Citrus Leaf Blotch Virus: A New Citrus Virus Associated with Bud Union Crease on Trifoliate Rootstocks

M. C. Vives, L. Galipienso, L. Navarro, P. Moreno, and J. Guerri

ABSTRACT. Citrus leaf blotch virus (CLBV) was first detected in a Nagami kumquat, clone SRA-153 from Corsica (France), showing bud union crease when propagated on Troyer citrange. Citranges are important rootstocks in Spain, presently being used for about 50% of all commercial citrus trees. Therefore, dispersal of a graft-transmissible pathogen causing bud union crease on this rootstock could potentially cause important economic losses. CLBV has filamentous particles about 900 × 14 nm in size, with a single-stranded, positive sense, genomic RNA (gRNA) of 8,747 nt, and a coat protein about 41 kDa. The gRNA contains three open reading frames (ORFs) and two untranslated regions of 73 and 541 nt at the 5' and 3' termini, respectively. Biological and molecular properties of CLBV support its inclusion in a new virus genus. The virus can be detected by graft-inoculation onto Dweet tangor seedlings, in which it induces chlorotic blotching in young leaves, but transmission is sometimes erratic and at least six indicator plants should be used in each test. We developed a quick detection procedure using RT-PCR with two sets of primers derived from sequences in ORF1 (a region containing motifs characteristic of an RNA polymerase) and ORF3 (the coat protein gene). Results with both sets of primers were similar. CLBV was readily detected in young leaves of infected Nagami kumquat or in Nules Clementine, Owari Satsuma, Eureka lemon, Marsh grapefruit or Newhall navel orange inoculated with kumquat SRA-153, but not in Pineapple sweet orange, a host that yielded more than 80% false negatives. Detection in field trees was less consistent, as the virus generally has low titer and is unevenly distributed. By this procedure CLBV was detected in two mandarins from Japan, a kumquat from New South Wales (Australia), and in various sweet orange trees showing bud union crease on citrange or citrumelo, from commercial citrus orchards in Valencia (Spain) and Florida (USA), but not in other trees in the same orchards showing similar symptoms. Our results indicate that CLBV is present in citrus varieties other than kumquat in several geographic areas. Failure to detect CLBV in some trees with bud union crease could be due to low titer or uneven distribution of the virus within the plant. Alternatively, a different agent could be involved.

Decline associated with bud union disorders on trifoliate orange, citrange or citrumelo have been described in several countries, but reports are often confusing and knowledge on these disorders is generally very limited. Some are thought to be of genetic origin (6, 10, 11, 15), others are caused by pathogens (13, 18) and several have no known cause. In Corsica, two clones of kumquat (K-124 and 125) showed bud union disorders when propagated on Troyer citrange (17). In Israel, bud union crease was observed in some lines of Shamouti sweet orange propagated on Troyer citrange or citrumelo, and in Nagami kumquat budded on Troyer citrange (2). Decline associated with bud union disorders on trifoliate orange, citrange or citrumelo rootstocks have also been detected in field trees of Navel sweet orange in California (1), Pera sweet orange in Brazil (14), Marsh grapefruit and Roble sweet orange in Florida (6), Nules Clementine plants carrying different pathogens in Italy (3), and Nules Clementine, Navelina and Navelate sweet orange in Spain.

Trifoliate rootstocks are important in many citrus growing areas of the world including Spain due to their tolerance to *Citrus tristeza virus* and to the good yield and fruit quality that they induce in different citrus varieties. Therefore, dispersal of a graft-transmissible pathogen causing bud union crease on these rootstocks could potentially cause important economic losses.

