Proteomic Analysis Reveals Suppression of Bark Chitinases and Proteinase Inhibitors in Citrus Plants Affected by the Citrus Sudden Death Disease

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Accepted for publication 3 June 2008.

ABSTRACT

Cantú, M. D., Mariano, A. G., Palma, M. S., Carrilho, E., and Wulff, N. A. 2008. Proteomic analysis reveals suppression of bark chitinases and proteinase inhibitors in citrus plants affected by the citrus sudden death disease. *Phytopathology* 98:1084-1092.

Citrus sudden death (CSD) is a disease of unknown etiology that greatly affects sweet oranges grafted on Rangpur lime rootstock, the most important rootstock in Brazilian citriculture. We performed a proteomic analysis to generate information related to this plant pathogen interaction. Protein profiles from healthy, CSD-affected and CSD-tolerant stem barks, were generated using two-dimensional gel electrophoresis. The protein spots were well distributed over a pI range of 3.26 to 9.97 and a molecular weight (MW) range from 7.1 to 120 kDa. The patterns of expressed

proteins on 2-DE gels made it possible to distinguish healthy barks from CSD-affected barks. Protein spots with MW around 30 kDa and pI values ranging from 4.5 to 5.2 were down-regulated in the CSD-affected rootstock bark. This set of protein spots was identified as chitinases. Another set of proteins, ranging in pI from 6.1 to 9.6 with an MW of about 20 kDa, were also suppressed in CSD-affected rootstock bark; these were identified as miraculin-like proteins, potential trypsin inhibitors. Down-regulation of chitinases and proteinase inhibitors in CSD-affected plants is relevant since chitinases are well-known pathogenesis-related protein, and their activity against plant pathogens is largely accepted.

Additional keywords: plant proteomics.

In 2001, the outbreak of a new citrus disease called citrus sudden death (CSD), which affects sweet oranges grafted on Rangpur lime plants, became a threat to the Brazilian citrus industry (16,38). Rangpur lime is the most prevalent rootstock in Brazil, and it is employed in about 85% of the 200 million citrus trees in São Paulo and Minas Gerais states, the largest citrus area in Brazil, which is responsible for more than 53% of the world citrus juice trade. CSD canopy symptoms are not specific, but they have the following typical characteristics: leaves become pale and yellow-green; the trees also show partial defoliation and have fewer new shoots than usual or even no internal shoots, which may culminate in the death of the tree (1). This behavior is related to rootstock phloem degeneration in the bud-union region, and the most specific symptom of the disease seems to be the yellow discoloration in the rootstock bark (16,38) (Fig. 1). Plants with CSD symptoms show aggregation at relatively low incidence; this pattern is suggestive of the action of a vector, as in the case of *Citrus tristeza virus* (CTV), which is vectored by *Toxoptera citricida* (1). Recent surveys estimate that more than 4 million citrus trees have been lost to CSD (2).

CSD is a bud-union disease that resembles CTV in its symptoms, epidemiological characteristics (1), and anatomical features (38). CSD has been found to be graft-transmissible (49), but it remains a disease of unconfirmed etiology. Several attempts have been made to discover CTV variants associated with CSD (1,11, 17,25,26,37). All such studies used canopy and bark symptoms as detection criteria, but they failed to identify CTV strains that were

