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Identification and Genomic Characterization of a New Virus (*Tymoviridae* Family) Associated with Citrus Sudden Death Disease

Walter Maccheroni,[1] Marcos C. Alegria,[1] Christian C. Greggio,[1] João Paulo Piazza,[1] Rachel F. Kamla,[1] Paula R. A. Zacharias,[1] Moshe Bar-Joseph,[1]† Elliot W. Kitajima,[2] Laura C. Assumpção,[1] Giovana Camarotte,[1] Jussara Cardozo,[1] Elaine C. Casagrande,[1] Fernanda Ferrari,[1] Sulamita F. Franco,[1] Poliana F. Giachetto,[1] Alessandra Girasol,[1] Hamilton Jordão, Jr.,[1] Vitor H. A. Silva,[1] Leonardo C. A. Souza,[1] Carlos I. Aguilar-Vildoso,[1] Almir S. Zanca,[1] Paulo Arruda,[1] João Paulo Kitajima,[1] Fernando C. Reinach,[1] Jesus A. Ferro,[1] and Ana C. R. da Silva[1]*

Alellyx Applied Genomics, Campinas,[1] and Department of Entomology, Phytopathology, and Zoological Agriculture, ESALQ/USP, Piracicaba,[2] São Paulo, Brazil

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Citrus sudden death (CSD) is a new disease that has killed approximately 1 million orange trees in Brazil. Here we report the identification of a new virus associated with the disease. RNAs isolated from CSD-affected and nonaffected trees were used to construct cDNA libraries. A set of viral sequences present exclusively in libraries of CSD-affected trees was used to obtain the complete genome sequence of the new virus. Phylogenetic analysis revealed that this virus is a new member of the genus *Marafivirus*. Antibodies raised against the putative viral coat proteins allowed detection of viral antigens of expected sizes in affected plants. Electron microscopy of purified virus confirmed the presence of typical isometric *Marafivirus* particles. The screening of 773 affected and nonaffected citrus trees for the presence of the virus showed a 99.7% correlation between disease symptoms and the presence of the virus. We also detected the virus in aphids feeding on affected trees. These results suggest that this virus is likely to be the causative agent of CSD. The virus was named *Citrus sudden death-associated virus*.

The outbreak of new diseases in citrus culture has always challenged the producers and researchers urging the development of new strategies for identification of the causative agents and disease control. Several citrus diseases have been related to virus infection (13). They caused severe economical losses for the world citrus industry in the past and are still a problem. Among these viruses the most important is *Citrus tristeza virus* (CTV), a member of the family *Closteroviridae*, which induces diseases such as quick decline, causing the death of trees grafted on sour orange (*Citrus aurantium*) rootstock and stem pitting of scion cultivars regardless of the rootstock type (5).

Citrus Tristeza was first detected in Brazil in later 1930s, killing millions of sweet orange (*C. sinensis*) trees grafted on sour orange. The problem was solved by exchanging the sensitive sour orange with a tolerant Rangpur lime (*C. limonia*) rootstock. Today, more than 85% of the 200 million trees in the country are grafted on Rangpur lime (18).

In 1999, a new citrus disease was identified in the state of Minas Gerais, Brazil, and was named citrus sudden death (CSD). The disease is mainly characterized by a rapid decline of scions grafted on Rangpur lime or Volkamer lemon (*C. volkameriana*). Rootstocks such as Cleopatra mandarin (*C. reshni*), Sunki mandarin (*C. sunki*), and trifoliates (*Poncirus trifoliata*) and their hybrids (citranges and citrumelos) are apparently tolerant to the causal agent, and these rootstocks are presently being used as alternatives to Rangpur lime and for inarching purposes as a measure to recover CSD-affected trees (6, 30). The number of dead trees affected by the disease dramatically increased from 500 trees in 1999 to at least 1 million in 2003. This number would be even greater if we considered those infected but asymptomatic. The disease has progressed rapidly from the original focus in the southwest of Minas Gerais state towards the northern part of São Paulo state (30).

Symptomatic plants present a pale green coloration of the whole canopy, different levels of defoliation, the absence of new shoots, and death of the root system. General decline symptoms and plant death has been related to rootstock phloem degeneration in the bud union region (30). CSD is also characterized by the development of a strong yellow stain in the phloem of the Rangpur lime and Volkamer lemon rootstocks. The plant physiological status is important in disease progression, since the severity of the symptoms increases under conditions of high water demand. Death of infected trees occurs between 1 and 12 months after the appearance of symptoms, depending on the season and citrus variety. The disease

* Corresponding author. Mailing address: Alellyx Applied Genomics, Techno Park (Rod. Anhanguera Km 104), Rua James Clerk Maxwell 320, 13069-380 Campinas, SP, Brazil. Phone: 55 19 3783 9406. Fax: 55 19 3783 9412. E-mail: ana.rasera@alellyx.com.br.

† Present address: The S. Tolkowsky Laboratory, Dept. of Virology, Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel.

has a period of incubation of at least 2 years before symptoms are detected (18, 6).

The spatial and temporal patterns of CSD dissemination has been remarkably similar to those seen with the spread of CTV, which is mainly transmitted by the aphid *Toxoptera citricida* in Brazil (6). The similarities between the symptoms of CSD and CTV decline lead researchers to suggest that the new disease is probably caused by a new strain of CTV. Alternatively, a different virus could be the causative agent of the CSD disease (6, 30). Several attempts to associate CSD with a CTV mutant have failed (14, 35).

Combining massive shotgun sequencing and bioinformatic tools, we were able to identify a new virus present only in plants from the affected area and strongly associated with CSD symptoms.

MATERIALS AND METHODS

Plant collection for cDNA library construction. A total of 16 sweet orange trees (8 trees of the Pera variety and 8 trees of the Hamlin variety) grafted on Rangpur lime rootstock were studied. All trees originated from the same nursery. Half of the plants of each variety were transported, planted, and grown in the CSD-affected area; the other half were transported, planted, and grown in unaffected areas. The two areas are about 300 km apart. The trees sampled in the affected area had clear disease symptoms (i.e., rootstock with yellow stained bark); those from the unaffected area were asymptomatic. This strategy was designed to allow a direct comparison between trees with similar genetic backgrounds and viral populations, grown in affected and unaffected regions.

Plant collection for epidemiological studies. Trees were selected primarily according to the color of rootstock bark and by several other parameters (see Table 3 and Table 4), and their exact locations were recorded for future analysis.

