained.

Inoculum sources for mechanical inoculations were Nagami kumquat and Etrog citron seedlings which had been graft-inoculated with three bark patches from a Nagami kumquat donor and grown in a greenhouse at 18-25°C. Inoculum consisted of young kumquat and citron leaves crushed in a chilled mortar in the presence of an equal amount (w:v) of either 0.1 M phosphate buffer pH 7.2, or 0.05 M potassium phosphate buffer pH 7.0 with 2.5% nicotine. Both extracts were separately rubbed on celite-dusted leaves of groups of six plants each of *Chenopodium quinoa*, *Nicotiana glutinosa*, *N. occidentalis*, *N. benthamiana*, *N. clevelandii*, *N. cavicola* and *Gomphrena globosa.* Negative controls were two plants of each host mock inoculated with buffer. All plants were maintained in a glasshouse at 22°C, observed daily and tested after inoculation by RT-PCR every 15 days. Dips in 2.5% uranyl acetate were made according to Milne (1993) from *N. cavicola* and observed under a Philips Morgagni 282D electron microscope.

None of the inoculated Etrog citron seedlings showed foliar symptoms. However, after about eighth months the plants were peeled and three of them showed a mild stem pitting (not shown). Although this result confirmed Navarro's (1984) observations, to make sure that symptomatic Etrog seedlings were indeed infected by CLBV, RT-PCR assays were carried out using the primers designed on CLBV sequence (accession No. AJ318061) to amplify RNA polymerase (sense primer KU 27, 5'-GAT-

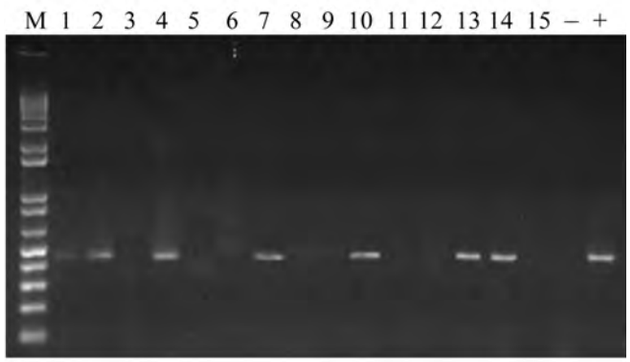

Fig. 1. Detection of CLBV by RT-PCR using primers KU 18 and KU 19 in different *Nicotiana* species. Lane 1, *N. glutinosa* inoculated with citron extract; lane 2, *N. glutinosa* inoculated with Kumquat extract; lane 3, mock inoculated *N. glutinosa*; lane 4, *N. cavicola* inoculated with citron extract; lane 5, *N. cavicola* inoculated with Kumquat extract; lane 6, mock inoculated *N. cavicola*; lane 7, *N. occidentalis* inoculated with citron extract; lane 8, *N. occidentalis* inoculated with Kumquat extract; lane 9, mock inoculated *N. occidentalis*; lane 10, *N. benthamiana* inoculated with citron extract; lane 11, *N. benthamiana* inoculated with Kumquat extract; lane 12, mock inoculated *N. benthamiana*; lane 13, *N. clevelandii* inoculated with citron extract; lane 14, *N. clevelandii* inoculated with Kumquat extract; lane 15, mock inoculated *N. clevelandii*; lane +, CLBV isolate ISA 10-CT-I; lane –, negative control (water); lane M, 1-KB plus DNA ladder.