associated with CSD symptoms. Román et al. (38) also used the criterion of the yellowing of rootstock bark as a diagnostic test of CSD-affected trees to make the claim that CSD is a bud-union disease and to characterize the pathological anatomy. More recently, the impact of CSD damage to fruit production and quality was assessed in a study (3) that also took visual symptoms into consideration. In 2005, a new member of the genus *Marafivirus* (*Tymoviridae*) was discovered in a search for a CTV variant in citrus plants suffering from CSD; it was characterized and named *Citrus sudden death-associated virus* (CSDaV) (25). The first report of a *Tymoviridae* virus infecting citrus was made in 2002, when RNA with high similarity to *Oat blue dwarf virus* (OBDV, genus *Maculavirus*) was described; it shared some characteristics of CSDaV, and it was reported to occur in citrus in the United States (19). To date, it has still not been demonstrated that CSDaV is, in fact, the etiological agent of the disease since Koch's postulates have not yet been fulfilled. CTV has been endemic to Brazil since the late 1930s, and mild CTV isolates have been employed as cross-protecting agents in a strategy to protect against CTV; all citrus plants in the field thus harbor CTV. Although all plants infected by CSDaV are co-infected by CTV, to date, no reports of a single inoculation of CSDaV causing CSD have been made. This implies that CSDaV may not be solely implicated in CSD etiology, as was already pointed out by Maccheroni et al. (25). Taking all these factors into consideration, we chose to rely most on symptoms as a diagnostic test, namely, the yellowing of rootstock bark associated with the canopy symptom, in order to confirm whether a citrus tree of sweet orange scion grafted on a Rangpur lime rootstock is suffering from CSD.

The proteomic approach involves studying protein content, including the identification of the amino acid sequence of the protein, evaluation of possible post-translational modifications (PTM),

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doi:10.1094/PHYTO-98-10-1084

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and further assignment to functional pathways in the cell. Usually, classical proteomic studies are performed by comparative analysis of proteins expressed by an organism under different conditions (e.g., healthy versus disease-affected individuals). Two-dimensional gel electrophoresis (2-DE) is a widely used method in proteomics because it generates a map of proteins that reflects changes in protein expression levels, isoforms, or PTMs. It also permits further structural analysis of isolated proteins by various mass spectrometry approaches (18). Matrix-assisted laser desorption and ionization coupled with tandem time of flight mass spectrometry (MALDI-TOF/TOF) analysis (46) of enzymatically cleaved peptides generates a peptide mass fingerprint (PMF) and tandem MS (MS/MS) fragmentation spectra of selected peptides, thereby enabling protein identification by dedicated software analysis (32). The great advantage of using proteomics to study plant-pathogen interactions is that it permits a focus on the interaction of two agents at the same time during the course of an infection.

Using the resolving power of 2-DE, we analyzed citrus bark in the search for clues about the biochemical status of diseased plants. Since rootstock bark is the plant organ that shows the conspicuous symptom of CSD (the yellow staining) (38) (Fig. 1), we analyzed protein extracted from the stem bark in the bud-union region.

MATERIALS AND METHODS

Plant samples. Citrus stem bark was sampled in 2004 from plants in commercial groves. The scion-rootstock combination that was susceptible to CSD (Rangpur lime grafted with 'Valencia' Sweet orange) was obtained from 9-year-old orchards, and tolerant combinations (Tahiti lime over Rangpur lime or Sweet orange over Cleopatra mandarin) were from 8- or 18-year-old orchards, respectively.

Bark samples were collected from five plants from each orchard described in Table 1. Healthy citrus trees were from a CSD-free area. CSD-symptomatic trees were from a grove affected by CSD in the endemic area. From the same CSD-affected grove, symptomless samples were also collected from plants without visible symptoms, found in the vicinity of affected plants (38). In addition, samples from a tolerant scion-rootstock combination were collected from the CSD endemic area. The location of epidemics is according to published data (1,38; Gavião Peixoto is a municipality adjacent to Araraquara and Matão, 200 km from Frutal, MG, and it is free of CSD). For scion (Sweet orange stem bark) and rootstock (Rangpur lime stem bark), the study had three treatments with five replicates each: healthy, CSD-affected, and CSD-tolerant.