Citrus leaf blotch virus (CLBV) was first detected in Nagami kumquat, clone SRA-153 from Corsica (France), showing bud union crease

when propagated on Troyer citrange (12). When this clone was inoculated to plants of Nules Clementine, Eureka lemon, Marsh grapefruit and Pineapple sweet orange and buds of these plants were propagated on citrange seedlings in the greenhouse, some plants of Clementine and lemon showed bud union crease 6 mo after propagation, whereas Marsh grapefruit and Pineapple sweet orange had normal bud union after 3 yr (4). Partial purification of CLBV showed that virions are filamentous particles about 900 × 14 nm in size, with a single-stranded, positive sense, genomic RNA (gRNA) of 8,747 nt, and a coat protein about 41 kDa. The gRNA contains three open reading frames (ORFs) and untranslated regions of 73 and 541 nt at the 5' and 3' termini, respectively. In morphology and genome organization of CLBV resembles trichoviruses, but biological and molecular differences with these viruses support its inclusion in a new virus genus (5, 16). CLBV-infected plants contain double-stranded RNA molecules corresponding to the gRNA and two sets of 3' and 5' co-terminal subgenomic RNAs (17).

Control of graft-transmissible pathogens can be achieved by sanitation, quarantine and certification programs (13), but these programs require reliable detection procedures. So far, CLBV can be detected only by biological indexing on Dweet Tangor, a host in which it induces chlorotic blotching in young leaves, but transmission is sometimes erratic and the test is not reliable (4). In addition, quick and sensitive detection procedures are urgently needed to assess potential association of CLBV with bud union disorders on trifoliate rootstocks observed in Spain and other countries, and to study the epidemiology of this virus.

Attempts at purification failed to yield enough virion concentration to prepare an antiserum (5). However, recent sequencing of the genome (16) opened new possibilities for diagnosis. Reverse transcription (RT) and PCR amplification (RT-PCR) have frequently been used to detect plant RNA viruses and viroids (7, 8, 9, 20) and are generally more sensitive than other detection techniques. In this work, we developed and evaluated a rapid and specific one-step RT-PCR assay for the detection of CLBV in greenhouse-grown plants and in field trees.

MATERIALS AND METHODS

Virus sources and hosts. The following sources were used: Nagami kumquat SRA-153; Washington navel, Navelina and Pineapple sweet oranges, Eureka lemon, Marsh grapefruit, Nules Clementine and Owari satsuma plants inoculated with kumquat SRA-153; two satsuma cultivars introduced from Japan through the National Citrus Quarantine Station; freeze-dried tissue from: Nagami kumquat from New South Wales, Australia (kindly provided by P. Barkley), navel and Moro Blood sweet orange from California, USA (kindly provided by L. Marais), showing bud union crease on citrange; freeze-dried tissue from field samples of Navel and Roble sweet orange showing bud union crease on Swingle citrumelo from different commercial orchards in Florida, USA (kindly provided by S. M. Garnsey); and field samples of different varieties of sweet orange, showing bud union crease on citrange rootstocks, collected in various citrus growing areas in Spain.

RNA extraction. Total RNA extracts were obtained from approximately 100 mg of thoroughly trimmed fresh young leaf tissue or 25 mg of freeze-dried leaf tissue, using TRIzol^{R} reagent (Invitrogene), which contains isothyocyanate, following the manufacturer's instructions for samples with high sugar content, and were finally resuspended in 25 µl of DEPC-treated distilled water.

