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Development of a full-genome cDNA clone of *Citrus leaf blotch virus* and infection of citrus plants

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SUMMARY

Citrus leaf blotch virus (CLBV), a member of the family Flexiviridae, has a ~9-kb single-stranded, positive-sense genomic RNA encapsidated by a 41-kDa coat protein. CLBV isolates are associated with symptom production in citrus including leaf blotching of Dweet tangor and stem pitting in Etrog citron (Dweet mottle disease), and some isolates are associated with bud union crease on trifoliate rootstocks, but Koch's postulates for this virus were not fulfilled. A full-genome cDNA of CLBV isolate SRA-153, which induces bud union crease, was placed under the T7 promoter (clone T7-CLBV), or between the 35S promoter and the *Nos-t* terminator, with or without a ribozyme sequence downstream of the CLBV sequence (clones 35SRbz-CLBV and 35S-CLBV). RNA transcripts from T7-CLBV failed to infect Etrog citron and *Nicotiana occidentalis* and *N. benthamiana* plants, whereas agro-inoculation with binary vectors carrying 35SRbz-CLBV or 35S-CLBV, and the *p19* silencing suppressor, caused systemic infection and production of normal CLBV virions. Virus accumulation was similar in citron plants directly agro-infiltrated, or mechanically inoculated with wild-type or 35SRbz-CLBV-derived virions from *Nicotiana*, and the three sources incited the symptoms characteristic of Dweet mottle disease, but not bud union crease. Our results show that (1) virions derived from an infectious clone show the same replication, movement and pathogenicity characteristics as the wild-type CLBV; (2) CLBV is the causal agent of Dweet mottle disease but not of the bud union crease syndrome; and (3) for the first time an RNA virus could be successfully agro-inoculated on citrus plants. This infectious clone may become a useful viral vector for citrus genomic studies.

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INTRODUCTION

Citrus leaf blotch virus (CLBV), a member of the putative genus *Citrivirus*, family Flexiviridae (Adams *et al.*, 2005; Martelli *et al.*, 2007), has filamentous virions about 960 × 14 nm in size composed of a single-stranded, positive-sense, genomic (g) RNA of 8747 nt and a 41-kDa coat protein (CP) (Galipienso *et al.*, 2001; Vives *et al.*, 2001). CLBV dispersal occurs primarily by propagation of infected buds, but seed transmission at low rates has been detected in at least three citrus species or hybrids (Guerri *et al.*, 2004). The CLBV gRNA has three open reading frames (ORFs) and untranslated regions (UTR) of 73 and 541 nt at its 5′ and 3′ termini, respectively. ORF1 potentially encodes a ~227-kDa polyprotein containing methyl-transferase, AlkB-like, Out-like peptidase, papain-like protease, helicase and RNA-dependent RNA polymerase motifs; ORF2 encodes a ~40-kDa polypeptide with a motif characteristic of cell-to-cell movement proteins (MP) of the 30k superfamily; and the ~41-kDa polypeptide encoded by ORF3 was identified as the CP (Martelli *et al.*, 2007; Vives *et al.*, 2001). In addition to the gRNA, CLBV-infected tissues contain two 3′-co-terminal and two 5′-co-terminal subgenomic RNAs (sgRNAs) produced during virus replication (Vives *et al.*, 2002a).

CLBV was first detected and characterized in a Nagami kumquat (*Fortunella margarita* (Lour.) Swing.), isolate SRA-153, showing bud union crease on Troyer citrange (*Citrus sinensis* (L.) Osb. × *P. trifoliata* (L.) Raf.) rootstock (Galipienso *et al.*, 2001; Navarro *et al.*, 1984). This isolate also induced vein clearing in Pineapple sweet orange (*Citrus sinensis* (L.) Osb.), chlorotic blotching in Dweet tangor (*C. tangerina* (Hort. ex Tan.) × *C. sinensis*) and stem pitting in Etrog citron (*C. medica* L.). However, while bark inoculum from plants of Nules clementine (*C. clementina* Hort. ex Tan.) or Eureka lemon (*C. limon* (L.) Burn. f.) graft-inoculated with SRA-153 induced the same symptoms as kumquat SRA-153, similar inoculum from Marsh grapefruit (*C. paradisi* Macf.) or Pineapple sweet orange (*Citrus sinensis* (L.) Osb.) also inoculated with SRA-153 induced chlorotic blotching in Dweet tangor and stem pitting in Etrog citron, but not vein clearing in Pineapple sweet orange or bud union crease on Troyer

citrange (Galipienso *et al.*, 2000). Similarly, kumquats 38-1 and 497-2, recovered after shoot-tip grafting *in vitro* (Navarro *et al.*, 1975) in kumquat SRA-153, induced chlorotic blotching in Dweet tangor and stem pitting in Etrog citron, but not vein clearing in Pineapple sweet orange or bud union crease on citrange (Navarro *et al.*, 1984). CLBV was later detected in two sources of Dweet mottle, a disease with symptoms identical to those incited by kumquats 38-1 and 497-2 (Roistacher and Blue, 1968; Vives *et al.*, 2005). These findings suggest the presence of more than one agent in kumquat SRA-153 and the elimination of some of them after shoot-tip grafting *in vitro* or after passage through Marsh grapefruit or Pineapple sweet orange (Galipienso *et al.*, 2000, 2001; Navarro *et al.*, 1984). Moreover, CLBV was detected in field trees from different geographical areas showing bud union crease on trifoliate rootstocks, but not in other trees with similar symptoms (Galipienso *et al.*, 2004; Vives *et al.*, 2002b). Ascertaining the role of CLBV in bud union crease syndrome is important because trifoliate rootstocks are widely used in areas affected by *Citrus tristeza virus* (CTV) (Moreno *et al.*, 2008), but so far the Koch's postulates for this disorder or for Dweet mottle disease have not been fulfilled due to low concentration of CLBV in citrus (Galipienso *et al.*, 2001).

We built full-genome cDNA clones of the CLBV gRNA that caused systemic infection in agro-infiltrated herbaceous and citrus host plants. Virions recovered from agro-inoculated plants (CLBV-IC) resembled those of the wild-type isolate (CLBV-wt). CLBV-IC induced chlorotic blotching in Dweet tangor and stem pitting in Etrog citron, but not vein clearing in Pineapple sweet orange or bud union crease on trifoliate rootstocks, indicating that CLBV is the causal agent of Dweet mottle disease, and that bud union crease and sweet orange symptoms must be caused by a different agent or result from an interaction between CLBV and this hypothetical agent.