The following CSD-tolerant citrus plants were used as controls: Tahiti lime grafted on Rangpur lime (i.e., CSD-tolerant rootstock control) and 'Valencia' sweet orange grafted on Cleopatra mandarin (i.e., CSD-tolerant scion control) were collected in CSD-affected areas and used as controls for rootstock and scion, respectively (38), since these scion-rootstock combinations do not develop CSD symptoms. CSD-tolerant plants seem to be good controls since they are grown in the same soil and climatic conditions as the CSD-affected plants and under the same vector and pathogen pressure (49); however, they do not develop symptoms (1), not even microscopic ones (38). Symptomless plants from the CSD-affected region cannot be used as controls since at the sampling date (2004), there were no records addressing the pathogenic agent causing CSD; thus, healthy citrus samples were collected far away from the CSD epidemics.

A section 2 cm in width and 10 cm in height spanning the bud-union line was excised from each plant. After washing the sample with tap water, the tissue was dried with paper towels and stored at -80°C . Barks from rootstock and scions were excised and analyzed separately (five plants for each treatment). Samples were ground in liquid nitrogen in a cryogenic mill (Freezer/Mill 6750

SPEX CertiPrep, Metuchen, NJ). Three grinding cycles of 3 min each were applied in order to obtain a fine homogeneous powder.

Rootstock random amplified polymorphism DNA analysis. Total DNA from rootstock bark samples was extracted (12), spectrophotometrically quantified and employed in random amplified polymorphism DNA (RAPD) analysis. To be sure on the nature of rootstock samples analyzed, samples collected as Rangpur lime and Cleopatra mandarin had their RAPD profiles compared (9) with the most common rootstock used by Brazilian citrus growers: Rangpur lime, Volkamerian lemon, Cleopatra Mandarin, sour orange (*C. aurantium* L.), *P. trifoliata*, citrumelo Swingle, and Sunki mandarin. RAPD primers were Operon RAPD 10-mer set (Alameda, CA) and polymerase chain reaction (PCR) was carried out in 25- μl reaction consisting of 100 ng of DNA, 0.2 mM dNTPs, 0.4 μM primer, 3 mM MgCl_2 in 20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 1 U of *Taq* polymerase from Invitrogen. Amplification was performed in an Eppendorf Mastercycler (Hamburg, Germany) after an initial 4 min denaturation step at 94°C , which was followed by 36 cycles of 2 min denaturing at 92°C , 1 min annealing at 37°C and 2 min extension at 72°C . A final extension step of 6 min at 72°C was taken. PCR-amplified DNA was visualized under UV light after electrophoresis in 1.4% agarose in 40 mM Tris-HCl, 40 mM acetic acid, and 1 mM EDTA. DNA amplicons profiles were visually compared after gel image capture and rootstock origin was determined from this comparison.

Protein extraction. One gram of ground bark and 50 mg of polyvinylpyrrolidone (PVPP) were mixed and used for protein extraction with 5 ml of 50 mM Tris-HCl, pH 8.9, containing 4 mM PMSF, 5% sodium dodecyl sulfate (SDS), and 15% glycerol. After gentle stirring, dithiothreitol (DTT) was added to a final concentration of 100 mM, and the mixture was centrifuged for 20 min ($11,000 \times g$) at 4°C . The supernatant was set aside, and the pellet was again extracted with 3 ml of the same buffer and centrifuged. Both supernatants were subjected to precipitation with three volumes of iced acetone with 0.07% (vol/vol) β -mercaptoethanol, and stored for 2 h at -80°C . The acetone-aqueous extract was centrifuged for 15 min ($5,000 \times g$) at 4°C . The pellet was washed twice with 80% (vol/vol) acetone containing 0.07% β -mercaptoethanol and washed twice more with iced acetone containing 0.07% β -mercaptoethanol. The pellets were dried and dissolved in 1.5 ml of buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 50 mM DTT in 5 mM Tris, pH 8.9. The protein concentration of the final supernatant was measured using bovine serum albumin (fraction V) as a protein standard (5). All protein extracts were stored at -80°C .