RT-PCR. Two sets of primers were designed based on the CLBV gRNA sequence (EMBL accession AJ318061) (Table 1). The first set of primers (KU-27 and KU-15) was used to amplify a 456-bp cDNA product containing the RNA-dependent RNA polymerase conserved domains within ORF 1 (16). The second set of primers (KU-18 and KU-19) yielded a 438-bp cDNA fragment containing the conserved C-terminal region of the coat protein gene. For cDNA synthesis, 1µl of total RNA preparation, equivalent to 4 mg of plant tissue, and 0.2 µM of each primer were denatured at 85°C for 5 min and chilled on ice. One-step RT-PCR was then performed in a 25-µl reaction volume adding 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50mM KCl), 3 mM MgCl2, 0.4 mM dNTPs, 4 units RnaseOUT™ ribonuclease inhibitor, 20 units SuperScript™ II Rnase H-reverse transcriptase and 1 unit *Taq* DNA polymerase (Invitrogene), and DEPC-treated water. The reaction mix was incubated at 42°C for 45 min for reverse transcription and then the enzyme inactivated at 94°C for 2 min. Thermocycling conditions included 40 cycles of 20 s at 94ºC, 20 s at 50°C (KU-27 and KU-15 primers) or 45°C (KU-18 and KU-19 primers) and 30 s at 72ºC, followed by a final extension of 5 min at 72°C. PCR reaction products (10 µl) were analyzed by electrophoresis in a 2% agarose gel in 1× TAE buffer (40 mM Tris-acetate pH 8.3, 1 mM EDTA) at 100 volts for 1 h. Gels were stained in ethidium bromide (0.5 µg/ml) for 15 min, rinsed in water and observed in a UV-transilluminator. The 1-Kb plus ladder (Invitrogene) was used for size estimation of the PCR products.

RESULTS

Detection of CLBV in greenhouse-grown plants. In order to compare different tissues as viral RNA source for RT-PCR detection, total RNA extracts from tender shoots, young bark, young expanded leaves, old bark and old leaves from kumquat, healthy or infected with isolate SRA-153, were used as templates with the two selected primer sets (Table 1). Single DNA bands of 456 or 438 nt were amplified from all CLBV-infected tissues using primers KU-27 and KU-15 or KU-18 and KU-19, respectively, whereas no amplification was obtained when analyzing equivalent tissues from healthy kumquat (Fig. 1). The size of the products was that expected for the selected primers, and their viral origin was confirmed by southern blot hybridization (data not shown). The amount of DNA amplified from all tissue sources was similar, but since leaves are easier to handle, these were used in subsequent experiments. Results with both sets of primers were parallel, therefore, only data obtained with primers KU-27/KU-15 will be presented.

To assess if CLBV detection by RT-PCR was affected by host variety, plants of Nagami kumquat, Washington navel, Navelina and Pineapple sweet oranges, Eureka lemon, Marsh grapefruit, Owari satsuma and Nules Clementine were graft

TABLE 1
NUCLEOTIDE SEQUENCE OF PRIMERS USED FOR CITRUS LEAF BLOTCH VIRUS (CLBV) DETECTION

Primer	Nucleotide sequence (5' to 3'	Genomic location		Predicted PCR product size
		nt position	ORF	
KU 27	GATGCAAGCCAGGATGAATAC	5321-5350	RNA polymerase	456 bp
KU 15	CAGACACTCCAAGACCTTTCC	5776-5756	Polymerase	
KU 18	TTAAGATTACAGACACGAAGG	7686-7706	Coat protein	438 bp
KU 19	CTGTTTTTGAATTTTGCTCG	8123-8104	Coat protein	

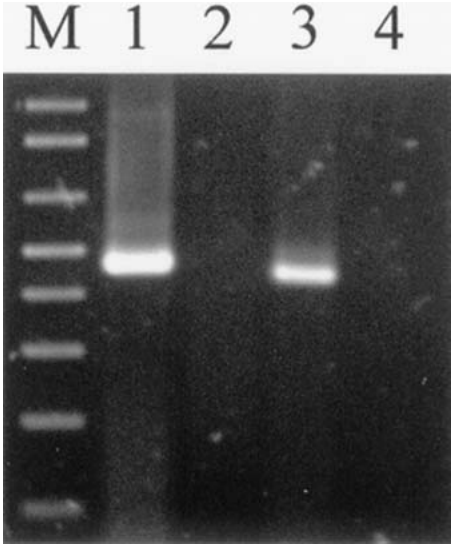

Fig. 1. Detection of Citrus leaf blotch virus (CLBV) by RT-PCR analysis of total RNA extracts from young leaves of kumquat SRA-153 (lanes 1 and 3) or healthy kumquat (lanes 2 and 4), using primers KU-27 and KU-15 (lanes 1 and 2), or KU-18 and KU-19 (lanes 3 and 4). Lane M, 1-Kb plus DNA ladder.