RESULTS AND DISCUSSION

Full-genome cDNA constructs

Inoculation of citrus with an RNA virus (*Citrus tristeza virus*, CTV) using a full-genome cDNA clone was a difficult challenge that required using an indirect procedure (Gowda *et al.*, 2005; Satyanarayana *et al.*, 1999, 2001). Attempts to infect citrus plants directly by biolistic or *Agrobacterium*-mediated inoculation with cDNA clones of CTV were unsuccessful (Gowda *et al.*, 2005 and our unpublished data), whereas inoculation of *Nicotiana benthamiana* protoplasts with RNA transcripts yielded CTV virions that, upon mechanical inoculation to citrus plants, were infectious and reproduced symptoms of the wild-type isolate (Satyanarayana *et al.*, 1999, 2001). Therefore, to develop an infectious clone of CLBV, we prepared cDNA constructs for both production of RNA transcripts and agro-inoculation.

Three overlapping cDNA fragments encompassing the full genome of the CLBV isolate SRA-153 were obtained by reverse transcription (RT) and PCR amplification with appropriate CLBV-specific primers (Table 1) using total RNA extracts from kumquat infected with this isolate (Vives *et al.*, 2001). Those fragments were used to build three different full-genome constructs: in one of them (clone 35SRbz-CLBV), the three DNA fragments were successively cloned in the plasmid vector pUC35S-8.1, so that the CLBV genome, followed by a ribozyme, was located between the 35S promoter of the *Caulifower mosaic virus* (CaMV) and the nopaline synthase terminator (*Nos-t*); in a second construct (clone 35S-CLBV), the CLBV genome was placed between the 35S promoter and the *Nos-t* in plasmid pUC35S-8.2, a modified version of pUC35S-8.1 with the ribozyme sequence deleted; in the third construct (clone T7-CLBV), the CLBV genome was under the T7 RNA polymerase promoter (Fig. 1). Clone T7-CLBV was used to

Table 1 Primers used to reverse-transcribe and PCR-amplify different fragments of the CLBV genomic RNA and to modify plasmid vectors.

Fragment/modification	Primer	Sequence 5′–3′*	Position (nt)†
f5′	KU52	GAAAAGCAACGAAAGCAACCTACACAACCC	1–30
	KU58	CCCCTTTTCCAAGAACCACACATTTTCCA	3224–3196
fC	KU59	GGTAACGGTGGCTCCATTGAAGATTG	3122–3147
	KU60	AATTCAAATTCATCAATAACAGCTGTTTCG	6341–6312
f3′	KU61	GAAATGAACAGATCTGGGGTAGATGCGCAC	6257–6286
	KU62	$(T)_{25}$GTCTAAAAGTTCTTAAAAGACATCC	8747–8723
	KU66	TTTCT**GGATCC**$(T)_{25}$GTCTAAAAG	8747–8739
Promoter substitution	KU63	TTCATAAGCT*TAATACGACTCACTATA*GAAAAGCAACGAAAGCAACC	1–20
	KU64	CTCTTTATTTTCCAAGCTTTTGATGAAATTCC	613–582
Rbz deletion	V5	TCGAGTCTT**CCCGGG**AGCT	NA
	V6	**CCCGGG**AAGAC	
Rbz and *Nos-t* deletion	V7	TCGAGTCTT**GGATCC**GC	NA
	V8	GGCCGC**GGATCC**AAGAC	

*Sequences in bold represent introduced restriction sites. The sequence of the T7 promoter is indicated in italics.
†Nucleotide positions are indicated on he sequence of the CLBV isolate SRA-153 (EMBL accession number AJ318061).
NA, not applicable.

