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Partial characterisation of citrus leaf blotch virus, a new virus from Nagami kumquat

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Summary. Citrus leaf blotch virus (CLBV) was purified from leaves of Nagami kumquat SRA-153 that showed bud union crease when propagated on Troyer citrange. Virions were filamentous particles (960 × 14 nm) containing a 42 kDa protein and a single-stranded RNA (ssRNA) of about 9,000 nt (Mr 3×10^6). Infected tissue contained three species of double-stranded RNA (dsRNA) of Mr 6, 4.5 and 3.4×10^6. The nucleotide sequence of several complementary DNA (cDNA) clones showed significant similarities with replication-related proteins from plant filamentous viruses in several genera. A digoxigenin-labelled probe from one of these cDNA clones hybridised in Northern blots with ssRNA from virions and with the three dsRNA species, suggesting that the ssRNA is the genomic RNA of the virus, the largest dsRNA is its replicative form, and the two smaller dsRNAs probably replicative forms of 5′ co-terminal subgenomic RNAs. CLBV was also detected in several citrus cultivars from Spain and Japan including Navelina sweet orange field trees propagated on Troyer citrange showing bud union crease; however, no virus could be detected in other citrus trees with similar symptoms. This indicates that CLBV is not restricted to kumquat SRA-153, but its involvement in causing the bud union disorder remains unclear.

Introduction

During routine indexing used in the Citrus Variety Improvement Programme at the Instituto Valenciano de Investigaciones Agrarias (IVIA) [23] a new graft transmissible disease was detected in Nagami kumquat (*Fortunella margarita* (Lour.) Swing), clone SRA-153, from Corsica. This clone showed bud union crease when propagated on Troyer citrange (*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf) but not on rough lemon (*C. Jambhiri* Lush.) [24]. When the isolate was inoculated to plants of Nules clementine (*C. clementina* Hort. ex Tan.),

Eureka lemon (*C. limon* (L.) Burm. f.), Marsh grapefruit (*C. paradisi* Macf.) and Pineapple sweet orange (*C. sinensis* (L.) Osb.) and these were propagated on citrange, some plants of clementine and lemon showed bud union crease six months after propagation, whereas Marsh grapefruit and Pineapple sweet orange plants showed normal bud union after one year [10]. Inoculum from species showing bud union crease on citrange induced vein clearing in Pineapple sweet orange, blotching in Dweet tangor (*C. tangerina* Hort. ex Tan. × *C. sinensis* (L.) Osb) and stem pitting in Etrog citron (*C. medica* L.) whereas those showing normal union only induced blotching on Dweet tangor and stem pitting on Etrog citron. These results suggested the presence of more than one virus or virus strains in kumquat SRA-153 and their separation after passage through Pineapple sweet orange or Marsh grapefruit. The disease in kumquat SRA-153 can be diagnosed by indexing on Dweet tangor or Etrog citron, but uneven distribution of the virus in other inoculum sources makes this procedure unreliable unless a sufficient number of indicator plants is used [10].

Bud union disorders in citrus have been described in several countries. Some are thought to be of genetic origin [20, 22, 31] whereas others are caused by pathogens [25, 36]. In Corsica, two clones of kumquat (K-124 and 125) showed bud union disorders when propagated on Troyer citrange [35]. In Israel, bud union crease was observed in some lines of Shamouti sweet orange propagated on Troyer citrange or citrumelo (*C. paradisi* Macf. × *P. trifoliata* (L.) Raf), and in Nagami kumquat budded on Troyer citrange [3]. Decline associated with bud union disorders on *P. trifoliata*, citrange or citrumelo rootstocks have also been detected in field trees of Navel sweet orange in California [1], Pera sweet orange in Brazil [29], Marsh grapefruit and Roble sweet orange in Florida (S. M. Garnsey, pers. comm.), Nules clementine plants carrying different pathogens in Italy [5], and Nules clementine, Navelina and Navelate sweet orange in Spain.

Citranges are important rootstocks in many citrus growing areas of the world including Spain, where they presently account for about 50% of all commercial trees. Dispersal of a graft-transmissible pathogen causing bud union crease on this rootstock could potentially cause important economic losses. Quick and reliable diagnostic procedures, needed for the sanitation, quarantine and certification programs currently in operation [23], require purification and characterisation of the pathogen. Here we report isolation and partial characterisation of a filamentous virus named citrus leaf blotch virus (CLBV) from kumquat SRA-153, and detection of this virus in other citrus species from several geographical origins.