2-DE and image analysis. IPG strips (13 cm) (GE Healthcare, Little Chalfont, UK) were hydrated for 14 h with 700 μg of protein extract dissolved in 7 M urea, 2 M thiourea, 4% CHAPS (wt/vol), bromophenol blue (BPB) 0.002% (wt/vol), and 50 mM DTT at room temperature. The IEF of 3 to 10 immobilized pH gradient (IPG) strips was performed for 30 min at 200 V, 30 min at a gradient from 200 to 1,000 V, 2.5 h at a gradient from 1,000 to 8,000 V, and then at 8,000 V until a total of 20 kVh was reached. The isoelectric focusing (IEF) of 4 to 7 IPG strips was as follows: 30 min at 200 V, 90 min at a gradient from 200 to 1,000 V, 3 h at a gradient from 1,000 to 8,000 V, and then 8,000 V until a total of 20 kVh. Focused strips were equilibrated using a first incubation step in equilibration buffer (50 mM Tris-HCl, pH 8.9, 6 M urea, 30% glycerol [vol/vol], SDS 2% [wt/vol], 0.002% BPB [wt/vol] containing 100 mM DTT for 15 min, followed by another 15 min incubation step in the same equilibration buffer containing 250 mM indole-3-acetic acid (IAA) instead of DTT.

The second dimension was performed on a 12.5% polyacrylamide SDS gel (Tris-glycine system; 20×20 cm) for IPG strips 3 to 10 (24). Tris-tricine 10% polyacrylamide SDS gels were employed for IPG strips 4 to 7 (41) using the Protean II system

(Bio-Rad, Hercules, CA). The BenchMark protein ladder from Invitrogen (Carlsbad, CA) was used as the molecular weight marker on all gels.

After SDS-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis (run at 4°C), gels were stained by CBB colloidal staining (6). The stained gels were scanned (all gels with the same parameters for intensity, brightness, and contrast) and analyzed using ImageMaster 2D Software (GE Healthcare). Each treatment (healthy, CSD-affected, and CSD-tolerant) group included the analysis of five plants with reproducible and high quality 2-DE gel patterns for each IPG range (five replicas for each treatment in pI 3 to 10 and five replicas in pI 4 to 7). ImageMaster 2D was used to measure spot volume from selected spots and to compare gel patterns between groups. The average value of five replicates followed by standard deviation is presented. The 2-DE profiles obtained for sweet orange scion bark of healthy, CSD-affected, and CSD-tolerant trees were compared to each other; 2-DE profiles of Rangpur lime rootstock bark from the three treatments (healthy, CSD-affected, and CSD-tolerant) were also compared.

In-gel protein digestion. Down-regulated proteins (spots numbered in Result section) were manually excised with pipette tips (cut according to the spot size) and destained in 300 µl of 50 mM NH₄HCO₃, 50% ACN, dehydrated with 50 µl of ACN and dried in a vacuum centrifuge. Twenty microliters of a trypsin solution (20 µg ml⁻¹ in 50 mM NH₄HCO₃, 5 mM CaCl₂) was added (400 ng of trypsin) and digestion was carried out at 37°C for 22 h under gentle shaking. After the gel pieces were spun down, the supernatant was transferred to another tube, and the gel

was extracted twice with 50 µl of a solution containing 60% ACN and 1% TFA (vol/vol) (44). After vortexing for 3 min at low speed, supernatants were collected, pooled, and desalted in a lab-made zip tip. A piece of glass wool was compacted at the bottom of a pipette tip (200 µl), and 100 µl of a suspension of C-18 particles (30 to 65 µm) in isopropyl alcohol at 100 mg ml⁻¹ was passed through the tip. The desalting process was initiated by conditioning the C-18 with 200 µl of methanol followed by 200 µl of 0.5% TFA. Samples were applied and the salt was washed out by passing 200 µl of 0.5% TFA through the tip. Elution of peptides was carried out with an 80% ACN/1% TFA solution; the volume of the extracted peptides was then reduced to 5 to 10 µl by centrifugal evaporation.