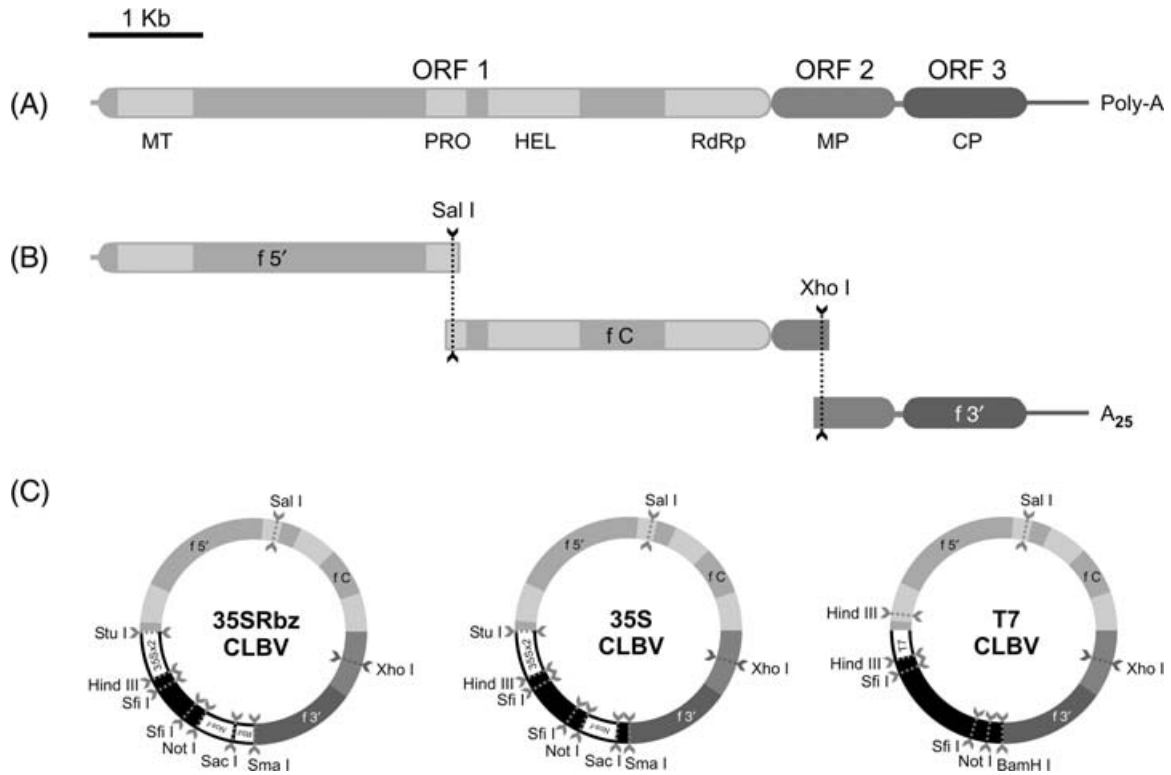

Fig. 1 (A) An outline of the CLBV genome, with shaded boxes representing the predicted open reading frames (ORFs). Proteins potentially encoded by ORFs 2 and 3, and functional domains in ORF 1 are indicated below the boxes (MT, methyl-transferase; PRO, protease, HEL, helicase; RdRp, RNA-dependent RNA polymerase; MP, movement protein; CP, coat protein). (B) Fragments reverse transcribed and PCR amplified from the CLBV genomic RNA, and restriction sites used to assemble full-genome cDNA clones. (C) Schematic representation of the three constructs prepared with a full-genome cDNA of the CLBV gRNA: clones 35SRbz-CLBV and 35S-CLBV, with the CLBV genome under the control of the double enhanced 35S promoter (35Sx2) of the *Cauliflower mosaic virus* and the nopaline synthase terminator (*Nos-t*), and clone T7-CLBV with the CLBV genome under the control of the T7 RNA polymerase promoter. In clone 35SRbz-CLBV, the antigenomic sequence of the *Hepatitis delta virus* ribozyme (Rbz) was juxtaposed to the CLBV genome (see details in the text).

obtain RNA transcripts for inoculation of plants and protoplasts, whereas clones 35SRbz-CLBV and 35S-CLBV were subcloned in the binary vector pBIN19-SfiI L3.7AfI II to yield clones pBIN35SRbz-CLBV and pBIN35S-CLBV, which were used for plant agro-inoculation.

Inoculation with CLBV RNA transcripts

CLBV-wt can be mechanically transmitted to *N. occidentalis* and *N. benthamiana* plants by leaf rubbing, causing a symptomless infection, and to citrus plants by stem-slash inoculation (Vives *et al*., 2008). Therefore, we first attempted to infect *N. occidentalis* and Etrog citron plants with CLBV-IC by mechanical inoculation of capped and uncapped *in vitro* RNA transcripts freshly prepared from T7-CLBV clones. Although UV absorption and electrophoresis analyses indicated that the quality and quantity of RNA transcripts were apparently appropriate (data not shown), none of the inoculated plants was infected, as deduced from the lack of RT-PCR amplification using CLBV-specific primers. Contrastingly, more than 50% of the control *N. occidentalis* plants rubbed, and about 100% of the Etrog citrons stem-slashed, with CLBV-wt virion extracts yielded an amplification band of the expected size with the same primers, indicating that they were CLBV infected. Previous attempts to inoculate citrus plants mechanically with CTV RNA transcripts, or with RNA purified from infectious virion preparations, using different additives and inoculation procedures, were also unsuccessful (Satyanarayana *et al*., 2001). However, *N. benthamiana* leaf protoplasts inoculated with similar transcripts from the same infectious cDNA clone supported CTV replication and yielded normal progeny virions (Satyanarayana *et al*., 1999, 2001).

To determine if RNA transcribed from T7-CLBV was able to replicate, mesophyll protoplasts from *N. occidentalis*, *N. benthamiana* and Etrog citron leaves were inoculated with similar capped and uncapped RNA transcripts. In some experiments, small amounts of viral gRNA and sgRNAs were detected in *N. benthamiana* protoplasts inoculated with capped transcripts by Northern blot analysis with CLBV-specific digoxigenin-labelled (DIG) riboprobes,

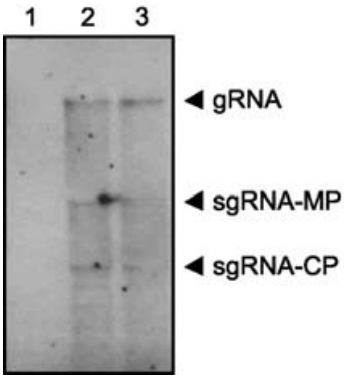

Fig. 2 Northern blot analysis of total RNA extracts prepared at 5 dpi from *N. benthamiana* protoplasts transfected with virion extracts from a healthy (lane 1) or a CLBV-infected (lane 2) kumquat plant, or with *in vitro* synthesized RNA transcripts from the T7-CLBV cDNA clone (lane 3), carrying the CLBV genomic sequence under the control of the T7 promoter of the RNA polymerase. The membrane was hybridized with a DIG-riboprobe specific for the CLBV coat protein (Vives *et al.*, 2002a). Arrowheads indicate positions of the CLBV genomic (g) and subgenomic (sg) RNAs of the movement (MP) and coat (CP) proteins.

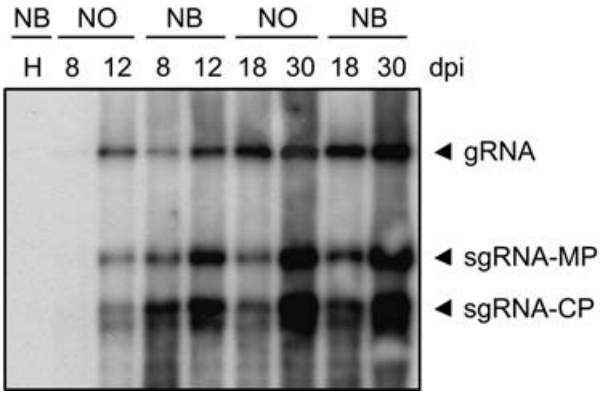

Fig. 3 Northern blot analysis of total RNA extracts from *N. benthamiana* (NB) or *N. occidentalis* (NO) plants agro-inoculated with the infectious cDNA clone pBIN35SRbz-CLBV. RNA extracts were prepared from leaves agro-infiltrated (8 and 12 dpi) or systemically infected (18 and 30 dpi). The membrane was hybridized as in Fig. 2. Arrowheads indicate positions of the CLBV genomic (g) and subgenomic (sg) RNAs of the movement (MP) and coat (CP) proteins. H, RNA extract from a healthy plant.

indicating CLBV replication, but viral RNAs were detected only at 5 days post-inoculation (dpi) (Fig. 2), a period close to the protoplast survival limit. Inefficient inoculation of protoplasts with RNA transcripts and slow replication of CLBV may be behind the weak and erratic detection observed. Tests with a green fluorescent protein (GFP)-labelled CTV transcript showed that only 0.01% of transcript-inoculated *N. benthamiana* protoplasts were infected; however, detection of CTV RNAs was consistently achieved at 3–4 dpi (Satyanarayana *et al.*, 2001). Overall, these results suggest that the T7-CLBV construct is indeed infectious, but CLBV RNA is not an efficient inoculum to infect plants or protoplasts.