Materials and methods

Virus isolates

The following inoculum sources were used for virus purification: i) Nagami kumquat SRA-153, ii) Kumquat plants 38-1 and 497-2 obtained by shoot-tip grafting in vitro from kumquat SRA-153, iii) Pineapple sweet orange, Dweet tangor, Etrog citron, Marsh grapefruit, Nules clementine and Eureka lemon plants pre-inoculated with kumquat SRA-153, iv) A Piña sweet orange plant from an old citrus collection at Burjassot, Valencia (Spain), v) Two mandarin varieties introduced from Japan through the National Citrus Quarantine Station, and vi) A

Navelina sweet orange grafted on Troyer citrange with bud union crease from a commercial orchard at Picassent, Valencia (Spain). All inoculum sources induced chlorotic blotching on Dweet tangor and stem pitting on Etrog citron, whereas Nagami kumquat SRA-153, and Nules clementine and Eureka lemon pre-inoculated with kumquat SRA-153, additionally induced vein clearing on Pineapple sweet orange and bud union crease on citrange. Healthy plants of all the above varieties were used as controls.

Nagami kumquat and Etrog citron were propagated on rough lemon; Pineapple sweet orange and Dweet tangor plants were seedlings, and the remaining varieties were propagated on either Troyer or Carrizo citrange seedlings. Plants were grown in 50% sand, 50% peat moss fertilised by a standard procedure [2] and kept in a greenhouse at 18–26 °C.

Virus purification

Several protocols for purification of isometric and flexuous virus particles were tried [12, 16]. In the one finally adopted, 10 g of bark or leaves from young shoots were pulverised in liquid nitrogen with a pestle and mortar, extracted with 4 volumes (40 ml) of TACM buffer (50 mM Tris-HCl, pH 8.0, 0.1% (w/v) ascorbic acid, 0.5% (w/v) cysteine, and 0.5% (v/v) 2-mercaptoethanol), filtered through cheesecloth, and the extract clarified by centrifugation at 12,000×**g** and 4 °C for 20 min. After precipitation in 4% (w/v) polyethyleneglycol and 1% (w/v) NaCl for 1.5 h, and centrifugation at 17,000×**g** for 25 min, the pellet was resuspended in 1 ml of TACM buffer, layered on a 20–50% (w/v) linear sucrose gradient in 50 mM Tris-HCl, pH 7.7 (Tris 7.7), and centrifuged at 170,000×**g** and 4 °C for 2.5 h. One-ml fractions were taken from the top using an ISCO gradient fractionator and individually examined in the electron microscope (EM) for the presence of virus particles. Fractions 7, 8 and 9, containing filamentous particles, were pooled and centrifuged at 225,000×**g** for 1.5 h at 4 °C. The resulting pellet was resuspended in 1.2 ml of Tris 7.7, mixed with 0.8 ml of 53% (w/v) Cs_2SO_4 in the same buffer, and centrifuged at 160,000×**g** for 24 h at 4 °C. The virus-containing band was collected and dialysed overnight in Tris 7.7 at 4 °C. The virus was precipitated by ultracentrifugation at 225,000×**g** for 1.5 h at 4 °C, and the pellet resuspended in 75 μl of the same buffer.

Electron microscopy

Carbon-Formvar coated grids were floated for 10–15 min on a drop of purified extract, and these were rinsed with 30 drops of distilled water, then 5 drops of 1% uranyl acetate, dried, and examined in a Philips CM 10 EM at 60 KV.

Protein analysis

The proteins in the purified extract were denatured with sodium dodecyl sulfate (SDS), separated by SDS-PAGE [7] using 14% acrylamide in the resolving gel, and stained with silver nitrate. Protein size was estimated using low range molecular weight markers (Bio-Rad).

Nucleic acid analysis

Genomic RNA was extracted with phenol/chloroform from purified virus preparations, precipitated with 3 volumes of ethanol in presence of 0.3 M sodium acetate, denatured with 1.8% formaldehyde and 70% formamide at 65 °C for 10 min, and electrophoresed in 1% agarose gel in MOPS buffer (20 mM morpholino-propanesulfonate, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0) containing 1.8% formaldehyde, at 60 V for 3 h. A 1 Kb DNA ladder (BRL) was used to estimate size. Gels were stained with ethidium bromide.

