Dweet Mottle Disease Probably is Caused by Citrus Leaf Blotch Virus

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ABSTRACT. Dweet mottle disease was first detected in Cleopatra mandarin CRC-270 in California and causes chlorotic blotching in Dweet tangor seedlings. Nagami kumquat SRA-153 induces similar symptoms in this indicator, but additionally causes vein clearing in several citrus species, stem pitting in Etrog citron, and bud-union crease when propagated on Troyer citrange. Citrus leaf blotch virus (CLBV) was found in this kumquat and its genomic RNA sequenced, thus allowing its detection by molecular hybridization and RT-PCR assays. Two Californian sources of Dweet mottle (DMV-931 and DMV-932) and kumquat SRA-153 were graft-inoculated on Pineapple sweet orange, Dweet tangor, Etrog citron and Nules Clementine and infected Clementine buds were then propagated on Carrizo citrange rootstock. Kumquat SRA-153 induced bud-union crease of Nules Clementine on citrange, vein clearing in Pineapple sweet orange, chlorotic blotching in Dweet tangor and stem pitting in Etrog citron, whereas Dweet mottle sources only induced blotching in Dweet tangor and stem pitting in Etrog citron. Fragments of the CLBV genome were RT-PCR amplified from indicator plants inoculated with either kumquat SRA-153 or Dweet mottle sources. The nucleotide identity between CLBV isolate SRA-153 and the DMV-931 and DMV-932 sources was 96.6% and 96.8% in a region of the RNA replicase gene, and 98.7% and 98.5% in the coat protein gene, respectively. These data and previous findings that Dweet mottle and CLBV are difficult to eliminate by shoot-tip grafting or thermotherapy, suggest that Dweet mottle may be caused by CLBV, and that, besides CLBV, a different pathogen causing bud-union crease and vein clearing may be present in kumquat SRA-153 but not in DMV-931 and DMV-932 sources.

Index words. Troyer Citrange, Etrog citron, virus characterization, RT-PCR.

In 1968, routine indexing carried out in the Citrus Variety Improvement Program developed in California, revealed a new pathogen in a Cleopatra mandarin tree from Florida (CRC 270) that induced chlorotic blotching in Dweet tangor and a mild exocortis reaction in Etrog citron (8). The parent tree showed no visible evidence of damage due to any known virus, the trunk appeared normal and no pitting or bark discoloration was evident, although the fruits were small, the twigs were dying back, and little new growth was apparent. The pathogen responsible for these symptoms was graft and mechanically transmitted to several citrus species and varieties but, since the pathogen caused a reaction only in Dweet tangor, it was named Dweet mottle virus (DMV) (8).

In 1984, in routine indexing done at the Citrus Variety Improvement Program developed in Spain, a graft-transmissible pathogen was found in Nagami kumquat, clone SRA-153, from Corsica (France). When graft-inoculated to Dweet tangor it induced chlorotic blotches resembling those induced by DMV, but additionally it caused stem pitting in Etrog citron, vein clearing in Pineapple sweet orange and other cultivars, and bud union crease when propagated on Troyer citrange rootstock (2, 7).

Citrus leaf blotch virus (CLBV) has been partially purified from Nagami kumquat SRA-153 (3) and its genomic RNA (gRNA) completely sequenced (12). The CLBV gRNA contains three open reading frames (ORFs) encoding a polyprotein involved in replication, a potential movement protein and the capsid protein. In addition to the gRNA, infected plants contain two sets of subgenomic RNAs that are 3' and 5' coterminal, respectively (13). Availability of the CLBV sequence allowed the development of rapid and specific methods for routine detection of the

virus based on RT-PCR, and dot-blot and tissue print hybridization protocols (4, 14). Using these procedures, CLBV has been detected in leaf samples of different citrus varieties from Spain, Australia, France, Japan and USA (3, 4, 14), confirming that this pathogen is widespread in many citrus growing areas.

In this work we report biological and molecular evidence that suggests Dweet mottle disease may be caused by Citrus leaf blotch virus.

MATERIALS AND METHODS

Virus isolates and citrus hosts. The CLBV isolate SRA-153 used in this work was maintained in plants of Nagami kumquat grafted on rough lemon. The Californian sources of Dweet mottle disease, DMV-931 and DMV-932, kindly provided by D. Gumpf and introduced through the citrus quarantine station in Moncada, Valencia (Spain), were on Pineapple sweet orange and Satsuma mandarin, respectively.