Inoculation with transfected *A. tumefaciens* cells

Agrobacterium-based binary vectors have been used for efficient delivery of viral genomes into plant cells (Chiba *et al.*, 2006; English *et al.*, 1997; Stephan and Maiss, 2006). To determine if 35S-CLBV and 35SRbz-CLBV cDNA clones could be properly transcribed in plants and initiate infection, *Agrobacterium tumefaciens* cultures carrying the recombinant binary vectors pBIN35S-CLBV or pBIN35SRbz-CLBV were infiltrated in leaves of four *N. occidentalis* and five *N. benthamiana* plants. Total RNA from agro-infiltrated leaves was analysed at 2, 4, 6, 8 and 12 dpi by Northern blot using CLBV-specific DIG-riboprobes. A weak hybridization signal for CLBV gRNA and sgRNAs was observed in one of the *N. benthamiana* plants infiltrated with pBIN35SRbz-CLBV at 12 dpi (data not shown), suggesting inefficient infection and/or replication probably due to a plant antiviral response. To overcome this problem, *N. benthamiana* and *N. occidentalis* leaves were co-infiltrated with *A. tumefaciens* cultures transfected with the recombinant binary vectors pBIN35S-CLBV (ten plants) or pBIN35SRbz-CLBV (ten plants), and with the vector pBIN35S-p19 expressing a silencing suppressor protein (Voinnet *et al.*, 2003). Northern blot analyses of agro-infiltrated leaves revealed earlier (8 dpi) and higher accumulation of CLBV-specific gRNA and sgRNAs in all inoculated plants, indicating that both pBIN35S-CLBV and pBIN35SRbz-CLBV cDNA clones can be properly transcribed and initiate infection in *N. benthamiana* and *N. occidentalis* cells, although *N. benthamiana* accumulated more CLBV RNA than *N. occidentalis* at this early stage (Fig. 3).

Analyses of new leaves at 15, 18, 20, 25 and 30 dpi by RT-PCR and Northern blot with CLBV-specific primers and a DIG-riboprobe, respectively, showed systemic infection in all agro-infiltrated plants. CLBV-IC was detected at 18 dpi in six *N. benthamiana* and in seven *N. occidentalis* plants out of the ten inoculated with pBIN35SRbz-CLBV, and at 20 dpi in the remaining plants, whereas in plants inoculated with pBIN35S-CLBV first detection of CLBV-IC occurred at 20 dpi in five *N. benthamiana* and four *N. occidentalis*, and at 25 dpi in the remaining plants. Virus accumulation in new leaves of *N. benthamiana* and *N. occidentalis* plants inoculated with the same construct was similar, as deduced from the intensity of the hybridization signal in Northern blots (Fig. 3), with this signal being slightly more intense in plants inoculated with pBIN35SRbz-CLBV than in those inoculated with pBIN35S-CLBV in the first detection. However, this early difference was not observed in new flush produced after the plants were pruned (data not shown). Electron microscope observation of crude extracts from systemically infected leaves showed that CLBV-IC and CLBV-wt virions were indistinguishable (Fig. 4). These results

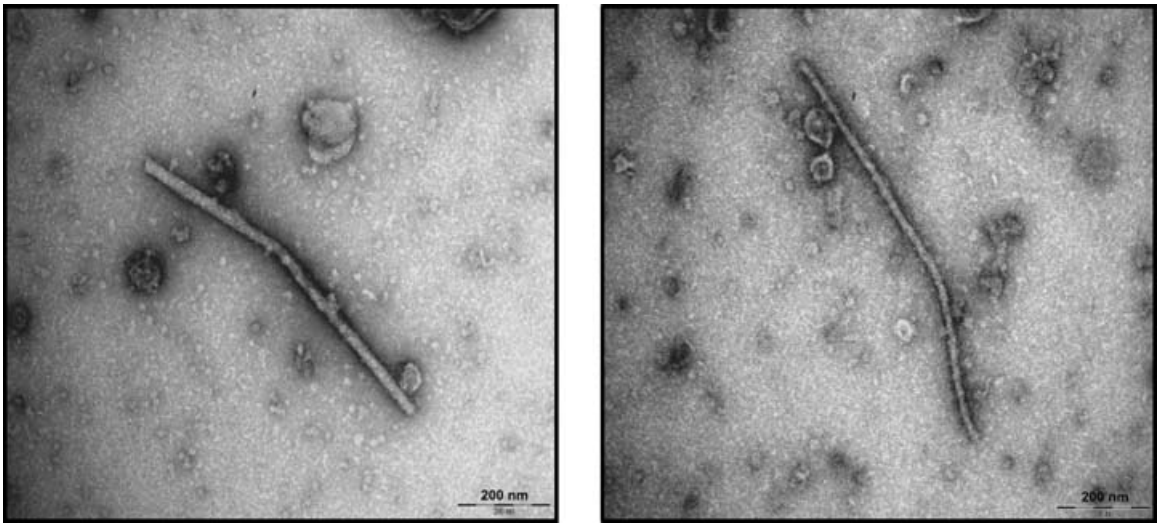

Fig. 4 CLBV virions observed in crude extracts from systemically infected *N. benthamiana* leaves agro-infiltrated with the pBIN35SRbz-CLBV cDNA clone (upper panel) (see Materials and methods), or from citrus infected with the wild-type virus (CLBV-wt) (lower panel). Preparations were negatively stained with 1% uranyl acetate and observed at 80 kV.

indicated that agro-inoculation with binary vectors pBIN35S-CLBV or pBIN35SRbz-CLBV causes infection in cells of the two *Nicotiana* species and that the CLBV-IC virions produced have a normal aspect and move systemically in both hosts. Faster movement and earlier accumulation of CLBV in plants agro-infiltrated with pBIN35SRbz-CLBV than in those agro-infiltrated with pBIN35S-CLBV might result from some replication advantage of transcripts derived from the first construct due to precise processing of their 3′ terminus by the ribozyme. This initial advantage apparently disappeared after systemic movement of CLBV virions, suggesting that virions derived from both constructs are equally infectious. As infection was detected earlier (at 18–20 dpi) and viral accumulation was faster in plants agro-inoculated with pBIN35SRbz-CLBV than in those inoculated with pBIN35S-CLBV (at 20–25 dpi), the first construct was used in subsequent experiments.