Double-stranded (ds) RNA from infected tissue was extracted with phenol-detergent and purified by non-ionic cellulose column chromatography in presence of 16% (v/v) ethanol, as described previously [21], but using 20 μg/ml glycogen (Boehringer Mannheim) as co-precipitant. dsRNAs were separated by PAGE (6% acrylamide) in TAE buffer (40 mM Tris-acetate, pH 7.5, 2 mM Na_2-EDTA) at 125 V for 3 h and stained with ethidium bromide or silver nitrate [13]. Molecular size markers were dsRNAs from citrus tissue infected with citrus tristeza virus isolate T-388 (Mr 13.3, 2, 0.8 and 0.5×10^6) [21] and dsRNAs from cucumber mosaic virus-infected *Nicotiana glauca* tissue (Mr 2.25, 2.06, 1.5, and 0.7×10^6).

The nature and strandedness of the nucleic acids were determined by digestion with DNAse I (Sigma) in the presence of 5 mM $MgCl_2$ or with RNAse I (Sigma) in distilled water or in 0.3 M NaCl [13].

cDNA cloning and sequencing

Approximately 1 μg of dsRNA, purified from about 1,000 g of kumquat SRA-153 bark tissue, was treated with DNAse I (Sigma) and denatured with methylmercuric hydroxide. The first cDNA strand was synthesised with random primers using avian myeloblastosis virus (AMV) reverse transcriptase, and the second strand with RNAse H and DNA polymerase I of *E. coli*, using the Time-saver cDNA synthesis kit (Pharmacia Biotech), according to the manufacturer's instructions. The cDNA was ligated to the *Eco*RI-digested plasmid pT7T3 18U EcoRI/BAP (Pharmacia Biotech) and used to transform DH5α-competent *E. coli* cells [30].

Selected clones were sequenced in both directions using an ABI PRISM DNA 377 sequencer (Perkin-Elmer). For analyses, the following programmes of the GCG package [9] were used: GAP to align sequences; ASSEMBLE to concatenate sequences from different cDNA fragments, and BLAST for seeking homologous sequences in databases. Multiple alignments were performed with CLUSTAL W [34] and bootstrap analysis and phylogenetic relationships with MEGA [14] programmes.

The following virus sequences were used for comparison: apple chlorotic leafspot virus (ACLSV A, D14996; ACLSV P, M31714), apple stem pitting virus (ASPV, D21829), grapevine rupestris stem pitting associated virus (GRSPaV, AF026278), cherry green ring mottle virus (CGRMV, AF017780), garlic latent virus (GLV, Z68502), blueberry scorch virus (BSV, L25658), potato virus M (PVM, X53062), cherry virus A (CVA, X82547), citrus tatter leaf virus (CTLV, D14455), apple stem grooving virus (ASGV, D14995), grapevine virus A (GVA, X75433) and grapevine virus B (GVB, X75448).

Hybridisation

Cloned cDNA was PCR-labelled using specific primers and the PCR DIG Labelling and Detection kit (Boehringer Mannheim).

For dot-blot hybridisation, 1 μl of the dsRNA extract was denatured at 94 °C with an equal volume of formamide and applied to a nylon membrane (Hybond-N, Amersham). For Northern blotting, genomic RNA denatured with formamide or dsRNA denatured with methylmercuric hydroxide was electrophoresed in 1% agarose gel in MOPS buffer containing 1.8% formaldehyde, and electro-blotted onto a nylon membrane using a Bio-Rad trans-blot cell. Finally, genomic DNA from citrus [8], digested with *Eco*RI and *Hin*d III, was electrophoresed in 2% agarose gel, transferred to a nylon membrane and used in Southern blots [33].

The membranes were baked for 1 h at 85 °C in a vacuum oven and hybridised (16 h at 68 °C), washed and developed using the PCR DIG Labelling and Detection kit (Boehringer Mannheim), according to the manufacturer's instructions.

Results

Virus purification and partial characterisation

EM observations of purified preparations obtained from kumquat SRA-153 revealed two types of filamentous particles. The first, present in sucrose gradient fractions 1 and 2, were rigid rods 1,000×8–10 nm in size. These particles, similar to those observed by others [6, 27] and present in extracts from both healthy and infected tissues, were probably host protein structures. The second type, present in fractions 7–9, were flexuous filaments 860–1,000 nm long and 14 nm wide, with a modal length of 960 nm (200 particles measured) that showed a faint cross banding (Fig. 1). These particles were seen in extracts from infected tissues of different sources, but not in similar extracts from healthy plants. Usually, several fields had to be searched on each grid to find particles, since they were scarce. No isometric virus-like particles were detected in the products of any of the purification protocols tested.