inoculated with the CLBV isolate SRA-153 and six young and six old leaves from each plant were analyzed. The original Nagami kumquat SRA-153 was used as control. CLBV was detected in all citrus cultivars, but differences were observed in the number of leaves yielding positive amplification and in the intensity of the PCR-amplified DNA band. Nagami kumquat, Navelina and Washington navel orange and Owari satsuma mandarin yielded DNA bands of similar intensity from all young and old leaves assayed. CLBV was also readily detected in all young leaves of Eureka lemon, Marsh grapefruit and Nules Clementine, but only some old leaves yielded amplification and the DNA bands obtained from them were usually less intense than those amplified from young leaves. Finally, CLBV was detected in only one out of six young leaves and in none of the old leaves of Pineapple sweet orange (Fig. 2 and data not shown).

Detection of CLBV in field trees. In order to optimize CLBV detection by RT-PCR in field trees, six old leaves and when available six young leaves from two infected Navelina orange plants grafted on Carrizo citrange, were analyzed in winter, spring and summer. Detection was less consistent in field trees than in greenhouse-grown plants, and the best results were obtained in spring and summer. In these seasons, one to four young leaves per plant yielded amplification with a band of variable intensity (Fig. 3). At any season, amplification from old leaves was erratic and bands were usually weaker than those obtained from young leaves. Sometimes, CLBV could not be detected in any of the six old leaves analyzed from a tree.

To assess reliability of the procedure for routine detection, 67 trees were analyzed from an orchard of Navelina orange grafted on Carrizo citrange that had some trees with bud union crease. Total RNA from a pool of six young leaves from each tree was analyzed by RT-PCR. CLBV was detected in 14 trees. All CLBV-positive trees showed severe symptoms of bud union crease and none of the trees with normal bud union yielded an amplification with CLBV-specific primers, however, CLBV could not be detected in some symptomatic trees. In a further assay, 10 trees with bud union crease, which had not yielded PCR amplification in the first experiment, were selected and six young leaves from each tree were individually analyzed. CLBV was then detected in one to four individual leaves from six of these trees.

Detection of CLBV in citrus from different geographic areas. Citrus tissue from several citrus growing areas in Australia, Japan and USA was also tested for CLBV infection (Table 2). CLBV was detected in two satsumas from Japan, a Nagami kumquat from New South Wales (Australia) and three Roble sweet orange grafted on