Mechanical inoculation to citrus plants and biological indexing

To determine if CLBV-IC virions generated in systemically infected *N. benthamiana* or *N. occidentalis* plants were infectious in citrus, six plants of each Etrog citron and *C. excelsa* Wester were slash inoculated with partially purified virion extracts (Galipienso *et al.*, 2000; Vives *et al.*, 2008) and then pruned at 15 dpi to induce new flush. An identical set of plants was inoculated with a similar virion preparation obtained from kumquat SRA-153 as control. All plants inoculated with either CLBV-IC or CLBV-wt virions became systemically infected, as detected by RT-PCR with RNA extracts from the first flush (40 dpi). The amount of viral RNAs detected by Northern blot analysis was similar in plants

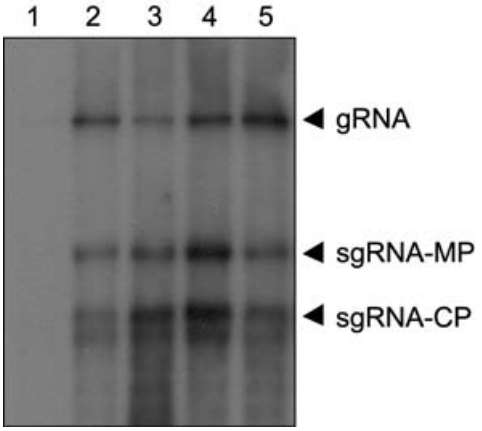

Fig. 5 Northern blot analysis of total RNA from leaf (lanes 2 and 3) or bark (lanes 1, 4 and 5) extracts from Etrog citron plants healthy (lane 1) or mechanically inoculated with CLBV (lanes 2–5). Inoculation was performed with semipurified virion extracts from the wild CLBV isolate SRA-153 (lanes 2 and 4) or from *N. benthamiana* plants agro-inoculated with the infectious cDNA clone pBIN35SRbz-CLBV (lanes 3 and 5). The membrane was hybridized as in Fig. 2. Arrowheads indicate positions of the CLBV genomic (g) and subgenomic (sg) RNAs of the movement (MP) and coat (CP) proteins.

infected with either virion source (Fig. 5), indicating that the CLBV-IC virions also replicate and move in citrus plants as the wild-type virions. Etrog citron plants infected with CLBV-IC virions showed stem pitting symptoms indistinguishable from those incited by CLBV-wt, whereas *C. excelsa* plants remained symptomless as expected (Galipienso *et al.*, 2000). For further biological characterization six plants of each Pineapple sweet orange, Dweet tangor, Etrog citron and Nules clementine were

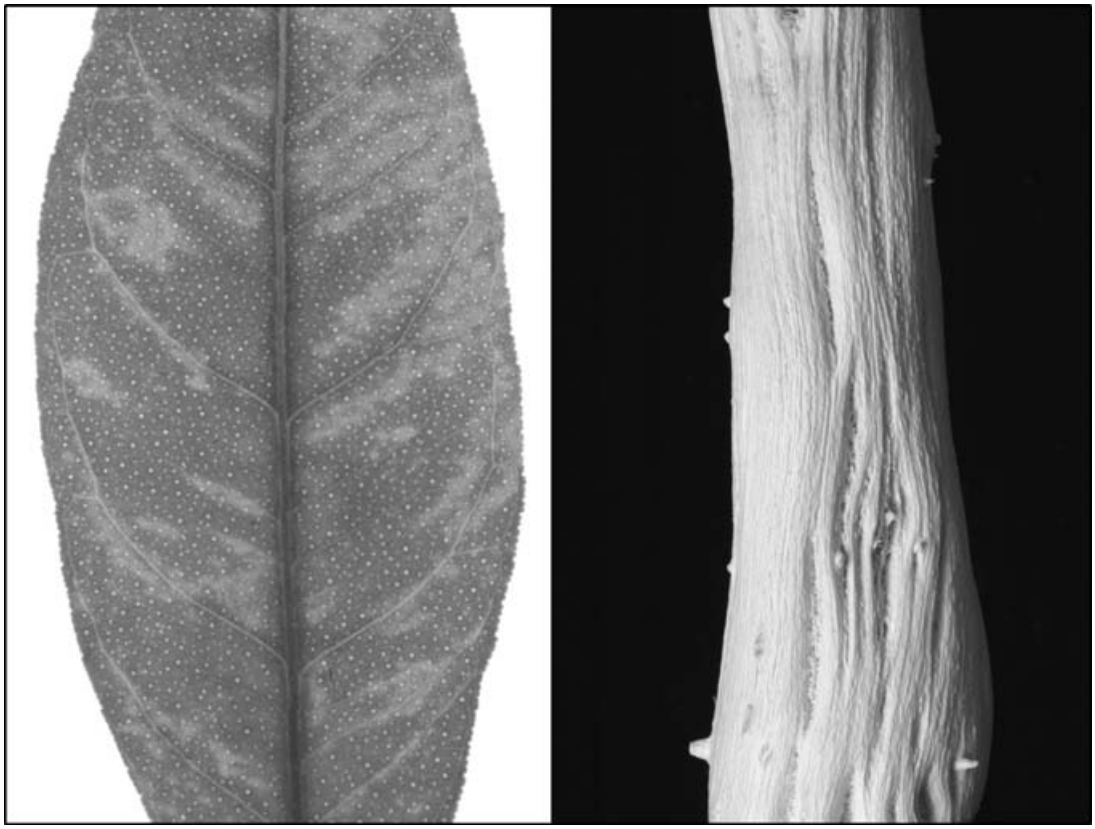

Fig. 6 Stem pitting in Etrog citron (right) and leaf blotching in a Dweet tangor leaf (left) induced by CLBV-IC. *N. benthamiana* plants were agro-inoculated with the infectious cDNA clone pBIN35SRbz-CLBV and virions purified from systemically infected leaves were mechanically inoculated to citron plants. Infected citron bark was used to graft inoculate Dweet tangor plants.