These three inoculum sources were indexed on Pineapple sweet orange, Dweet tangor, Nules Clementine, and Etrog citron. Pineapple sweet orange and Dweet tangor plants were seedlings, and Nules Clementine and Etrog citron were propagated on Carrizo citrange and rough lemon seedlings, respectively.

Two to four bark patches from each virus source were graft inoculated onto six plants of each indicator. Plants were grown in an artificial potting mix (50% sand and 50% peat moss) in a temperature-controlled (18-26°C) greenhouse and fertilized by a standard procedure (1).

RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR) amplification. Total RNA was extracted from approximately 100 mg of thoroughly trimmed fresh young leaf tissue using TRIzol[R] reagent (Invitrogen), which contains isothyocyanate, following the manufacturer's instructions for samples with high sugar content, and

was then resuspended in 25 μl of DEPC-treated distilled water. Total RNA was used as template to amplify by PCR two genome regions (R and C) as previously described (4, 14). Region R, located in ORF 1 was amplified with primers KU-54 (5'-ACTTGCAGAAATGATCAGACCG-3', positions 2260-2281) and KU-55 (5'-TGCCTCATAGAAATTTATTAATG-CAC-3', positions 2728-2703). Region C, containing the conserved C-terminal region of the coat protein gene, was amplified with primers KU-18 (5'-TTAAGATTACAGACACGAAGG-3', positions 7686-7706) and KU-19 (5'-CTGTTTTTGAATTTTGCTCG-3', positions 8123-8104), based on the CLBV sequence (12).

Cloning, SSCP analysis, sequencing, and nucleotide sequence analysis. The RT-PCR products were cloned into the pGEM-T plasmid vector (Promega) using standard protocols (10), and ten randomly selected clones from each region and isolate were PCR-amplified with the corresponding primers. The PCR products were analyzed by SSCP with electrophoresis in 10% polyacrylamide gels at 300V for 2.5 h as previously described (9). The SSCP pattern of individual clones was compared with that of the corresponding RT-PCR product. The nucleotide sequence of the predominant haplotype for each region and isolate was determined with an ABI PRISM DNA sequencer 377 (PE Biosystems). Sequence alignment was done with the CLUSTAL W program (11) and estimation of the nucleotide and amino acidic identities were done with the MEGA program (6).

RESULTS

Biological indexing in indicator plants. Six plants of Pineapple sweet orange, Dweet tangor, Etrog citron and Nules Clementine were graft inoculated with bark patches of Dweet mottle sources (DMV-931 and DMV-932), kumquat SRA-153

or healthy kumquat. Six-months-old infected Clementine buds were then propagated on Carrizo citrange seedlings (Fig. 1). The first two flushes of Pineapple sweet orange and Dweet tangor were observed for leaf symptoms. Etrog citron was observed for stem pitting 3 to 10 mo after inoculation. Budlings of infected Nules Clementine on Carrizo citrange rootstock were examined 6 and 12 mo after propagation, by lifting a piece of bark to check for the presence of bud union crease.

In all plants tested Kumquat SRA-153 source induced intense vein clearing in Pineapple sweet orange, intense chlorotic blotching in Dweet tangor, stem pitting in Etrog citron, and bud union crease of Nules Clementine grafted on Carrizo citrange, whereas Dweet mottle sources DMV-931 and DMV-932 caused only mild chlorotic blotching in 3-4 Dweet tangor plants, and stem pitting in 4-5 Etrog citron plants, but no vein clearing or bud union crease (Fig. 1). Indicator plants budded from healthy kumquat showed no symptoms.

Detection of CLBV in Dweet mottle sources. To determine if CLBV was present in Dweet mottle sources, total RNA extracts from DMV-931, DMV-932, kumquat SRA-153 and healthy kumquat were used as templates with the two selected primer sets. Extracts from DMV-931, DMV-932, and kumquat SRA-153 sources yielded single RT-PCR products of the expected size for region R (469 nt) or for region C (438 nt), whereas no amplification was obtained using equivalent extracts from healthy kumquat (Fig. 2A). CLBV was also detected by RT-PCR in all symptomatic plants inoculated with kumquat SRA-153, DMV-931, or DMV-932, respectively (data not shown).