Aphid and leafhopper collection. Insects were collected in the orchards and transported on ice in plastic bags to the lab, where they were killed by freezing (10 min at −20°C). Aphids were collected as a colony from young flushes. Colonies coming from different flushes in the same tree were considered a single sample. Leafhoppers were collected from the grass and weeds grown between the rows of citrus trees with the aid of a net.

cDNA library construction. The enriched fractions of double-stranded RNAs (dsRNAs) were isolated from 10 g of bark samples by two rounds of CF-11 chromatography (37). The resulting material was further treated to eliminate single-stranded RNAs (ssRNAs) by precipitation with LiCl (2 M) and resuspended in a total volume of 10 μl of DEPC (diethylpyrocarbonate)-treated sterile water.

cDNA was synthesized using 2 μg of dsRNAs as the template and 1 μg of random primers in accordance with the instructions of the Superscript III cDNA kit manufacturer (Invitrogen). After size fractionation of cDNA, larger fragments were ligated in pCR4Blunt-TOPO (Invitrogen) and electroporated into *Escherichia coli* DH10B (Invitrogen).

Sequencing and genome assembly. cDNA inserts from libraries or other cloning products were sequenced in both orientations by use of the dideoxynucleotide termination method (33) and an automatic sequencer (3730xl DNA analyzer; Applied Biosystems). Complete *Citrus sudden death-associated virus* (CSDaV) genome sequence determination was achieved by PCR or reverse transcriptase PCR (RT-PCR) using the cDNA libraries or total RNA from CSD-affected plants as templates, a combination of internal forward and reverse primers (designed on the basis of the sequences from the initial reads), vector universal primers, and degenerated primers designed on the basis of *Marafivirus* genomes (*Oat blue dwarf virus* [OBDV], NC_001793; *Maize rayado fino virus*, [MRFV], NC_002786). The 3′ end was determined by RT-PCR using oligo(dT)–first-strand synthesis, and the 5′ end was determined by 5′ rapid amplification of cDNA ends (Invitrogen) of RNA obtained from partially purified virus preparation.

Viral detection by RT-PCR. Total RNA was extracted from plants (100 mg of tissue) or insects (50 aphid individuals and 10 leafhopper individuals) by use of TRIZOL with a slight modification of the instructions of the manufacturer (Invitrogen); i.e., after TRIZOL extraction, two extractions with phenol-chloroform-isoamyl alcohol and ethanol precipitation were performed. The resulting RNA was suspended in 20 μl of sterile diethyl pyrocarbonate-treated water. The leafhopper total RNA was subjected to treatment with DNase I to eliminate genomic DNA, and a control reaction mixture without reverse transcriptase was included in the experiment.

First-strand cDNA was synthesized with 200 U of reverse transcriptase Superscript II (Invitrogen)–8 μg of total RNA–1 μg of random primers in a final volume of 15 μl. PCR was performed using 1 μl of the synthesized cDNA, 0.5 mM concentrations of each forward and reverse primers, 0.125 mM dNTP, 2.5 mM $MgCl_2$, 1× reaction buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), and 1 U of *Taq* DNA polymerase (Invitrogen) in a final 20-μl reaction mixture. The PCR conditions were as follows: a denaturation step at 94°C for 2 min and 40 amplification cycles (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C). Amplicons were resolved in ethidium bromide-stained agarose gels and checked under UV light.

Detection of CSDaV in samples from plant tissues and insects was performed using the primers TYMOF2 (5′-GTCAGCTGTCCAACCAGTTCC-3′) and TYMORR (5′-GTGAAGATCAATGAGAGCCTG-3′) designed on the basis of the nucleotide sequence of the CSDaV RNA-dependent RNA polymerase (RdRp) domain. As internal controls, the following pairs of primers were used: primer pair p600F1 (5′-TCGCAGTCCACCCTCTCCG-3′) and p600R1 (5′GA CGCTCCTCGAGACGATGG-3′), which amplifies a 557-bp fragment of the CTV genome; primer pair CHSF1 (5′-CCAGCCCAAGTCTAAGATCACC-3′) and CHSR1 (5′-CGCAAGTGTCCGTCAATTGC-3′), which amplifies a 423-bp fragment from citrus chalcone synthase gene; and primer pair pCIF1 (5′-GTA TTGCGACGTTAGAGGTG-3′) and pCIR1 (5′-CATCACAGACCTGTTATT GCTC-3′), which amplifies a 548-bp fragment of 18S rRNA from leafhopper ribosomal gene.

Virus purification. CSDaV particles were partially purified from CSD-affected citrus plants according to the methodology described by Bar-Joseph et al. (4). The morphology of isolated virus particles was determined by electron microscopy using a negative-staining methodology with 2% uranyl acetate (16).

Production of CP antibodies. Peptides were designed on the basis of the deduced coat proteins (CPs) p21 and p22.5 of CSDaV, taking into consideration β-turn structure prediction (11) and hydrophobicity level (24). Peptides corresponding to amino acids (aa) 7 to 20 (PepA [AGPAPSRDDRVDRQ]), 11 to 24 (PepB [PSRDDRVDRQPRLP]), 51 to 64 (PepC [DGSEAKNLSDDLSG]), 111 to 124 (PepD [PASASETSYYGGRL]), and 161 to 175 (PepE [RFSYSVYSNG GTKGT]) of the putative p21 coat protein were synthesized, keyhole limpet hemocyanin conjugated, and used to immunize rabbits (Genscript Corp., Piscataway, N.J.). Polyclonal antibodies were supplied by Genscript Corp. as antisera (AntiPepA, -B, -C, -D, and -E) and used without further treatment for Western blot analysis.

Polyclonal antibodies against the recombinant coat proteins p21 and p22.5 were also obtained. The CSDaV genomic sequences between nucleotide positions 6033 to 6680 (p22.5) and 6090 to 6680 (p21) were cloned into the pET-28a(+) expression vector (ClonTech), and expression was conduced with the *E. coli* BL21(DE3) strain. p22.5 was expressed fused to a His tag and a thrombin site at the N terminus (His-p22.5), and p21 was expressed fused to a His tag at the C terminus (p21-His) and also in a nonfused form (NFp21). Large-scale expression of His-tagged proteins was induced by IPTG (isopropyl-α-D-thiogalactopyranoside), bacterial cells were disrupted using a French press, and the soluble fractions of the recombinant proteins were isolated using a His-Trap purification kit (Amersham). The p21-His and His-p22.5 proteins were used to raise polyclonal antibodies in rabbits.