graft inoculated with bark patches from citron plants infected with CLBV-IC, with CLBV-wt (SRA-153 isolate) or healthy. Two months after inoculation, clementine buds from the three sources were propagated on Carrizo citrange seedlings to examine effects on the bud union. The CLBV-wt source induced intense vein clearing in Pineapple sweet orange, chlorotic blotching in Dweet tangor, stem pitting in Etrog citron and bud union crease in Nules clementine grafted on Carrizo citrange rootstock (Galipienso *et al.*, 2000), whereas CLBV-IC only incited chlorotic blotching in Dweet tangor and stem pitting in Etrog citron plants (Fig. 6). Control plants inoculated with healthy kumquat showed no symptoms. These findings indicate that CLBV is the causal agent of Dweet mottle disease (Roistacher and Blue, 1968; Vives *et al.*, 2005), characterized by leaf blotching in Dweet tangor and stem pitting in Etrog citron, and that bud union crease and vein clearing in Pineapple sweet orange must be caused by a different pathogen or by an interaction between CLBV and another agent, thus supporting previous suggestions based on biological indexing of different CLBV isolates (Galipienso *et al.*, 2000, 2001).

Agro-inoculation of citrus plants

To examine if direct agro-inoculation of citrus was feasible, young fully expanded leaves of five plants of each Etrog citron, rough lemon (*C. jambhiri* Lush) and alemow (*C. macrophylla* Wester), were co-infiltrated with *A. tumefaciens* cultures transfected with the binary vectors pBIN35SRbz-CLBV and pBIN35S-p19. This inoculation system did not allow pruning the plants after inoculation and analyses for CLBV detection were performed in agro-infiltrated leaves (at 7, 10 and 15 dpi) or in the following flush (about 60 dpi). While RT-PCR amplification failed to detect CLBV-IC in agro-infiltrated leaves of the three citrus species, or in the new shoots of rough lemon or alemow, systemic infection was detected in the five Etrog citron plants (Fig. 7). These plants showed stem pitting symptoms similar to those observed in citron plants inoculated with CBLV-wt or with CLBV-IC from agro-infiltrated *Nicotiana* plants (Fig. 6), as were their Northern blot hybridization patterns with a CLBV-specific DIG-probe (Fig. 5), further confirming CLBV infection. In subsequent agro-infiltration experiments with pBIN35SRbz-CLBV and pBIN35S-p19 vectors,

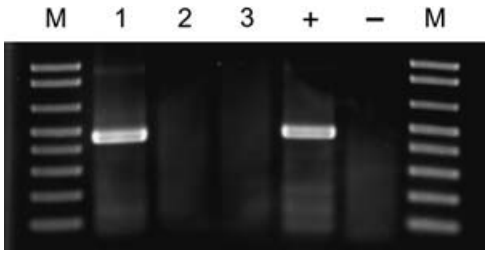

Fig. 7 Detection of CLBV in new shoots of Etrog citron (lane 1), rough lemon (lane 2) and alemow (lane 3) plants agro-inoculated with the infectious cDNA clone pBIN35SRbz-CLBV by RT-PCR amplification with CLBV-specific primers KU27 and KU15 (Vives *et al.*, 2002b, 2008). Healthy citron plants (–) or graft-inoculated with the CLBV isolate SRA-153 (+) were used as control. M, molecular size marker (1-kb plus DNA ladder).

about 100% of the Etrog citron plants were also systemically infected. Again, CLBV-IC and CLBV-wt accumulated at similar level and incited the same symptoms in graft-inoculated Etrog citron and Dweet tangor indicator plants (Fig. 6), indicating that direct agro-infiltration of Etrog citron plants with the pBIN35SRbz-CLBV construct mimics infection with CLBV-wt.

Successful delivery of plant viruses, including phloem-restricted viruses, through agro-infiltration of plants has been achieved for many years (Hanley-Bowdoin *et al.*, 1988; Leiser *et al.*, 1992; Prufer *et al.*, 1995; Rochester *et al.*, 1990), but direct agro-infection of woody plants has not yet been reported. Trials to agro-inoculate alemow or Mexican lime (*C. aurantifolia* (Christ.) Swing.) plants with *A. tumefaciens* EHA105 and binary vectors carrying a cDNA clone of the CTV isolate T36 were unsuccessful, even when these constructs were co-infiltrated with others encoding a silencing suppressor protein (Gowda *et al.*, 2005 and our unpublished data). The presence of potential splicing signals in the CTV sequence (S. Ambrós, unpublished data) might reduce the number of active CTV gRNA molecules delivered to the cytoplasm, as observed with other viruses (Marillonnet *et al.*, 2005). Then, RNA silencing might further act on the large CTV gRNA, thus impairing infectivity. Drastic reduction of infectivity of another closterovirus due to RNA silencing was observed in agro-infiltrated *N. benthamiana* leaves (Chiba *et al.*, 2006). Consistent infection of citron plants after agro-infiltration with a CLBV-derived binary vector might be due to factors such as (1) the small size of the CLBV genome in comparison with CTV (about half size), (2) the use of an *Agrobacterium* strain carrying the helper plasmid pCH32 with additional virulence genes, (3) a lower number of potential splicing signals recognized by citron cells in the CLBV gRNA, (4) a less active RNA silencing machinery in citron or (5) a combination of these factors. Failure to agro-infect rough lemon and alemow plants might be related to differences between citrus species in their RNA splicing activity, as previously observed between *Nicotiana* species (Marillonnet *et al.*, 2005), or in their capacity to limit virus replication. In this regard, it is worth noting that citron has been used as experimental host due to its capacity to accumulate citrus viroids and viruses, including CLBV, to high level (Duran-Vila *et al.*, 1991 and our unpublished data).