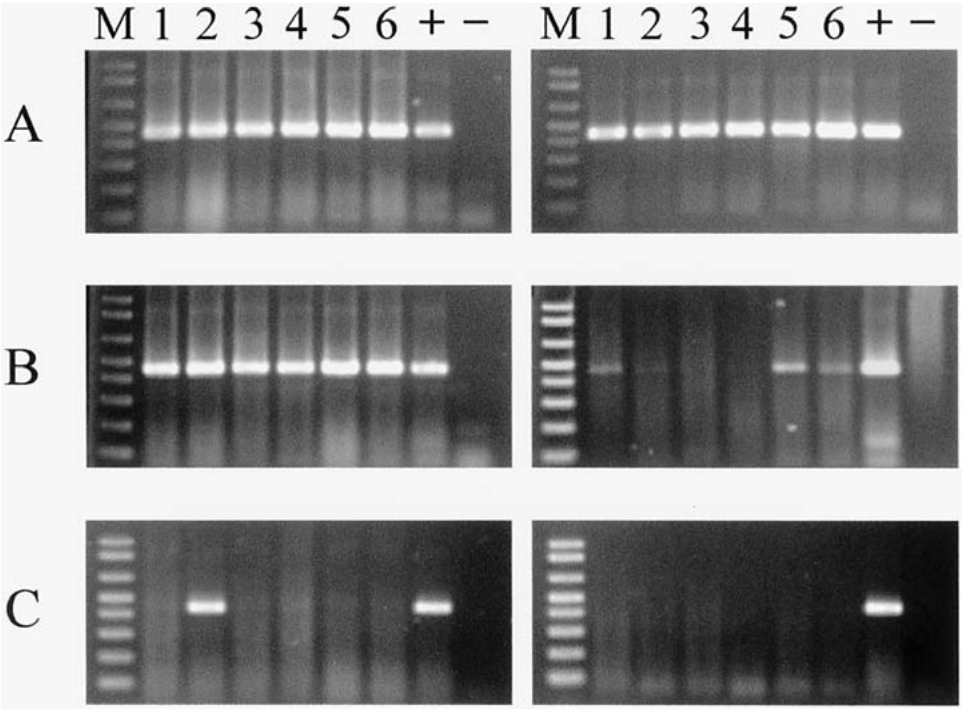

Fig. 2. RT-PCR detection of Citrus leaf blotch virus (CLBV) in six young (left panels) and old (right panels) leaves from Nagami kumquat (A), Marsh grapefruit (B) and Pineapple sweet orange (C) plants inoculated with kumquat SRA-153. Lane +, kumquat SRA-153; lane -, healthy kumquat; lane M, 1-Kb plus DNA ladder.

Swingle citrumelo from two different commercial orchards at Haines City and Lake Wales, Florida (USA). The samples of the two latter origins were from trees showing bud union crease on trifoliate rootstocks. However, CLBV was not detected in some trees from the same orchards in Florida, which also showed bud union crease symptoms, nor in different origins of Navel orange and Moro sweet orange from Florida and California (USA) also showing bud union crease (Table 2).

DISCUSSION

Our results show that CLBV can be specifically detected by one-step RT-PCR in different citrus species and varieties, grown under greenhouse conditions or in the field. However, analysis of different leaves from the same tree revealed that in some varieties CLBV is unevenly distributed within the plant, and this may be a drawback for virus diagnostics. While CLBV was consistently detected in young and old leaves of infected Nagami kumquat, Owari satsuma, Navelina and Navel orange grown in the greenhouse, detection in old leaves of other citrus species (Eureka lemon, Marsh grapefruit and Nules Clementine) was not consistent and did not allow a trustworthy result. Particularly difficult was CLBV detection in Pineapple sweet orange, in which only one out of six young leaves and none of the six old leaves gave amplification. These data indicate that reliable detection of CLBV by RT-PCR requires the use of young leaves as RNA source, and that in some varieties like Pineapple sweet orange, at least 10 individual young leaves per tree should be analyzed to reduce the risk of false negatives. These results agree with previous