Northern and Western blotting. Total RNA was obtained from enriched preparation of virus particles by phenol extraction, resolved in a 1% agarose gel, transferred to Hybond-N+ nylon membranes (Amersham), and hybridized against the coat protein p21 nucleotide sequence labeled with [^{32}P]dCTP. Recombinant coat proteins and crude protein extracts from citrus trees were separated in sodium dodecyl sulfate–12% (wt/vol) polyacrylamide gel (25), and proteins were transferred to a nitrocellulose membrane (Hybond-C extra; Amersham) by semidry electroblotting (Bio-Rad apparatus). The membrane was blocked overnight with a Blotto solution (phosphate-buffered saline [PBS] [pH 7.2] containing 0.1% Tween 20 and 5% [wt/vol] nonfat milk powder), incubated with the polyclonal antibodies (1:1,000 in PBS) for 1 h at room temperature, washed three times with PBS-Tween 20 (0.05%), and incubated for 1 h with anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Bio-Rad) (1:2,000 in PBS). Color development was carried out in freshly prepared substrate solution (1.5 mg of Nitro Blue Tetrazolium, 3 mg of 5-bromo-4-chloro-3-indolylphosphate[BCIP] in 20 ml of carbonate buffer [pH 9.2]).

Bioinformatics. Sequence transfer from the automatic sequencer, sequence analysis, and alignment were performed using intramural programs based on BLAST (1), Cross_match (20), and CAP3 (22). CSDaV sequence was assembled with Phrap (20) and edited using Consed (19) programs. Open reading frame (ORF) inspection and annotation resulted from BLAST analysis in comparisons

TABLE 1. Classification of valid reads from cDNA libraries constructed using CSD-symptomatic and asymptomatic citrus trees[a]

Category	Total no. of valid reads	% of valid reads
CTV	53,545	87.01
Citrus mRNA	4,651	7.56
rRNA genes	3,230	5.25
Other viruses	109	0.18

[a] Valid reads are sequences (i) for which a cloning vector was screened; (ii) with an estimated error rate of less than 1 base per 100 bp; and (iii) that were 30 nucleotides in length or longer.

using other virus genomes. Additional alignments were generated using ClustalW (36), mainly to input phylogenetic analyses, and the neighbor-joining method (29). Searches for the putative cleavage sites in the CSDaV polyprotein were done by determinations of amino acid sequence similarities to sequences in the OBDV (15) and MRFV (21) cleavage sites.

Nucleotide sequence accession number. The complete CSDaV nucleotide sequence was submitted to GenBank and has been assigned accession no. AY884005. Utility applications of this sequence are under the protection of international patent laws (26).

RESULTS

Analysis of shotgun sequencing data. A total of 16 cDNA libraries (one library per tree) were constructed from dsRNA-enriched preparations from eight CSD-symptomatic and eight asymptomatic trees. From each cDNA library, about 2,000 randomly selected clones were sequenced from both ends, compared to GenBank nonredundant sequences through the use of BLASTN and BLASTX tools (1), and classified into four categories: rRNA genes, citrus mRNA, CTV, and other viruses (Table 1). The overall number of valid reads sequenced was 61,535; 55.3% of these were from symptomatic trees. The average insert size was 978 nucleotides. Although the dsRNAs were passed twice in CF-11 columns and differentially precipitated with LiCl, a significant amount of residual contamination of plant single-stranded RNA (rRNA and mRNA), representing 12.8% of the valid reads, was still present at the end of the procedure. The majority of the reads were CTV sequences (87%), which were assembled into contigs. Bioinformatic tools were developed to discriminate the sequences coming from affected regions from those coming from nonaffected regions. Although it was possible to observe a significant variability within the CTV genome, mostly at the polyprotein region (unpublished data), we were unable to locate a consistent difference between populations of CTV from infected and those from noninfected areas, which would support the idea of the association of CTV variants as the causative agent of CSD.

TABLE 2. Classification of the 109 reads with similarity to known viruses (except CTV)

Category	No. of reads	No. of trees[a]		Virus family[b]
		Symptomatic	Asymptomatic	
A	84	5	5	*Chrysoviridae* (dsRNA)
B	9	1	4	*Partitiviridae* (dsRNA)
C	6	1	4	*Caulimoviridae* (dsDNA)
D	6	2	0	*Tymoviridae* (+ssRNA)
E	2	0	1	*Bunyaviridae* (−ssRNA)
F	2	0	1	*Totiviridae* (dsRNA)

[a] Numbers of symptomatic trees (out of eight) and/or asymptomatic trees (out of eight) with the virus sequence obtained from the cDNA library source.
[b] +ssRNA, positive single-stranded RNA viruses; −ssRNA, negative single-stranded RNA viruses.

Sequences from viruses, other than CTV, represented 0.18% of all valid reads in a total of 109 sequences. These sequences were clustered into 34 contigs, leaving 21 reads as singlets. These clusters and singlets were further analyzed and categorized into virus families (Table 2). The majority (87.2%) of these sequences matched viruses with dsRNA genomes, which were distributed in three families (*Chrysoviridae*, *Partitiviridae*, and *Totiviridae*). Two families have ssRNA genomes, which comprise 7.3% of these sequences (*Tymoviridae* and *Bunyaviridae*), and 5.5% have a dsDNA genome (*Caulimoviridae*). Most categories of viruses were found in both groups of trees or exclusively in nonaffected trees (Table 2) except the *Tymoviridae*-matching group, which was present only in libraries from CSD-affected materials.

Association between CSD and a new *Tymoviridae* virus. To correlate the presence of the *Tymoviridae* family virus and CSD, a pair of primers was designed on the basis of the sequence obtained during the shotgun sequencing and was used to test 14 trees (7 symptomatic and 7 asymptomatic plants) from our initial collection by RT-PCR. All symptomatic plants tested positive, whereas asymptomatic plants from the nonaffected area tested negative.

To verify the apparent association between CSD and the new *Tymoviridae* virus, a total of 773 plants were sampled from different parts of the states of Minas Gerais and São Paulo (Table 3 and Table 4) and tested by RT-PCR for the presence of the new virus. From 351 plants grafted on Rangpur lime showing disease symptoms and sampled from the affected area, 350 plants were positive for the new virus, resulting in a 99.7% association (Table 3), whereas of 161 trees sampled from nonaffected areas of São Paulo state at various distances from the affected area (50, 100, and 300 km), all were CSDaV RT-PCR

TABLE 3. RT-PCR detection of CSDaV in citrus trees grafted on Rangpur lime rootstock, grown in the affected area (region A) or in unaffected areas (regions B, C, and D)

Region	Type of tree	No. of trees	No. (%) of positive samples
A	CSD-symptomatic trees	351	350 (99.7)
B	Asymptomatic trees grown 50 km away from affected Region A	46	0 (0.0)
C	Asymptomatic trees grown 150 km away from affected Region A	58	0 (0.0)
D	Asymptomatic trees grown 300 km away from affected Region A	57	0 (0.0)
Total		512	350

TABLE 4. RT-PCR detection of CSDaV in asymptomatic citrus trees grown in the CSD-affected area