When fractions 7–9 of the sucrose gradient were pooled and further purified by isopycnic centrifugation in Cs_2SO_4, a large band in the middle of the tube was seen in extracts from both healthy and infected tissue and contained cell debris, whereas a thinner band 4–5 mm below was detected only in extracts from infected plants and contained virus particles. The larger band served as reference to locate the virus-containing band, since the latter was barely visible or not visible at all in most experiments due to the low virus concentration. EM observation again revealed flexuous particles, 8–10 per grid opening, identical to those in the sucrose gradient fractions. Sometimes particles with similar morphology but 1,800–2,000 nm long were observed, suggesting end-to-end aggregation.

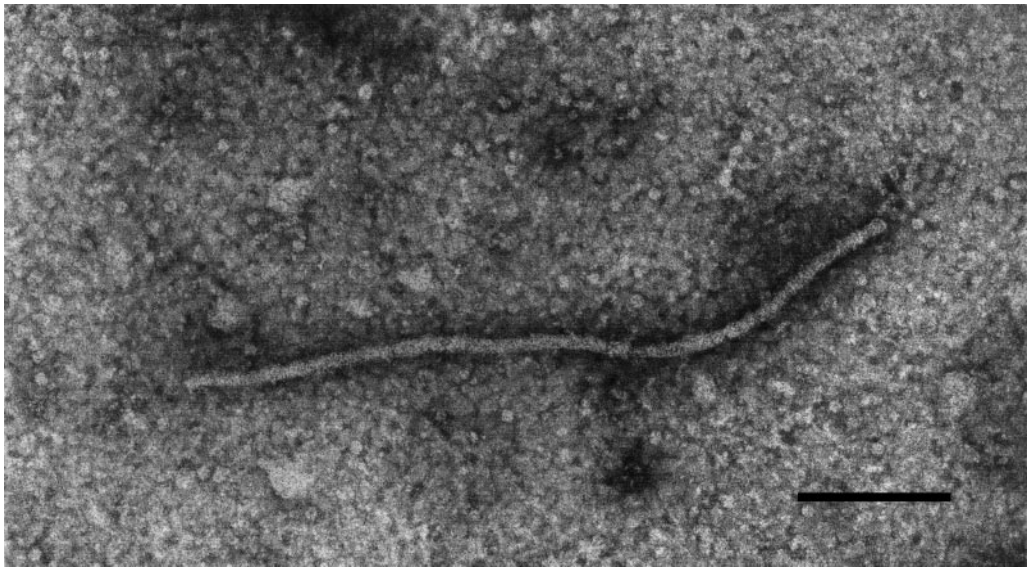

Fig. 1. Electron micrograph of a CLBV virion purified from kumquat SRA-153, stained with 1% uranyl acetate. Bar: 200 nm

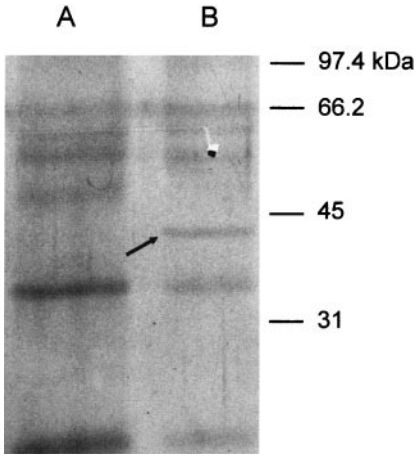

Fig. 2. SDS-PAGE of preparations purified from healthy kumquat (**A**) or kumquat SRA-153 (**B**). Silver stain. The arrow indicates the 42-kDa protein associated with the virion

No difference between healthy and infected preparations was seen in the SDS-PAGE results from sucrose density gradient fractions. However, after isopycnic centrifugation, a 42-kDa band was seen only in extracts from infected plants (Fig. 2). It could be detected after silver staining but not with Coomassie Blue. The 42-kDa protein was presumably the viral coat protein, but its low titre did not allow us to prepare an antiserum.