MALDI-TOF-TOF and database query. For each protein spot, 1 ml of the digested protein solution was mixed with 1 µl of an ACN solution containing 5 mg ml⁻¹ α-cyano-4-hydroxycinnamic acid, and 0.5 µl was spotted in a 192-well MALDI plate, dried and analyzed in a mass spectrometer (4700 Proteomics Discovery System, Applied Biosystems, Foster City, CA). The laser power (Nd:Yag – 355 nm) was adjusted to produce a better signal for both MS and MS/MS experiments. PMF of singly charged peptide ions (21) was collected from monoisotopic peaks falling in the *m/z* range from 980 to 4,000 Da with a resolution of about 13,000 and mass accuracy better than 15 ppm. The resulting mass spectrum of the five most intense peptides (MS/MS) was obtained over the mass range from 20 up to the mass of the parent ion, with a resolution and mass accuracy of about 2,000 and 50, respectively. The reflectron mode was used in all analyses. After

Fig. 1. Citrus sudden death (CSD) symptom in a ‘Valencia’ sweet orange grafted on Rangpur lime rootstock. **A**, A longitudinal section through the stem bark of a CSD-affected tree, showing the yellow discoloration in the phloem tissue in the rootstock. **B**, Cambial face of bark scratched from a healthy citrus tree (left) and a CSD-affected (right). The black line indicates the union between the scion and rootstock.

TABLE 1. Description of samples, organs, location, and disease status

Citrus tree (scion/rootstock)	Taxonomic origin	Organ sampled	Location	Citrus sudden death (CSD) epidemics	CSD status grove
Sweet orange/Rangpur lime	<i>C. sinensis/C. limonia</i>	Scion bark/rootstock bark	Gavião Peixoto, SP	Free	Healthy
Sweet orange/Rangpur lime	<i>C. sinensis/C. limonia</i>	Scion bark/rootstock bark	Frutal, MG	Endemic	CSD-affected (symptomatic and symptomless)
Tahiti lime/Rangpur lime	<i>C. latifolia/C. limonia</i>	Rootstock bark	Prata, MG	Endemic	CSD-tolerant (healthy)
Sweet orange/Cleopatra mandarin	<i>C. latifolia/C. reshui</i>	Scion bark	Prata, MG	Endemic	CSD-tolerant (healthy)

the analyses, the data from each spot (MS and MS/MS) were exported in a text file format. The algorithm used for the sequence database search was the MASCOT MS/MS Ion Search version 2.0 (32), against the National Center for Biotechnology Information (NCBI) all entries database using the following parameters: missed cleavage = 1, peptide mass tolerance = 0.1 Da, and MS/MS mass tolerance = 0.3 Da (33). Carbamidomethylcysteine was set as a fixed modification since the sulfhydryl groups of cysteine residues react with IAA. Methionine oxidation was evaluated as a variable modification due to the oxidative environment of sample preparation.

RESULTS

The RAPD profiles confirmed that all analyzed rootstock samples were Rangpur lime and Cleopatra mandarin (9), as indicated by the farm managers. Scions were identified according to their morphologic phenotype since no molecular markers for sweet orange varieties were available.

The yield of total protein extraction was about 3.2% from fresh bark tissue. Proteins with pI values ranging from 3.27 to 9.97 and MW ranging from 7.27 to more than 100 kDa were resolved. These values are consistent with the theoretical range expected for 10 to 12.5% polyacrylamide gels. In addition, only a few spots over 100 kDa were resolved, which is also in agreement with the limited range of resolution of the IPG strips used for IEF.

IPG strips 3 to 10 were employed to compare CSD-affected, healthy, and CSD-tolerant patterns of proteins extracted from scions and rootstocks. Figure 2 shows the 2-DE maps of proteins

from both the scions and the rootstocks from CSD-affected, healthy, and CSD-tolerant trees. All 2-DE gels showed a broad distribution of spots in a pI range from 3.2 to 9.45 and a mass range of 8 to 127 kDa. There was a remarkable difference between the 2-DE patterns of healthy and diseased plants, indicating that the composition of proteins of the rootstock bark from plants affected by CSD is altered (Fig. 2D to F). Similar, but less pronounced, differences were also observed in scion bark (mainly in the 20 kDa region) (Fig. 2A to C). In addition, the protein pattern obtained for CSD-tolerant plants was consistent with the pattern of healthy plants; this is expected since the control plants are tolerant to CSD disease. Considering the intrinsic biological variability among plants under natural conditions, in which citrus scions are clonally propagated from buds, versus citrus rootstocks, which are obtained from seeds, the pattern exhibited a high level of reproducibility given that five samples were evaluated for each treatment.