Type of tree[a]	No. of trees	No. (%) of positive samples
Tree grafted on Rangpur lime rootstock having a CSD-yellow bark symptomatic neighbor tree	176	32 (16.2)
Tree grafted on Rangpur lime rootstock from a CSD-affected block without a symptomatic neighbor tree	16	1 (6.2)
Tree grafted on Rangpur lime rootstock from an unaffected block in a CSD-affected farm	33	0 (0.0)
Tree grafted on Rangpur lime rootstock from an unaffected farm located 1 Km away from a CSD-affected farm	18	0 (0.0)
Tree grafted on Cleopatra rootstock grown in a CSD-affected farm	18	15 (83.3)
Total	261	48

[a] Condition of the tree at the time of sampling.

negative. On the other hand, 33 asymptomatic trees (grafted on Rangpur lime) that were grown in the vicinity of symptomatic trees or within affected blocks tested positive for the virus (Table 4). An inspection performed 7 months after tissue sampling of these asymptomatic CSDaV-positive trees showed that eight trees became symptomatic for the disease (rootstock with yellow stained bark), seven had been eradicated, probably due to the development of the disease, and nine were inarched with tolerant rootstocks. These data corroborate the hypothesis of a strong association between the presence of the new virus and the symptoms of CSD. Therefore, we named the new *Tymoviridae* virus *Citrus sudden death-associated virus* (CSDaV). Asymptomatic trees within affected blocks grafted on tolerant rootstocks such as Cleopatra mandarin and *P. trifoliata* hybrids also tested positive for the virus and probably function as a virus source to propagate the infection, since they do not develop the disease.

Genome sequence and organization of CSDaV. The complete CSDaV genome sequence was obtained by primer walking performed using the cDNA clones from a shotgun library or RT-PCR from total plant RNA. The CSDaV genome is 6,805 nucleotides in length, excluding the poly(A) track. The nucleotide contents of the genomic RNA (gRNA) are high in cytosine and low in guanine (A, 19.7%; T, 23.1%; C, 37.4%; G, 19.8%). The poly(A) track has been identified by synthesis of first-strand cDNA primed with oligo(dT). A stretch of at least 15 adenines was observed, but the actual length of the poly(A) track remains undetermined. A putative eukaryotic polyadenylation signal, AAUAAA (7), is observed starting at nucleotide 6803. Figure 1 shows the CSDaV genome, its organization, and its comparison to *Tymoviridae* representatives belonging to the genera *Tymovirus* (*Turnip yellow mosaic virus* [TYMV]), *Maculavirus* (*Grapevine fleck virus* [GFkV]), and *Marafivirus* (*Maize rayado fino virus* [MRFV], *Oat blue dwarf virus* [OBDV], and *Grapevine asteroid mosaic-associated virus* [GAMaV]). A large ORF (ORF1) between nucleotides 109 and 6675 encodes a protein of 2,188 aa having a predicted molecular mass of 240 kDa (p240). Searches for protein similarities revealed conserved signature domains for methyltransferase (MT) (nucleotide positions 487 to 1119), papain-like protease (PRO) (nucleotide positions 2797 to 3114), NTP binding helicase (HEL) (nucleotide positions 3358 to 4053), RdRp (nucleotide positions 4528 to 5776), and a CP (nucleotide positions 6028 to 6675). Further analysis revealed a small ORF at the 3′ end of the CSDaV genome (nucleotides 6260 to 6724) that encodes a putative protein (p16) of 154 aa with 42% identity with the N-terminal portion of a putative movement protein (p31) from GFkV. There is also, at the 5′end of the CSDaV genome, a reading frame (nucleotide positions 566 to 1621) interrupted by 7 stop codons which translated short polypeptides that have an overall identity of 43% to the putative movement protein (p43) from MRFV (15). A similar reading frame interrupted by stop codons is also found in OBDV and GFkV.

In *Tymoviridae* viruses, the polyprotein translated from the large ORF is processed by its own proteolytic activity at a specific site, separating the RdRp domain from the MT, PRO, and HEL domains (Fig. 1), which remain combined in a single N-terminal polypeptide (27). In addition, members of the genus *Marafivirus* have a large ORF that encodes the replication and structural proteins in the same polypeptide. Thus, an additional proteolysis is necessary to separate the RdRp domain from the CP domain (Fig. 1). A search for proteolytic cleavage sites around positions of the CSDaV p240 that were expected to be processed identified two putative regions. One is located

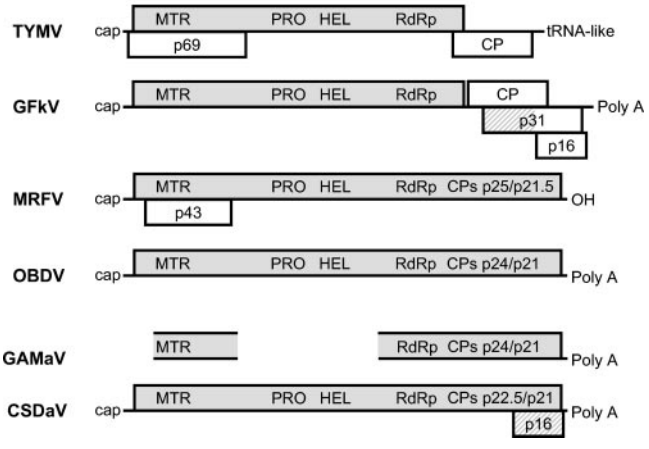

FIG. 1. Schematic representation of the genome organization of CSDaV and viruses of the genera *Tymovirus* (TYMV), *Maculavirus* (GFkV), and *Marafivirus* (MRFV, OBDV, and GAMaV). Each solid gray-shaded box represents a single ORF. The polyprotein domains of methyltransferase (MTR), protease (PRO), helicase (HEL), polymerase (RdRp), and coat proteins (CP) are shown. A movement protein (p69) and putative movement proteins (p43, GFkV p31, GFkV p16, and CSDaV p16) are shown. Regions of CSDaV p16 and GFkV p31 sharing sequence similarities are indicated with gray diagonal lines.