When selected fraction of the sucrose gradient were concentrated and virion nucleic acid was phenol-extracted, a single band of about 9,000 nt (Mr 3×10^6) was observed by agarose gel electrophoresis (Fig. 3, left). This band was resistant to DNAse but digested by RNAse in distilled water or in 0.3 M NaCl, indicating that it was ssRNA.

Similar virus particles and also the 42-kDa protein could be purified from kumquats 38-1 and 497-2, and from Dweet tangor, Pineapple sweet orange, Etrog citron, Nules clementine, Eureka lemon and Marsh grapefruit plants inoculated with kumquat SRA-153 and maintained in the greenhouse. In all cases, the number of particles observed by EM (2–4 per grid opening) and the amount of coat protein detected by SDS-PAGE, were smaller than those obtained from kumquat SRA-

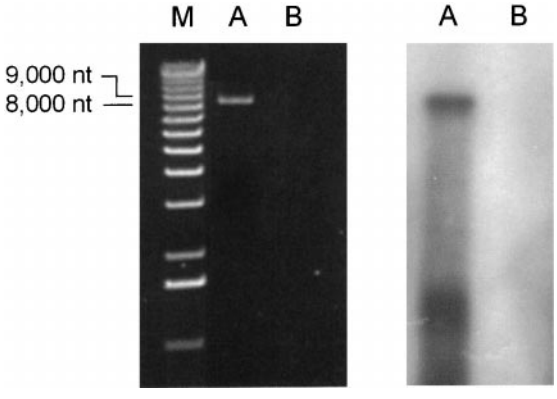

Fig. 3. Characterisation of genomic RNA extracted from partially purified virions from kumquat SRA-153 (*A*) or from healthy kumquat (*B*). *Left*: Electrophoresis in a 1% denaturing agarose gel stained with ethidium bromide. A 1 kb DNA Ladder (BRL) was used to estimate RNA size (*M*). *Right*: Northern blot analysis with a DIG-labelled probe prepared from the clone LG17. Reaction was developed with a chemiluminiscent substrate

153. Virion RNA could be detected from these sources, but only after Northern blotting with a specific DIG-labelled probe.

Purified extracts from two mandarin varieties introduced from Japan, a field tree of Piña sweet orange, and various field trees of Navelina sweet orange from Picassent, Valencia (Spain), also contained particles (0–2 per grid opening) of the same morphology. The 42-kDa protein could not be detected by SDS-PAGE but the genomic RNA was detected in Northern blots.

Analysis of dsRNA

Extracts of dsRNA from bark or leaves of kumquat SRA-153 contained molecules with Mr 6, 4.5 and 3.4×10^6, the second being most abundant, whereas no dsRNA was detected in extracts from healthy plants (Fig. 4, left). The dsRNAs were rarely observed with ethidium bromide, and silver staining was necessary. The bands were digested only after treatment with RNAse I in distilled water, confirming that they were dsRNA.

Processing of other citrus species inoculated with kumquat SRA-153, or kumquats 38-1 or 497-2, yielded the same pattern of bands, but at much lower intensity (not shown).

Identification and sequencing of viral cDNA clones

Several recombinant clones containing inserts of different size were analysed. Clones LG17 (420 nt), LG55 (813 nt) and LG70 (234 nt), which hybridised in dot blots with dsRNA extracts from diseased plants, but not in Southern blots with genomic DNA from citrus plants, were sequenced. Clones LG17 and LG55 overlapped in 202 nucleotides and showed identical sequences in the overlapping region. Blast analysis of the 1031 nt segment resulting from alignment of LG17

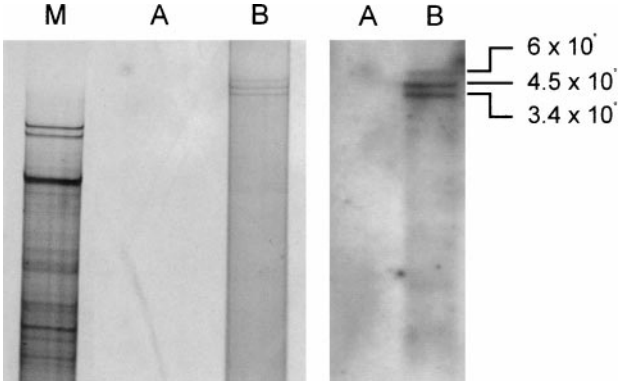

Fig. 4. Characterisation of dsRNA extracted from healthy kumquat (*A*) or kumquat SRA-153 (*B*). *Left*: DsRNA separated in a polyacrylamide gel (6% acrylamide) and silver stained. DsRNA from cucumber mosaic virus was used as marker (*M*). *Right*: Northern blot analysis of dsRNA separated in a 1% denaturing agarose gel and electroblotted onto a Nylon membrane. Hybridisation was as in Fig. 3