Sequence analysis. To characterize the population structure of CLBV in Dweet mottle isolates DMV-931 and DMV-932, the RT-PCR products from genomic regions R and C were analyzed by SSCP. All SSCP patterns showed only one or two intense bands, suggesting that each RT-PCR product was most likely composed of a single predominant sequence. To estimate more accurately the population composition of these isolates, the RT-PCR products generated from both genomic regions were cloned into pGEM-T vector and ten randomly selected clones were PCR amplified with the corresponding primers and
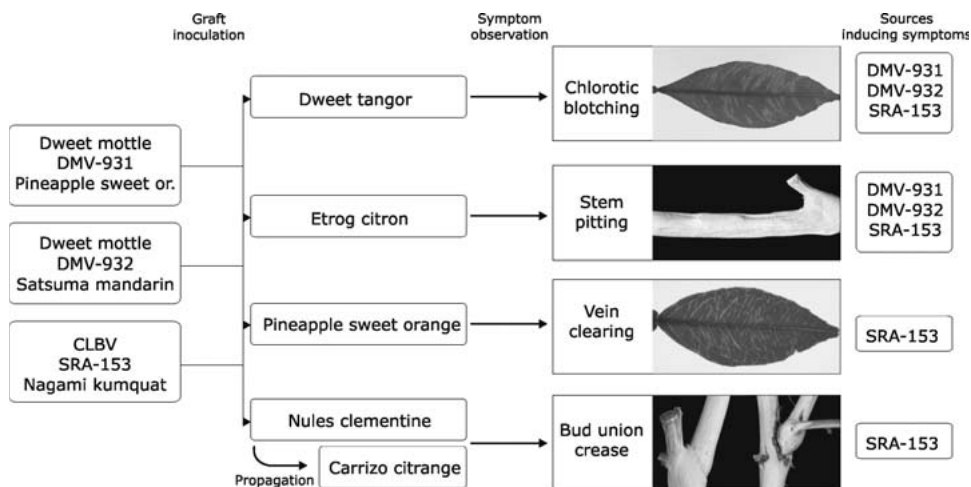

Fig. 1. Outline of the inoculations performed and symptoms caused by DMV-931, DMV-932, and SRA-153 inoculum sources.
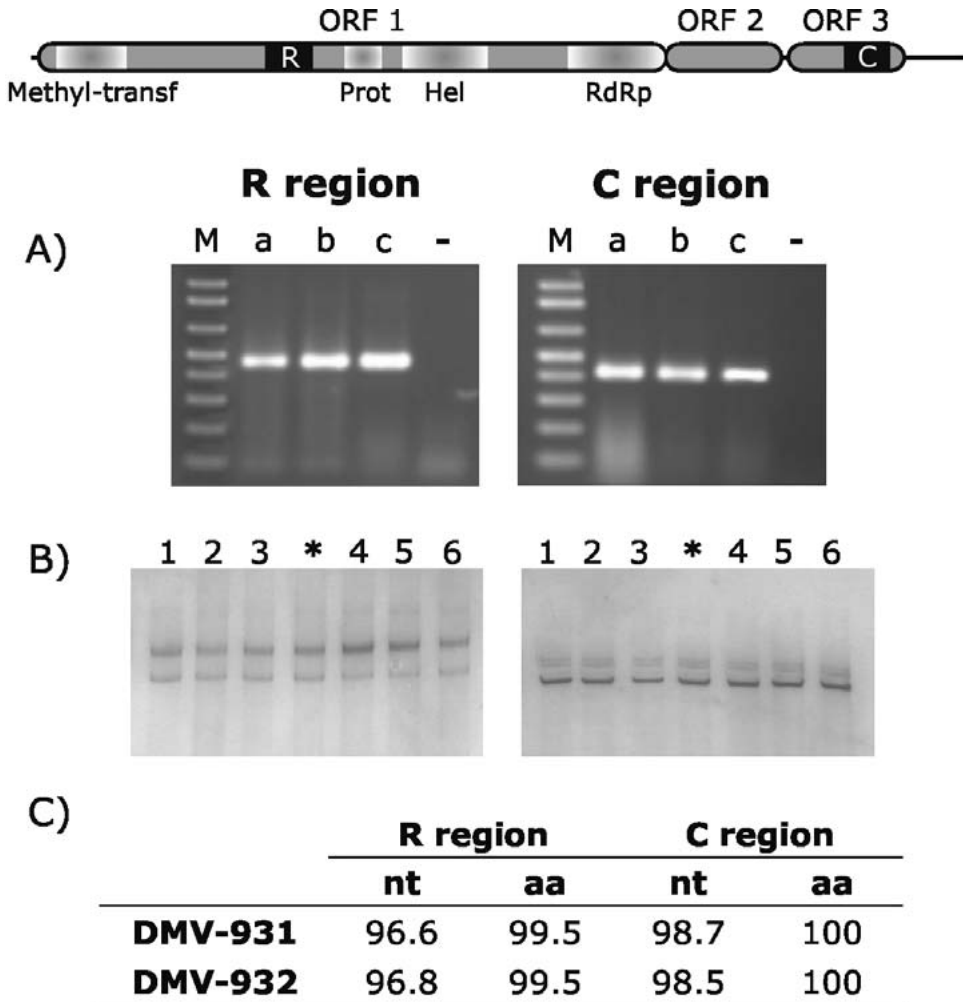
Fig. 2. RT-PCR detection of CLBV from kumquat SRA-153 and Dweet mottle sources, and SSCP analysis of the genomic regions R (left panels) and C (right panels). An outline of the CLBV genome is at the top. Black boxes indicate the genomic regions analyzed. A) RT-PCR detection of CLBV in total RNA extracts from DMV-931 (lane a), DMV-932 (lane b), and SRA-153 (lane c) sources, healthy kumquat (lane (-)), and 1-Kb plus DNA ladder (lane M). B) SSCP analysis of 6 cDNA clones (lanes 1-6) of each region from isolate DMV-931. Asterisks indicate the SSCP pattern of the RT-PCR products from which the clones were obtained. C) Nucleotide and amino acid identities (%) between CLBV SRA-153 isolate and Dweet mottle sources DMV-931 and DMV-932.