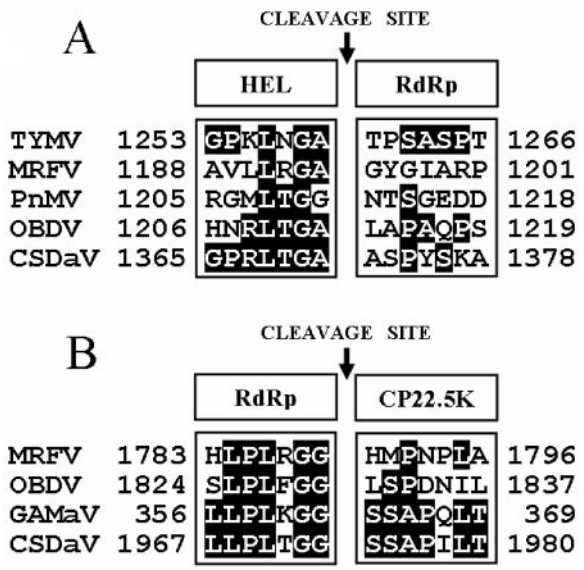

FIG. 2. Putative papain-like protease cleavage sites between the HEL and the RdRp domains (A) and between the RdRp and the CP domains (B). Amino acid numbers with respect to the polyproteins are given. Low numbering for the GAMaV sequence is due to incomplete genome information. Conserved residues are shown in white characters on a black background.

between the HEL and RdRp domains (Fig. 2A), with an Ala residue at position 1371 and another Ala residue at position 1372. Alignment of this CSDaV region with the corresponding region of TYMV (10) and predicted regions of MRFV (21), OBDV (15), and *Poinsettia mosaic virus* [PnMV] (9) shows considerable sequence conservation among the viruses, especially at the left side of the cleavage site (Fig. 2A). A second putative cleavage site was located between the RdRp and CP domains, at aa residues 1973 (Gly) and 1974 (Ser). Alignment of this CSDaV region with the corresponding predicted regions of MRFV (21), OBDV (15), and GAMaV (17) reveals that this region is also highly conserved among marafiviruses, especially between CSDaV and GAMaV, with only two discrepancies out of 14 amino acids. The Gly^{1973} is present in all viruses, and conservation is also more pronounced in the left side of the cleavage site at the RdRp C terminus (Fig. 2B).

The 5′ end of the CSDaV genome has been determined by rapid amplification of cDNA ends from partially purified preparation of viral particles. The presence of an MT domain and a genome starting with a guanidine suggests that CSDaV genomic RNA is probably capped, as has been demonstrated for all members of the family *Tymoviridae* so far analyzed (27). The 5′ nontranslated region (NTR) has 108 nucleotides and is comparable in length to the corresponding regions of other marafiviruses (OBDV, 114 nt; MRFV, 96 nt). The alignment of the CSDaV 5′ NTR with these corresponding sequences revealed no significant identity (data not shown).

Phylogenetic classification of CSDaV. Phylogenetic analyses of the CSDaV polyprotein reveal that this virus is related to members of the *Tymoviridae* family, especially those of the *Marafivirus* genus. The highest levels of identity observed were to MRFV (58%) and OBDV (64%). A comparison of the CP (215 aa) and RdRp (602 aa) domains of CSDaV to corresponding sequences from other viruses within this family confirmed the similarity (data not shown). In both comparisons, identities were higher between CSDaV and members of the genus *Marafivirus* (GAMaV, OBDV, and MRFV nucleotides).

Phylogenetic analysis of the CSDaV CP and RdRp domains described above and of comparable sequences from representatives of the three genera also supports the classification of CSDaV as a new member of the genus *Marafivirus* (Fig. 3). Indeed, the CSDaV virus clustered in a clade clearly separated from the genera *Maculavirus* and *Tymovirus*. Within the genus *Marafivirus*, CSDaV formed a separated branch with the grapevine virus GAMaV and is closer to OBDV than MRFV.

Virus distribution, partial purification, and electron microscopy. The concentration of virus particles is very low, as it was not possible to detect any virus particles in plant tissue by electron microscopy. However, CSDaV was detected by RT-PCR in roots, young flushes, bark (scion and rootstock), fruit peduncles, and leaves. This information enable us to isolate similar amounts of viral particles from roots and young flushes of CSDaV-infected sweet orange trees grafted onto Rangpur lime rootstocks or from asymptomatic sweet orange trees grafted onto Cleopatra mandarin rootstocks grown in CSD-affected groves. Electron microscopy observations of partially purified preparations showed the presence of isometric virions about 30 nm in diameter with a rounded contour and prominent surface structure (Fig. 4A). In similarity to other *Marafivirus*, CSDaV showed the presence of two types of particles: one apparently penetrated by negative staining (the T form, which is similar to the noninfectious empty shell of *Tymoviridae*) and intact particles (similar to the B form). The final confirmation that this virus particle is CSDaV was done by Northern blot analysis of RNA extracts from partially purified virus particle (Fig. 4B).

Production of coat proteins (CPs). Coat protein production in the genus *Marafivirus* is a result of the autoproteolytic processing of the polyprotein encoded by the large ORF and translation from a 3′ coterminal subgenomic RNA (sgRNA) (27). In CSDaV we found p22.5 CP, presumably generated by the proteolytic cleavage of the genomic RNA (gRNA) (Fig. 2B) and p21 CP, encoded by the sgRNA (Fig. 5). Synthesis of sgRNA in TYMV is driven by internal transcription initiation on a promoter located at the complementary minus-sense strand (34). This promoter is characterized by a conserved 16-nucleotide consensus sequence known as a tymobox (for tymoviruses) or a marafibox (for marafiviruses) followed downstream by a transcription initiation site. A putative marafibox is present in CSDaV at positions 5956 to 5971, and a transcription start site is predicted at nucleotides 5980 to 5983. This is well conserved compared to the corresponding domains in GAMaV, OBDV, MRFV, PnMV, and TYMV (Fig. 6). Sequence conservation among marafiviruses is complete in the promoter region, with the exception of CSDaV, which shows a single discrepancy at the 5′ end, where a guanine is exchanged for an adenine. The transcription initiation site seems to be divided into two consensus sequences, preferentially CAAG or CAAU. Transcription of an sgRNA from this putative start site would produce an sgRNA of about 0.9 kb. Northern blots of total RNA extracted from partially purified CSDaV particles were hybridized with the complementary sequence to the 3′ end of the genomic RNA (Fig. 4B). Two distinct bands were