CONCLUSIONS

We developed for the first time a full-genome cDNA clone of CLBV that, upon agro-infiltration, caused systemic infection in *N. benthamiana*, *N. occidentalis* and citrus plants and showed the same biological activity as the wild-type virus. To our knowledge, this is also the first report on virus infection of a woody plant with an RNA virus by direct agro-inoculation of a full-genome cDNA clone. Using citron as a receptor plant and perhaps the use of an *Agrobacterium* strain provided with additional virulence genes might have contributed to the high rate of infection obtained in our experiments. Failure to agro-infect other citrus species directly with CLBV constructs was easily overcome by graft inoculation with bark pieces from infected citron plants.

The infectious cDNA clone developed here was used to demonstrate the etiology of Dweet mottle, a graft-transmissible disease of citrus described 40 years ago, and to show that the syndrome of bud union crease on trifoliate rootstocks is probably caused by a different agent or results from an interaction between CLBV and a different factor. This CLBV clone will be also a helpful tool to (1) analyse sequence motifs involved in different steps of the CLBV infectious cycle in citrus, and (2) engineer a viral vector for gene expression or silencing in citrus plants. In this regard, a collection of more than 230 000 expressed sequence tags, generated from several citrus species, tissues at different developmental stages, and plants subjected to diverse biotic and abiotic stresses, is presently available (Forment *et al.*, 2005; and http://int-citrusgenomics.org/). Furthermore, a decision has been made to sequence completely a citrus genome. A viral vector would be helpful to test the actual function of different citrus genes. A CTV-based vector for citrus has recently been developed and shown to be stable (Folimonov *et al.*, 2007). However, in comparison with this, a CLBV-based vector may have the following potential advantages: (1) CLBV causes a symptomless infection in most citrus species and cultivars, and therefore phenotypic expression of silenced genes would not be masked; (2) contrasting with CTV, which is phloem limited, CLBV also replicates in parenchyma cells; (3) it accumulates in meristematic tissues, as deduced from the difficulty to recover CLBV-free plants by shoot-tip grafting *in vitro* (Navarro *et al.*, 1984), and thus it could be useful to study genes involved in leaf and fruit development; (4) its 8747-nt genome with only three ORFs might be easy to manipulate; (5) in contrast to CTV, CLBV is not transmitted by vectors and therefore it could be safely used in future field experiments; and (6) as CLBV infects *N. benthamiana* and

N. occidentalis, replication and systemic movement of new chimeric constructs could be quickly tested in these herbaceous hosts before assaying them in citrus plants.

EXPERIMENTAL PROCEDURES

Virus source and RNA extraction

The CLBV isolate SRA-153 (Navarro *et al.*, 1984) used in this work was maintained in plants of Nagami kumquat grafted on rough lemon, grown in an artificial potting mix (50% sand and 50% peat moss) in a temperature-controlled greenhouse (18/26 °C night/day), and fertilized by a standard procedure (Arregui *et al.*, 1982). Total RNA was extracted with TRIzol® reagent (Invitrogen) from 100 mg of leaf tissue, following the manufacturer's instructions for samples with high sugar content, and re-suspended in 25 μL diethyl pyrocarbonate (DEPC)-treated distilled water.

Construction of full-genome cDNA clones

The cDNA clones of CLBV were built with three overlapping cDNA fragments spanning the complete gRNA (Fig. 1A,B). These were obtained by RT-PCR amplification of total RNA extracts from plants infected with the CLBV isolate SRA-153 using appropriate primers based on the CLBV sequence (EMBL accession no. AJ318061) (Table 1). RT was performed with the ThermoScript™ RT-PCR system (Invitrogen) using oligonucleotide primers KU58, KU60 and KU62 (Table 1), to synthesize cDNA of the 5′-terminal (f5′), middle (fC) and 3′-terminal (f3′) regions, respectively. The cDNAs were PCR amplified with primer pairs KU52/KU58, KU59/KU60 and KU61/KU62 (Table 1) for the f5′, fC and f3′ fragments, respectively, using AccuPrime™ Pfx DNA polymerase (Invitrogen) and the following thermocycling conditions: one cycle of 94 °C for 2 min, 35 cycles of 94 °C for 20 s, 50 °C for 30 s and 68 °C for 5 min, and a final step at 68 °C for 5 min. The three DNA products synthesized were successively cloned in a modified pUC19 plasmid, the pUC35S-8.1 vector (S. Ambrós, unpublished data), that contains a double enhanced 35S promoter (35Sx2) of CaMV, the antigenomic sequence of the *Hepatitis delta virus* ribozyme from the vector 2.0 (kindly provided by Dr Andrew Ball, University of Alabama, Birmingham), the *Nos-t*, *Sal*I and *Xho*I restriction sites added to the multicloning site located between the 35S promoter and the ribozyme, to obtain the 35SRbz-CLBV clone (Fig. 1C). The amplified fragment f5′ was digested with *Sal*I, gel purified, and ligated into the *Stu*I and *Sal*I-digested vector to obtain pUCf5′ clones, with the first G of the CLBV gRNA juxtaposed to the transcription start site of the 35S promoter. A mix of ten pUCf5′ clones was digested with *Sal*I and *Xho*I and ligated with the PCR-amplified fC fragment previously digested with the same enzymes, to obtain clones pUCf5′C that contained the 5′-terminal and the central regions of the CLBV gRNA. Finally, the f3′ fragment digested with *Xho*I was ligated with a mix of ten pUCf5′C clones digested with *Xho*I and *Sma*I.

To obtain the 35S-CLBV clone (Fig. 1C), the pUC35S-8.1 vector was digested with *Xho*I and *Sac*I and the ribozyme sequence between these restriction sites was replaced with a minilinker *Xho*I–*Sma*I–*Sac*I, constructed with oligos V5 and V6 (Table 1). The new vector (pUC35S-8.2) was used to clone successively the three fragments of the CLBV gRNA as described above.

Finally, the clone T7-CLBV (Fig. 1C) was obtained by substituting the T7 RNA polymerase promoter of lambda phage for the 35S promoter in clone pUCf5′, and a minilinker *Xho*I–*BamH*I–*Not*I for the sequence of the ribozyme and the *Nos-t*. For the first substitution, a mix of ten pUCf5′ clones was digested with *Hind*III, and the excised fragment replaced with the PCR amplification product obtained from pUCf5′ with primers KU63 (containing the sequence of the T7 promoter followed by the first nucleotides of the CLBV gRNA) and KU64 (Table 1) after *Hind*III restriction (clones pUCT7f5′). For the second substitution, the *Xho*I–*BamH*I–*Not*I minilinker obtained with oligos V7 and V8 (Table 1) was ligated to a mix of ten pUCT7f5′ clones digested with *Xho*I and *Not*I (clones pUCT7f5′B). The fC and f3′ fragments of the CLBV gRNA were successively added to clones pUCT7f5′B as described for the 35SRbz-CLBV construct, except that the fragment f3′ was PCR amplified with primers KU61 and KU66 (Table 1) and cloned using the *Xho*I and *BamH*I restriction sites (Fig. 1C).