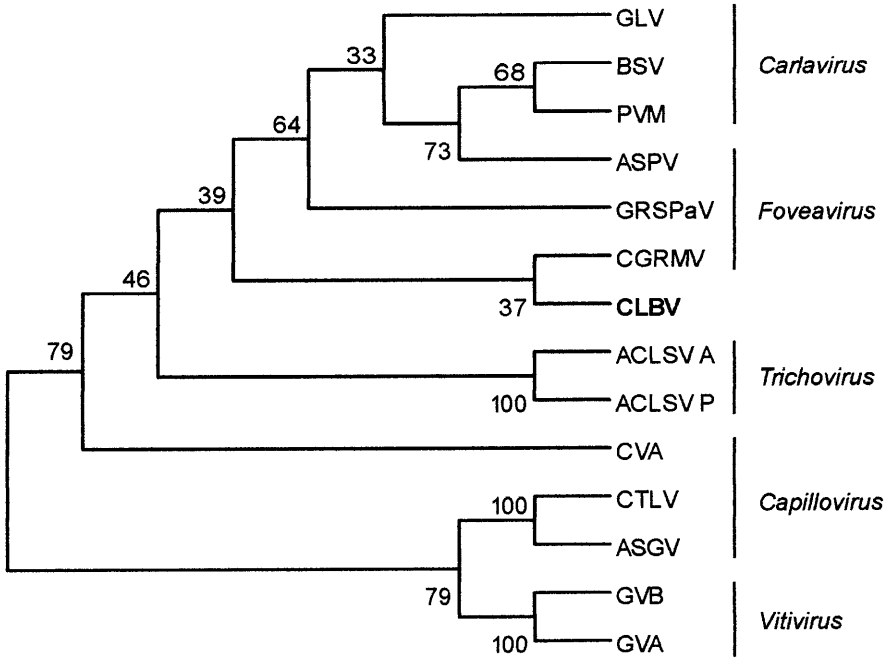

Fig. 5. Phylogenetic analysis using amino acid signatures of the LG73 segment of CBLV and the homologous region of several carla-, fovea-, tricho-, capillo- and vitiviruses

and LG55 (named LG73), showed its deduced amino acid sequence had significant similarities with replication-related proteins of viruses in the genera *Capillovirus, Carlavirus, Foveavirus, Trichovirus* and *Vitivirus*. Multiple alignment with those viruses indicated that this segment was located between methyl transferase and protease domains. Phylogenetic analysis using amino acid signature of the LG73 segment showed that CLBV was more related with fovea-, tricho- and carlaviruses than with viti- and capilloviruses (Fig. 5). The sequence of clone LG70 did not present similarity with other known sequences, possibly due to its limited size or location in a variable region of the genome. The sequence of segment LG73 has been deposited in EMBL (accession number AJ401103).

Northern blots

A DIG-labelled probe of clone LG17 hybridised in Northern blots with ssRNA from purified virions and with dsRNA extracts from kumquat SRA-153. Hybridisation was observed with the 3×10^6 ssRNA from purified virions (Fig. 3, right), and with the dsRNAs of 6, 4.5 and 3.4×10^6 (Fig. 4, right).

Discussion

CLBV particles were partially purified from Nagami kumquat SRA-153, which showed bud union crease on citrange rootstock, and induced vein clearing in Pineapple sweet orange, chlorotic blotching in Dweet tangor and stem pitting in

Etrog citron. Similar particles were also purified from kumquats 38-1 and 497-2, obtained from kumquat SRA-153 by shoot-tip grafting in vitro, and from various citrus varieties inoculated with kumquat SRA-153. Some of these varieties and kumquats 38-1 and 497-2, however, induced leaf blotching in Dweet tangor and stem pitting in Etrog citron, but not vein clearing in Pineapple sweet orange or bud union crease on citrange. The number of particles and the amount of coat protein obtained from these latter hosts were lower than those in extracts from kumquat SRA-153.