analyzed by SSCP. The SSCP patterns of these clones were compared with those of the corresponding RT-PCR products and found to be identical, indicating that both isolates are composed of a predominant sequence variant (Fig. 2B).

To estimate the nucleotide and amino acidic identities between CLBV isolates from kumquat SRA-153 and Dweet mottle sources DMV-931 and DMV-932, the nucleotide sequences of two clones from each genomic region and source were determined. The comparative nucleotide identities of CLBV isolate SRA-153 and DMV-931 and DMV-932 were 96.6 and 96.8% in R region, and 98.7 and 98.5% in C region, respectively, whereas the

amino acid identities with both Dweet mottle sources were 99.5% and 100% in the R and C regions, respectively (Fig. 2C).

DISCUSSION

The results obtained in this work suggest that CLBV is the causal agent of Dweet mottle disease. The symptoms induced by CLBV isolate SRA-153 in Dweet tangor and Etrog citron are indistinguishable from those caused by both Dweet mottle sources in the same indicators, and like CLBV, the pathogen responsible for Dweet mottle disease was also difficult to eliminate by shoot-tip grafting (7) or thermotherapy (unpublished results). Furthermore, CLBV was detected by RT-PCR in both Dweet mottle sources as well as in different indicator plants inoculated with them. However, while CLBV SRA-153 induced bud union crease of Nules clementine grafted on Carrizo citrange, vein clearing in Pineapple sweet orange, chlorotic blotching in Dweet tangor, and stem pitting in Etrog citron, Dweet mottle sources only induced chlorotic blotching in Dweet tangor and stem pitting in Etrog citron. These differences in symptom expression suggest that CLBV would be responsible only for blotching in Dweet tangor and stem pitting in Etrog citron, and symptoms of bud union crease and vein clearing would be caused by a different pathogen or by an interaction between CLBV and other biotic factors. Our results agree with previous detection of CLBV in kumquat lines 38-1 and 497-2, obtained by shoot-tip grafting from kumquat

SRA-153, that also induced blotching in Dweet tangor and stem pitting in Etrog citron, but not vein clearing in Pineapple sweet orange or bud union crease on Carrizo citrange (2, 3).

In a previous study, low genetic variation was found between CLBV isolates from different host species and geographical origins (15). Sequence comparisons between CLBV found in Dweet mottle sources and the isolate SRA-153, showed nucleotide identities over 96.6% and 98.5% and amino acid identities of 99.5% and 100% for the R and C regions, respectively, indicating that Dweet mottle isolates of CLBV were closely related to all other isolates characterized and confirming genetic stability of CLBV.

In addition to Dweet mottle sources, CLBV has been detected in samples from Corsica (France), Valencia (Spain), Florida (USA), Japan and Australia, indicating that this virus must be widespread (3, 4, 14). Recently we have reported seed transmission of CLBV in Nagami kumquat, Carrizo citrange and sour orange (5), a finding that could explain in part the dissemination of this virus throughout the world. To control CLBV spread during citrus propagation is necessary by not only the use of virus free buds, but also by rootstock seedlings originating from CLBV-free seed sources.

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