For agro-inoculation, the clones pBIN35SRbz-CLBV and pBIN35S-CLBV were prepared digesting the 35SRbz-CLBV and 35S-CLBV clones with *Sfi*I, and subcloning the excised fragments into the *Sfi*I-restricted binary vector pBIN19-SfiI L3.7AfI II. This construct was obtained by deletion of the *Sfi*I site (positions 3716–3728) and addition of an *Afl*II site (positions 3735–3740) into the pBIN19-SfiI L3.7 vector (S. Ambrós, unpublished data), a mutated version of the pBIN19-sGFP (Chiu *et al.*, 1996) in which an extra *Sfi*I site was previously included in the polylinker region.

In vitro transcription and inoculation with RNA transcripts

Eight clones of the T7-CLBV construct were linearized at the unique *BamH*I site located downstream of the CLBV cDNA and then transcribed *in vitro* using the MEGAscript™ T7 high-yield transcription kit (Ambion), with or without addition of ARCA-Anti Reverse Cap Analog (Ambion). The quality and quantity of RNA transcript preparations were assessed by UV absorption and 1% agarose gel electrophoresis.

Carborundum-dusted leaves of *N. occidentalis* and Etrog citron plants were mechanically inoculated with ~5 μg of RNA transcripts and the plants were incubated in a temperature-controlled greenhouse (18/26 °C night/day) and tested for CLBV infection at 7, 15, 30 and 60 dpi by RT-PCR amplification (Vives *et al.*, 2002b, 2008).

Leaf mesophyll protoplasts from *N. benthamiana* (Satyanarayana *et al.*, 1999), *N. occidentalis* (Satoh *et al.*, 2000) and Etrog citron plants (Albiach-Martí *et al.*, 2004) were also transfected with ~10 μg of RNA transcripts or with CLBV-wt virions semipurified according to Galipienso *et al.* (2001), and incubated at 28 °C in the dark. At 1–5 dpi, total RNA from protoplasts was denatured at 94 °C for 5 min in 50% formamide, chilled on ice, separated by electrophoresis in formamide-formaldehyde denaturing 1.2% agarose gels in MOPS buffer, and electroblotted onto positively charged nylon membranes (Roche) at 250 mA for 1 h and 1 A for 15 h, using 25 mM phosphate buffer, pH 6.45. Membranes were hybridized with a DIG-riboprobe specific for the CLBV coat protein, washed and developed as previously reported (Vives *et al.*, 2002a).

Agrobacterium-mediated inoculation

Agrobacterium tumefaciens cells, strain COR 308 carrying the helper plasmid pCH32 (kindly provided by Dr Hamilton, Cornell Research Foundation), were transformed by electroporation with ~5 ng of pBIN35SRbz-CLBV, pBIN35S-CLBV or pBIN35S-p19 plasmid DNA using a Gene Pulser (Bio-Rad) and then selected in Luria–Bertani medium (LB) containing 50 mg/L kanamicin and 5 mg/L tetracycline. Individual colonies were grown overnight in 5 mL of LB at 28 °C and each of these cultures was used to inoculate 50 mL of induction medium (LB with 10 mM MES and 20 μM acetosyringone) that was also incubated overnight at 28 °C. Bacteria were collected by centrifugation at 6000 ***g*** for 15 min at room temperature, re-suspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 μM acetosyringone) and left in this medium for 5 h after adjusting concentration to 1 OD_{600}. Bacterial suspensions were infiltrated in the intercellular spaces of *N. benthamiana*, *N. occidentalis* and Etrog citron leaves with a syringe directly placed on the leaf surface, or in the case of citron, on small scratches made on the abaxial leaf side. In most experiments plants were infiltrated with a mixture of two *A. tumefaciens* cultures transfected with binary vectors carrying (1) the CLBV-derived clones (pBIN35SRbz-CLBV or pBIN35S-CLBV), and (2) the *p19* gene of *Tomato bushy stunt virus* (TBSV) (pBIN35S-p19) that encodes a strong silencing suppressor protein (Voinnet *et al.*, 2003). CLBV infection was determined by RT-PCR and Northern blot analyses (Vives *et al.*, 2002a,b, 2008) at 2, 4, 6, 8 and 12 dpi in the agro-infiltrated leaves, or at 15, 18, 20, 25 and 30 dpi in the upper leaves of *N. benthamiana* and *N. occidentalis*, and at 7, 10 and 15 dpi in the agro-infiltrated leaves, or in the following flush (about 60 dpi) of Etrog citron plants.

Indexing in indicator plants

To test infectivity in citrus of virions generated in agro-infiltrated *N. benthamiana* or *N. occidentalis* plants (CLBV-IC), virion extracts from these plants (Galipienso *et al.*, 2000) were mechanically inoculated to six plants of each Etrog citron and *C. excelsa* by stem slashing (Garnsey *et al.*, 1977) with scalpel blades dipped in partially purified virion extracts (Galipienso *et al.*, 2000; Vives *et al.*, 2008). The same number of plants was inoculated with virion extracts of the CLBV-wt isolate SRA-153 as control. The inoculated plants were pruned to induce new flush, and systemic infection with CLBV-IC or CLBV-wt was analysed in new leaves at 30, 40 and 60 dpi by RT-PCR (Vives *et al.*, 2002b, 2008).

For further biological characterization six plants of each Pineapple sweet orange, Dweet tangor, Etrog citron and Nules clementine were graft inoculated with bark patches from healthy Etrog citron plants or infected with CLBV-IC or CLBV-wt. Two months later, infected clementine buds were propagated on Carrizo citrange seedlings. Symptom observation included vein clearing in Pineapple sweet orange, chlorotic blotching in Dweet tangor, stem pitting in Etrog citron and bud union crease in Nules clementine grafted on Carrizo citrange.

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