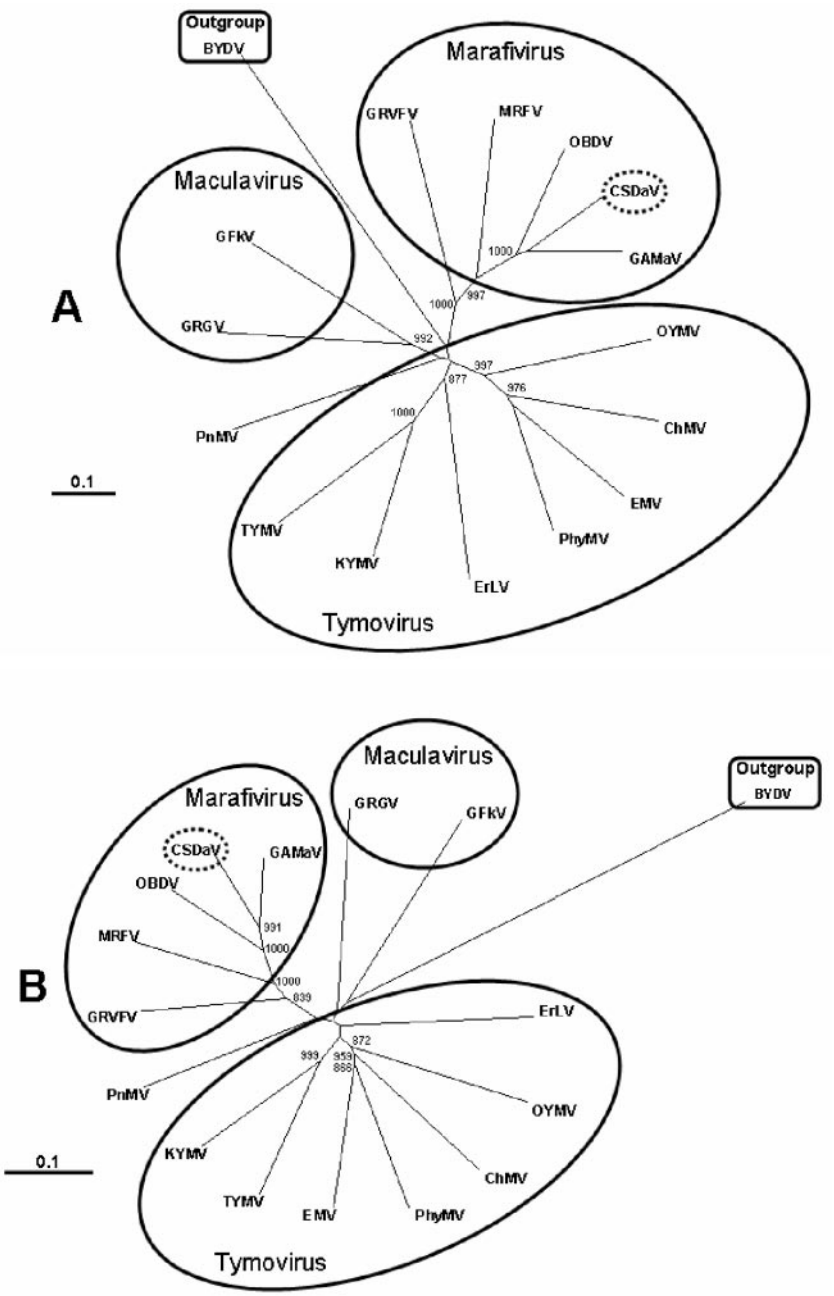

FIG. 3. Phylogenetic relationships among the sequences of the coat protein (A) and RNA-dependent RNA polymerase (B) of the *Citrus sudden death-associated virus* (CSDaV) and viruses belonging to the genera *Maculavirus*, *Marafivirus*, and *Tymovirus* within the *Tymoviridae* family. The numbers on the nodes represent the percentages of 1,000 bootstrap samples of a given species that grouped together. Virus species and EMBL or GenBank accession numbers for the respective coat and RdRp sequences used in the analysis are as follows: *Chayote mosaic virus* (ChMV [NP_067738 and AAF09240]), *Eggplant mosaic virus* (EMV [NP_040969 and AAA43039]), *Erysimum latent virus* (ErLV [NP_047921 and AAC80555]), *Grapevine asteroid mosaic-associated virus* (GAMaV [CAC10493]), *Grapevine fleck virus* (GFkV [NP_542613 and CAC84400]), *Grapevine red globe virus* (GRGV [AAQ08826 and AAQ08825]), *Grapevine rupestris vein feathering virus* (GRVFV [AAN52168]), *Kennedya yellow mosaic virus* (KYMV [NP_044329 and BAA00532]), *Maize rayado fino virus* (MRFV [NP_734077 and AAK52838]), *Oat blue dwarf virus* (OBDV [NP_044448 and NP_044447]), *Ononis yellow mosaic virus* (OYMV [NP_041258 and AAA46796]), *Physalis mottle virus* (PhyMV [P36351 and CAA76071]), *Poinsettia mosaic virus* (PnMV [NP_037648 and CAB70969]), and *Turnip yellow mosaic virus* (TYMV [P03608 and AAB92649]). *Barley yellow dwarf virus* (BYDV [AAF26425 and NP_037635]) was used as an out group.

observed: a 7.0-kb band that may correspond to the CSDaV genomic RNA (gRNA) and a smaller band of about 0.9 kb, corresponding to the sgRNA, that starts at nucleotide position 5980. By Western blot analysis of affected plant extracts developed with antibody against the coat proteins (Fig. 5) we deduced that the sgRNA is translated from the second methionine codon (nucleotide 6082), since one band recognized by the antibody has a size of 21 kDa (p21), which corresponds to