GCAAGCCAGGATGAATAC-3' genomic positions 5321-5341 and antisense primer KU 15, 5'-CAGACACTCCAAGACCTTTCC-3' genomic positions 5776-5756), and coat protein sequences (sense primer KU18, 5'-TTAAGATTACAGACACGAAGG-3' genomic positions 7686-7706, and antisense primer KU 19, 5'-CTGTTTTTGAATTTTGCTCG-3' genomic positions 8123-8104) (Vives *et al.*, 2002). Total RNA was extracted from citron leaves with Qiagen RNeasy Plant mini Kit (Qiagen, Milano, Italy). For cDNA synthesis 2 µl total RNA mixed with 2.5 µl of 1x PCR Buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3 mM $MgCl_2$, 0.4 mM dNTPs and 0.2 µM of each primer were denatured at 85°C for 5 min and chilled on ice. Amplification was carried out in a 25 µl reaction volume adding 20 units of Superscript II RNase H-reverse transcriptase and 1 unit Platinum Taq DNA Polymerase (Invitrogen, USA), 4 units RNaseOUT ribonuclease inhibitor (Amersham, USA) and DEPC treated water. The reaction was incubated at 42°C for 45 min for reverse transcription. The PCR conditions included 40 cycles of 20 sec at 94°C, 20 sec at 50°C (primers KU 27 and KU 15) or 44°C (primers KU 18 and KU 19) and 30 sec at 72°C, followed by a final extension of 5 min at 72°C (Vives *et al.*, 2002). PCR products (8 µl) were analysed in a 2% agarose gel.

In all assays the expected amplicons of 456 bp and 438 bp were obtained with primers KU27 and KU15 and KU18 and KU19, respectively (not shown), confirming the successful transmission of CLBV. To verify the virus distribution in Etrog, six leaves were collected at random from one seedling and tested by RT-PCR. A positive response was obtained only from four leaves, which may be due to the uneven distribution of the virus within the plant as reported by Galipienso *et al.* (2004).

PCR products were cleaned by wizard SV gel and PCR clean up system (Promega, USA) and cloned into pGEM-T Easy vector (Promega, USA) according to the manufacturer's instructions. Four clones for each DNA fragments were sequenced in both directions using an ABI Prism 3130 automatic sequencer (Applied Biosystem, USA). Nucleotide sequence were aligned with the Clustal W program (Thompson *et al.*, 1994) and compared with sequence available in the NCBI databas with the BLAST programme (Altschul *et al.*, 1997). Analysed amplicons showed identity of 100% at the amino acid level with the comparable sequences of the CLBV isolate (ISA 10-CT-I) used for inoculation. Consensus sequences of the two genes: RNA polymerase and coat protein (accession Nos. FJ449704 and EU877531) had 95% and 98% nucleotide identity, respectively, with the previously reported CLBV sequence (accession No. AJ318061).

None of the inoculated herbaceous hosts developed overt symptoms and no difference was observed between plants inoculated with infected citrus donors and the mock inoculated controls. Nonetheless, 15 and 30 days after inoculation three plants of each herbaceous species, two inoculated with extracts from diseased citrus and one mock inoculated, were tested by RT-PCR following the protocol described above.

Positive amplifications were consistently obtained only one month after inoculation from *Nicotiana* species (Fig. 1), which were symptomless like the other hosts, and remained so for the months that followed. In fact, the plants were checked by RT-PCR until tree months after inoculation giving always the same reaction. Amplicons were of the expected size (438 and 458 bp) and

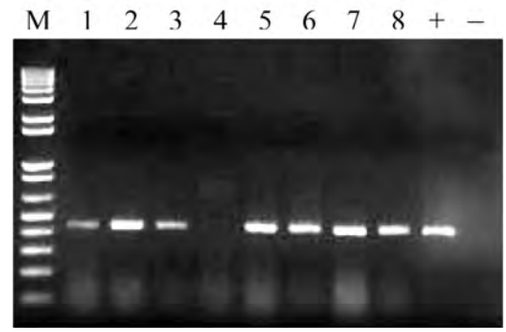

Fig. 2. Lanes 1-8, positive RT-PCR detection of *Citrus leaf blotch virus* using primers KU 18 and KU 19 from seven out eight mechanically inoculated but symptomless *N. cavicola* plants, Lane +, CLBV isolate ISA 10-CT-I; lane –, healthy *N. cavicola;* lane M, 1-KB plus DNA ladder.

the results with both sets of primers were consistent. Their sequence was 100% identical to that of the isolate ISA 10-CT-I. However, there was a difference in the efficiency of the inoculum for a higher number of PCR positives was found among plants inoculated with citron than kumquat extracts. PCR-positive reactions were given by all *Nicotiana* species and, occasionally, by inoculated Chenopodia and *G. globosa* (not shown), but this needs to be confirmed because virus particles were seen only in leaf dips from *N. cavicola*. It should be noted, however, that *N. occidentalis* and *N. benthamiana* were successfully infected by sap inoculation with CLBV SRA-153 isolate (Vives *et al*., 2008a).