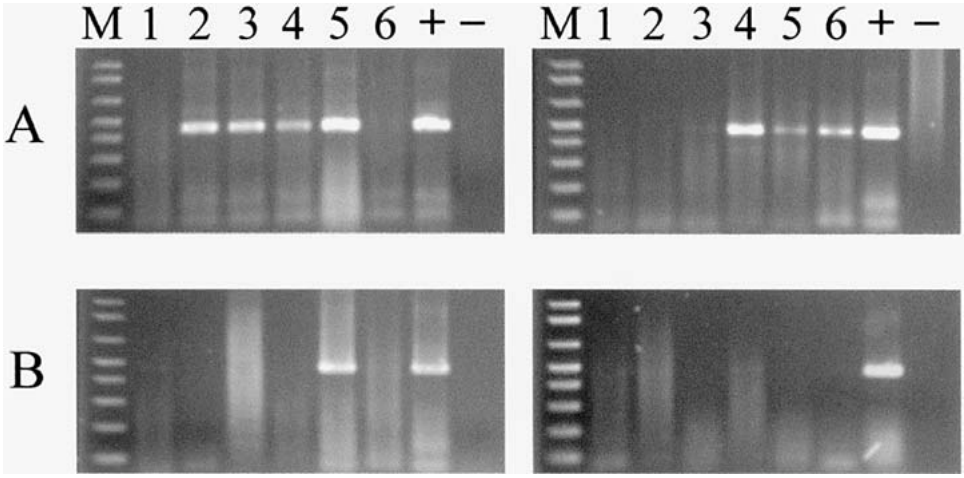

Fig. 3. RT-PCR detection of Citrus leaf blotch virus (CLBV) in six young (left panels) and old (right panels) leaves collected in spring from two field trees of Navelina orange (A and B). Lane +, kumquat SRA-153; lane -, healthy kumquat; lane M, 1-Kb plus DNA ladder.

data on CLBV detection by biological indexing in which it was observed that inoculation from kumquat SRA-153 always caused intense foliar symptoms in most Dweer tangor and Etrog citron plants, whereas transmission from other citrus species was more erratic and generally caused milder symptoms (4).

Detection of CLBV in field trees was less consistent. Thus, while CLBV was readily detected in both young and old leaves of infected Navelina orange in the greenhouse, detection in field trees of the same variety was achieved in only one to four out of six young leaves and often in none of the old leaves. This suggests low virus titer and/or uneven distribution of CLBV in field trees of Navelina orange, which is one of the easiest varieties for detec-

TABLE 2
DETECTION OF CLBV IN CITRUS TREES OF DIFFERENT GEOGRAPHICAL ORIGIN

Cultivar	Location	Budunion symptom s	PCR
Roble sw. o.*/citrumelo	Haines City, Florida, USA	+	+
Roble sw. o./citrumelo	Haines City, Florida, USA	+	+
Roble sw. o./citrumelo	Haines City, Florida, USA	+	—
Roble sw. o./citrumelo	Lake Wales, Florida, USA	+	+
Navel sw. o./citrumelo	Haines City, Florida, USA	+	—
Navel sw. o./citrumelo	Haines City, Florida, USA	+	—
Navel sw. o./citrange	California, USA	+	—
Navel sw. o./citrange	California, USA	+	—
Navel sw. o./citrange	California, USA	+	—
Navel sw. o./citrange	California, USA	+	—
Moro Blood sw. o./citrange	California, USA	+	—
Nagami kumquat/citrange	New South Wales, Australia	+	+
Satsuma mandarin	Japan	?	+
Satsuma mandarin	Japan	?	+

*sw. o.: sweet orange

tion in the greenhouse; however, detection in field trees of other citrus varieties should be tested.

CLBV detection by RT-PCR is fast and cheap and should be used in addition to biological indexing for routine testing in sanitation, quarantine and certification programs. In addition, the technique is very useful for epidemiological studies and the virus could be detected in desiccated tissues from other countries, which opens possibilities for international cooperation in CLBV detection without the risk of spreading this pathogen in areas where it is not yet present.

CLBV was detected in two Satsuma mandarins from Japan, a Nagami kumquat from Australia, and several sweet orange trees showing bud union crease on citrange or citrumelo from commercial citrus orchards in Spain and Florida, which indicates that CLBV is present in citrus varieties other than Nagami kumquat in several geographic areas. However, the virus could not be detected in neighbor trees showing similar symptoms. Failure to detect CLBV in some trees with bud union crease could be due to low titer or uneven distribution of the virus within the plant. Alternatively, the symptom could be caused by a different pathogen or by an interaction between genetic and other biotic or abiotic factors. Indeed, high incidence of bud union crease has been observed in Roble sweet orange trees propagated on Swingle citrumelo and other trifoliate rootstocks in Florida, irrespective of whether propagated buds were from old lines or from shoot-tip-grafted or seedling sources (6).

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