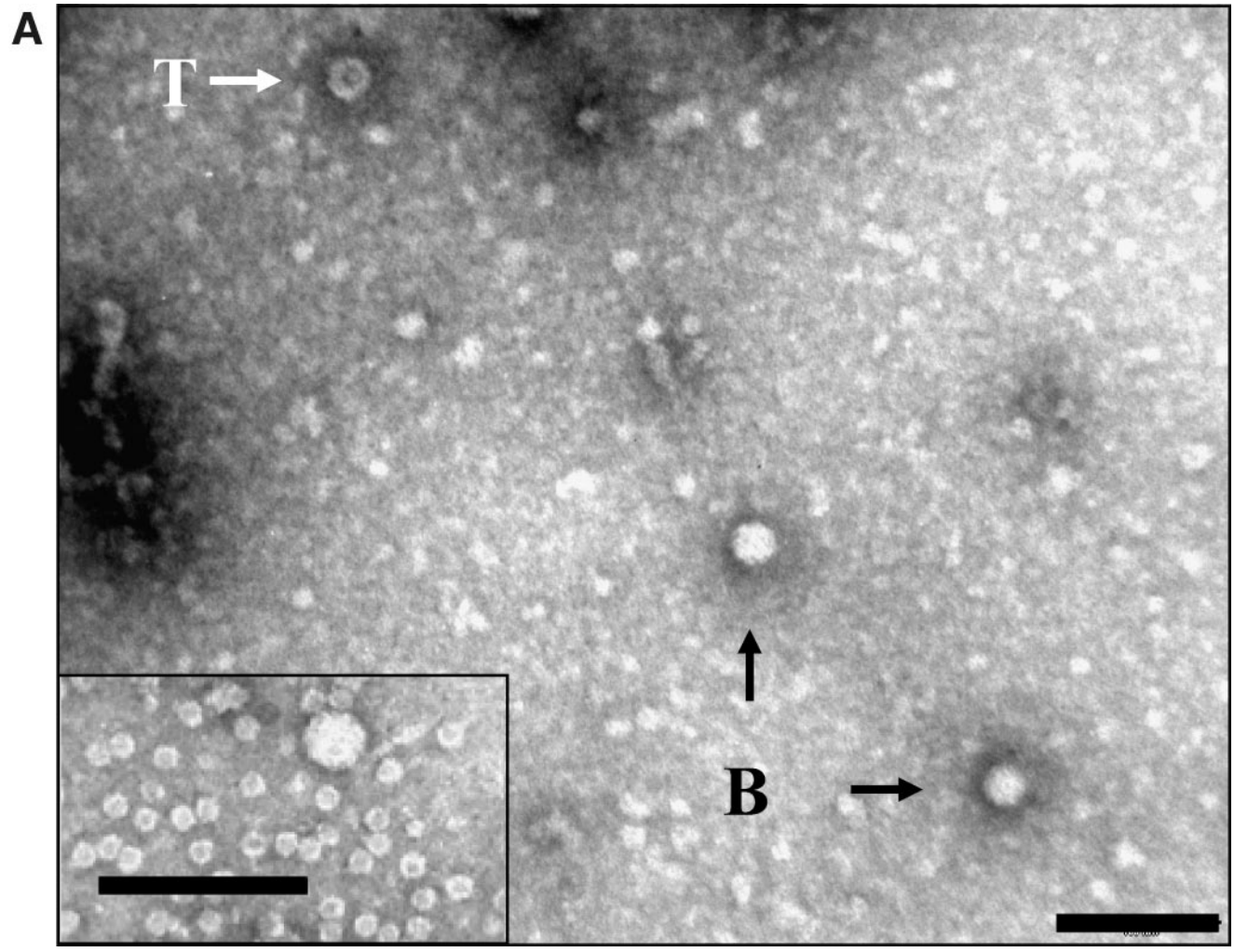

FIG. 4. (A) Electron microscopy of CSDaV purified from infected citrus young flushes. Grids were negative stained with 1% uranyl acetate. T, empty protein shells; B, RNA-containing shells. Bars at the bottom correspond to 100 nm. Magnification, ×23,700 and ×35,550 (insert). (B) Northern blot hybridization of total nucleic acids extracts from three CSDaV preparations of infected trees. The presence of genomic RNA (gRNA) and subgenomic RNA (sgRNA) of expected sizes can be observed. Molecular markers are indicated at the right of the figure.

the size of a protein translated from the second methionine codon. Translation from the first methionine codon would generate a protein of 24 kDa. The other band visualized on the Western blot of affected plant is approximately 22.5 kDa and is likely to correspond to p22.5 coat protein (Fig. 5).

Detection of CSDaV in insects. Leafhoppers are the natural vectors for marafiviruses (27). Thus, a collection of leafhoppers representing members of the subfamilies *Cercopidae*, *Cicadellidae*, and *Deltocephalinae* were sampled from heavily CSD-affected groves and screened by RT-PCR for the presence of CSDaV. All groups tested negative for the presence of CSDaV, whereas internal control amplification of the 18S rRNA gave a band of the expected size for all leafhopper samples analyzed.

Epidemiological studies have suggested that CSD may be transmitted by aphids (6). Although none of the *Tymoviridae* viruses are known to be transmitted by this type of insect, a screening was carried out for the presence of CSDaV in aphids found in citrus groves. Colonies of *Toxoptera citricida*, *Aphis spiraecola*, and *A. gossypii* were collected from citrus trees and

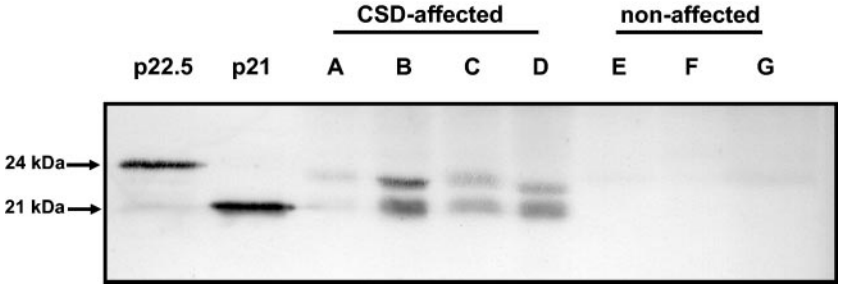

FIG. 5. Western blot detection of the CSDaV CPs from the crude protein extracts of CSD-affected and unaffected citrus trees. Recombinant CPs His-p22.5 (p22.5) and NFp21 (p21) were used as standards. Lanes A, B, C, and D correspond to crude protein extracts obtained from CSD-affected citrus trees. Lanes E, F, and G correspond to crude protein extracts of unaffected citrus trees. A mixture of antisera obtained against synthetic peptides was used for this immunoassay. The recombinant p22.5 (His-p22.5) runs at 24 kDa, because it was fused to a His tag and thrombin site at the N terminus.

assayed by RT-PCR for the presence of CSDaV (Table 5). All samples were positive for the internal controls. None of the colonies and respective host plants collected from unaffected areas tested positive for CSDaV, whereas most of the aphid samples that came from plants that tested CSDaV positive were also positive for the virus. This result indicates that CSDaV could be carried by aphids from affected areas. Further transmission experiments are in progress to clarify the actual ability of aphids to transmit CSDaV, but preliminary transmission results indicate that the virus is probably transmitted by aphids (W. Maccheroni and M. C. Alegria [Alellyx] and P. T. Yamamoto [Fundecitrus], unpublished results).

DISCUSSION

There are two main hypotheses prevalent among researchers to explain the etiological origin of CSD, namely, that it is (i) a variant of CTV (as all the Brazilian citrus trees are preimmunized with mild CTV isolates [12], a new variant might have appeared in Minas Gerais state) or (ii) a new virus of unknown origin (30). Until now, however, electron microscopy or any other methodology has failed to identify a new etiological agent.

In an experimental attempt to test these hypotheses, we used a genomic approach to characterize the sequences of RNA virus population from plants from CSD-affected and nonaffected regions.

Although our strategy could not detect significant differences in the CTV populations in the two groups of trees, we were able to identify a new virus of the *Tymoviridae* family that is strongly associated to CSD. It was named *Citrus sudden death-associated virus* (CSDaV).