To find out whether transmission from *N. cavicola* to *N. cavicola* would improve the efficiency of transmission, thus resulting in an increased virus concentration and, possibly in symptom expression, three subinoculations were made in succession to groups of 60 plants about 40 days apart from one another and checked by RT-PCR following the protocol described above using only the set of primers for coat protein (Fig. 2). The percentage of infection was 32.8% in the first inoculation carried out on 13/02/08, became 62.22% in the second inoculation (21/03/08) and reached 94.5% at the end of experiment (last inoculation made on 05/05/08). None of the plants showed symptoms (Fig. 3).

As a final check, dips from symptomless citron leaves and from leaves, stems and roots of *N. cavicola* were observed under the electron microscope. Slightly flexuous virus particles resembling those of CLBV (Galipienso *et al*., 2001) were seen in all samples (Fig. 4). A lower number of particles was present in dips from citron than *N. cavicola*. As to the latter, apparently more particles were extracted from stems than from leaves or roots.

The positive transmission of CLBV to *N. cavicola*, and other hosts, should in principle facilitate laboratory investigations, as it provides an additional source of virus alternative to and more manageable than citrus.

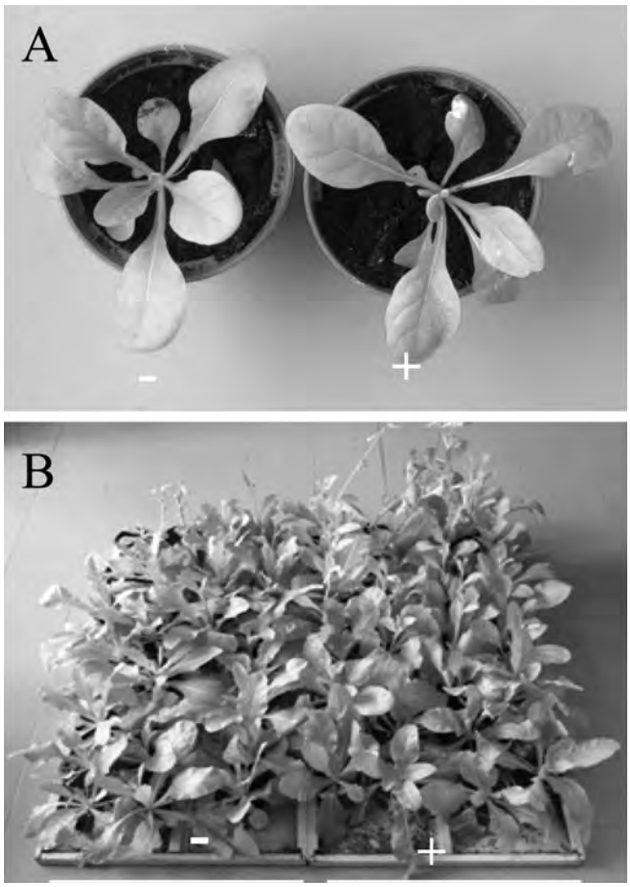

Fig. 3. Comparison between non inoculated *N. cavicola* (-) and infected *N. cavicola* (+) three weeks (A) and 2 months (B) after inoculation.

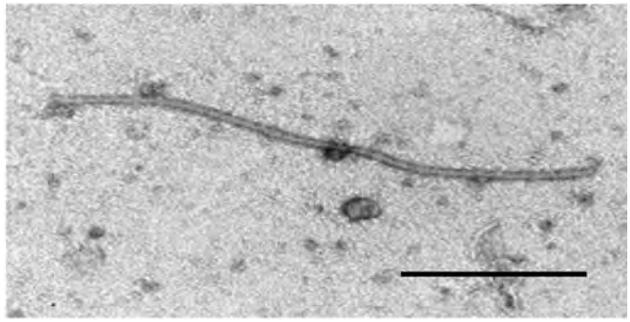

Fig. 4. A CLBV particle in a leaf dip from an inoculated but symptomless *N. cavicola* plant. Preparation was negatively stained with 2% uranyl acetate. Bar = 300 nm

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