Two explanations seem possible for these findings: i) CLBV would be responsible only for blotching in Dweet tangor and stem pitting in Etrog citron, and a different undetected virus would cause the bud union crease and vein clearing. This second virus, present in kumquat SRA-153, but eliminated by shoot-tip grafting in vitro, or after passage through certain hosts, would be synergistic with CLBV and would increase its titre in kumquat SRA-153. ii) Two strains of CLBV, one causing only blotching in Dweet tangor and pitting in Etrog citron, would be present in kumquat SRA-153. The more virulent strain would be eliminated, or its titre drastically reduced, after shoot tip grafting in vitro or passage through Pineapple sweet orange or Marsh grapefruit.

The second possibility is supported by the finding that so far no isolate inducing only vein clearing in Pineapple sweet orange and/or bud union crease on citrange has been found, and no other virus could be detected in kumquat SRA-153. Previous observations that symptoms induced by kumquat SRA-153 in indicator plants are more intense and less erratic than those induced by the other inoculum sources used here [10], are in agreement with the higher concentrations of virions, coat protein and genomic RNA, observed in extracts purified from kumquat SRA-153.

Virus particles with morphology somewhat similar to CLBV have been reported in citrus. Flexuous particles of 660–665 × 12 nm were found in a plant infected with psorosis isolate Ps-203m from California [15]. However, these particles had associated a 29-kDa protein and infected tissues contained dsRNA species of Mr = 5.3, 4.5 and 4.1×10^6. Additionally, this virus was transmitted to *Chenopodium quinoa*, whereas trials to mechanically transmit CLBV to this and other herbaceous hosts were unsuccessful [10].

Indian citrus ringspot virus (CRSV) has filamentous particles of 640–650 × 15 nm and induced in various indicator plants symptoms different from those produced by CLBV [4, 10, 28]. Additionally the CRSV coat protein (35 kDa) and its genomic RNA are smaller than those of CLBV and both are serologically unrelated (unpublished results).

Finally, the virions of citrus tatter leaf virus are filamentous particles of 650 × 15 nm with a 27-kDa coat protein and a genomic RNA of Mr 2.7×10^6 [26, 32]. Symptoms induced by CTLV and CLBV in various indicator species are also different [10].

Plants infected with CLBV contained three dsRNAs, one of them about double the mass of the genomic ssRNA, suggesting that it is the replicative form. This was confirmed by finding that probe LG17, obtained from dsRNA, hybridised with the

genomic RNA. Since this probe also hybridised with all three dsRNAs, the two smaller ones must also contain genomic sequences and are probably replicative forms of subgenomic RNAs of the virus.

The most abundant dsRNA was that of Mr 4.5×10^6, rather than the full length species. A similar situation is reported for apple chlorotic leaf spot virus, in which dsRNAs of 6.4 and 5.4 kbp were more abundant than that of 7.5 kbp, the replicative form of the genomic RNA [11]. These authors showed by Northern blotting with probes corresponding to several regions of the genome that the two subgenomic RNAs were 5′ co-terminal, and they proposed a new mechanism for expression of the genome of this virus. Probe LG17 is located in the 5′ half of the genomic RNA (M. C. Vives, unpublished), suggesting that the two subgenomic RNAs of CLBV are also 5′ co-terminal.

Virion morphology of CLBV resembles that of other viruses in the genera *Trichovirus*, *Foveavirus*, *Capilloviurs* and *Vitivirus*. Its particle is longer than that of capilloviruses (640 nm) [37], trichoviruses (640 to 800 nm) [17] and ICRSV [28] and similar to that of vitiviruses [19] and foveaviruses [18]. The size of its coat protein is only similar to that reported for apple stem pitting virus, recently included in the genus *Foveavirus* [18]. However, the presence of subgenomic RNAs 5′-coterminal might relate CLBV with trichoviruses. Additional data on sequence and organization of the genome will be necessary before CLBV can be placed in any of these genera or assigned to a new genus.

The world distribution of CLBV is presently unknown. However, particles identical to those found in kumquat SRA-153 and similar dsRNA species were detected in preparations from two mandarins imported from Japan through the National Citrus Quarantine Station, from a field tree of Piña sweet orange, probably introduced from Florida (USA), and from some trees showing bud union crease on citrange in a commercial citrus orchard at Picassent, Valencia (Spain). This suggests that CLBV is present in citrus varieties other than Nagami kumquat and in several geographic areas. Failure to detect virus particles in other trees with bud union crease in the same orchard at Picassent could be due to very low titre or uneven distribution of this virus within the plant. Alternatively, a different pathogen might be involved.

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