The family *Tymoviridae* comprises three genera, *Tymovirus*, *Marafivirus*, and *Maculavirus* (27). Comparison to members of the three genera in terms of genome organization and phylogenetic sequence analysis placed CSDaV in the genus *Marafivirus* and showed that it is more closely related to GAMaV and OBDV. Most distinct features of the genus *Marafivirus* can be identified in CSDaV, such as the presence of a single large ORF translated into a polyprotein with replication complex-protease domains fused to the CP domain and of a sgRNA promoter consensus sequence known as a marafibox and transcription of a 3′ coterminal sgRNA and two CPs, one produced from the cleavage of the C-terminal of the large polyprotein and other translated from the sgRNA. However, some features unique to this new virus should be stressed. CSDaV has a genome size of 6.8 kb, while the genome size in the genus *Marafivirus* ranges from 6.0 to 6.5 kb. An additional ORF was identified in the 3′ end of the CSDaV genome, and the translated putative protein p16 showed significant (42%) identity with the N-terminal half of the putative movement protein p31 of GFkV, a member of the genus *Maculavirus* (32). The putative proteins p16 and p31 of GFkV have been identified on the basis of low similarity with other movement proteins of tymoviruses (32), and no significant similarity was observed between the p16 proteins of GFkV and CSDaV. The in vivo translation of CSDaV p16 or GFkV p31 has not been verified, and the biological implications of the conservation between these putative proteins are still unknown.

In general, tymoviruses accumulate particles in the parenchyma cells and are mechanically transmitted whereas most maculaviruses and marafiviruses are phloem-limited viruses that are not mechanically transmitted (27). Members of the

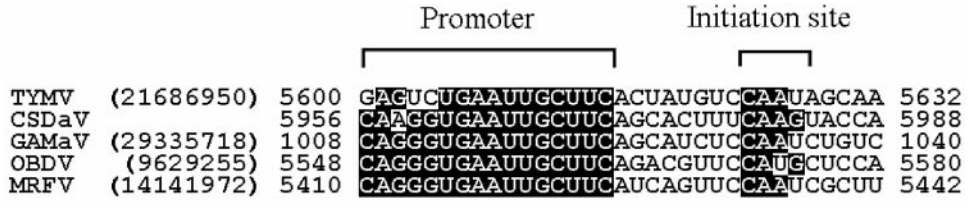

FIG. 6. Sequence alignment of the 16-bp marafibox and the putative sgRNA transcription initiation site of CSDaV and the corresponding regions of other *Marafivirus* species (GAMaV, OBDV, PnMV, and MRFV) and the tymobox of TYMV. Low numbering for the GAMaV sequence is due to incomplete genome information. Conserved nucleotides are shown with white characters on a black background.

TABLE 5. RT-PCR detection of CSDaV in aphids and their feeding citrus plants from CSD-affected and unaffected areas

Aphid species	CSD-affected area result[a]			Unaffected area result[a]		
	Plant	Aphid RNA	Plant RNA	Plant	Aphid RNA	Plant RNA
T. citricida	01	+	+	19	−	−
	02	+	+	20	−	−
	03	−	−	21	−	−
	04	+	+	22	−	−
	05	+	+	23	−	−
	06	+	+	24	−	−
	07	+	+			
	08	+	+			
	09	+	+			
	10	+	+			
A. gossypii	11	−	+	25	−	−
	12	−	−	26	−	−
	13	+	+	27	−	−
	14	−	−	28	−	−
A. spiraecola	15	+	+	29	−	−
	16	+	−	30	−	−
	17	−	−	31	−	−
	18	−	−	32	−	−

[a] +, CSDaV detected; −, CSDaV not detected.

genus *Tymovirus* such as TYMV possess a functional movement protein (MP) (p69) overlapping the large ORF; this supports the idea of a systemic spread of the virus in host plants (8). In MRFV, a putative MP of 43 kDa (p43) was described on the basis of weak sequence similarity with the TYMV p69 (21), and MRFV is the only *Marafivirus* that is capable of invading parenchyma cells (27), even though it is not mechanically transmitted (23). In the genomes of CSDaV, OBDV, and GFkV, a degenerated p43-like ORF interrupted by several stop codons was identified in the present work. Although this is speculative, it is reasonable to hypothesize that the truncated MPs of the three viruses are the remains of an ancestral MP that had degenerated during evolution as these viruses became restricted to the phloem cells.

CSDaV has been partially purified from infected citrus tissues, and the particles showed characteristics that fit well with the descriptions for this family. After infection, CSDaV seems to spread throughout the canopy and root system and can be detected by RT-PCR in most tissues as the plant becomes symptomatic. However, purification procedures resulted in a low yield, probably a reflection of very low titers of the virus in infected tissues. Indeed, all attempts to use electron microscopy to observe virus particles in thin sections of plant tissues or in negatively stained "dip" preparations of crude sap have failed so far (E. W. Kitajima, unpublished data). These observations agree with studies conducted with GAMaV and OBDV, which are marafiviruses phylogenetically related to CSDaV. Low titers of OBDV had been reported for infected host tissues (2), and electron microscopy observations were unable to identify GAMaV particles in infected grapevine cells (31).

Plant tissues infected with high titers of members of the *Tymoviridae* family, such as those of the *Tymovirus* genus, show typical cytopathic structures characterized by mitochondria and/or chloroplasts decorated with vesicles produced by peripheral membrane invaginations (27). In the case of infections with low titers of members of the *Marafivirus* genus, infected tissues present almost imperceptible alterations of mitochondria and/or chloroplasts (31) or hyperplasia and hypertrophy of phloem and adjacent parenchyma cells (3, 38), as seen with GAMaV and OBDV. Anatomical analyses of the bark tissues from CSD-affected trees showed, below the graft union, overproduction and degradation of phloem and collapse and necrosis of sieve tubes and companion cells, but there was no reference to mitochondria or chloroplast morphological alterations (30), which is in agreement with expectations with respect to morphological alterations for marafiviruses. The only exception is that grafting incompatibility has never been described for other marafiviruses.

The CSDaV sequences were readily found in aphids from infected citrus trees and were absent from leafhopper species collected from these areas. This could support previous epidemiological studies which suggested that CSD is disseminated by an aphidborne vector (6). Indeed, preliminary aphid transmission experiments (Maccheroni and Alegria [Alellyx] and Yamamoto [Fundecitrus], unpublished) resulted in transmission of CSDaV from infected to healthy plants. However, as in the case of the *Groundnut rossete umbravirus*, which is dependent on *Groundnut rossete assistor virus* for aphid transmission (28), there is a possibility that CSDaV is using CTV as a helper virus (30).

To our knowledge, all attempts to identify a CTV variant associated with CSD have produced inconclusive or negative results (14, 35). The present work has shown a very strong association between the presence of CSDaV and disease symptoms, strongly suggesting that the presence of the CSDaV is a necessary if not sufficient condition for the development of the disease.

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