This comparative proteomic analysis allowed us to conclude that there was a strong difference in the levels of specific proteins, especially in the two regions where the differences in the levels of proteins were remarkable: such differences are more pronounced in rootstock than in scion. Taking this previous result, we preceded with rootstock bark samples for further analysis. The expression level of a set of proteins with pIs between 6.1 and 9.6 and an MW of about 20 kDa in CSD-affected plants (Fig. 2A and D) was lower than in healthy plants (Fig. 2B and E). CSD-tolerant samples (Fig. 2C and F) showed relative volumes that were slightly higher than healthy one, although this difference is not significant, which indicates that the observed changes between

Fig. 2. Protein profiles in two-dimensional gel electrophoresis gels obtained for the scion bark of 'Valencia' sweet oranges: **A**, CSD-affected, **B**, healthy, and **C**, CSD-tolerant; and for the rootstock bark of Rangpur lime: **D**, CSD-affected, **E**, healthy, and **F**, CSD-tolerant. Samples were analyzed by employing immobilized pH gradient strips, pH 3 to 10, and 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

healthy and CSD-affected plants are related to the diseased status of the samples and should not be attributed to the environment (Fig. 3). It is also important to note that CSD-tolerant trees are a different combination of scion and rootstock than Sweet orange on Rangpur lime (Table 1). The effect of different scion–rootstock combinations on protein composition is unknown. Figure 2 corresponds to the most representative gel (pI from 3 to 10) from each treatment, and a detailed section of a representative gel from each treatment is shown in Figure 3.

When analyzing rootstock profiles, the comparison of the relative volume of nine selected spots in Figure 3 indicates that CSD-affected plants have a reduced content of such proteins. Although the difference is visually striking, when measuring relative spot volume, spots 6, 7, and 9 have a pronounced reduction in expression in CSD-affected bark; spots 1, 2, and 4 have lower means in CSD-affected plants, but overlapping standard deviation with healthy plants. Spots 3, 5, and 8 have the same mean value between healthy and diseased plants (Fig. 3).

The level of proteins with pI values ranging from 4.5 to 5.2 and with MW from 28 to 40 kDa in CSD-affected plants was lower than in healthy samples (Fig. 4). Such alterations were more pronounced in barks from rootstocks than from scions (Fig. 2). In order to better evaluate the number of spots in which the proteins were down-regulated in CSD-affected plants, we performed the IEF step with a narrow pH range between 4 and 7, aiming to achieve better resolution of the acidic proteins extracted from rootstocks (Fig. 4). This approach proved to be essential in order to resolve proteins in the 4.5 to 5.2 pI range. As stated above, the differences between healthy and CSD-affected protein spots were remarkable, and we clearly detected 13 spots in the 28 to 32 kDa region (Fig. 5). Spots 2 to 6 and 8 had a significantly lower spot volume in CSD-affected bark, whereas the remaining spots contained similar amounts of protein in healthy and CSD-affected plants. Figure 4 corresponds to the most representative gel (pI from 4 to 7) from each treatment, and a detailed section of a representative gel from each treatment is shown in Figure 5.

Fig. 3. Enlargement of a specific region of the two-dimensional gel electrophoresis gels/immobilized pH gradient to 10 from Figure 2, for citrus sudden death (CSD)-affected, healthy, and CSD-tolerant rootstock bark. The enlarged region shows proteins with pIs ranging from 6.1 to 9.6 and molecular weight around 20 kDa, and 9 of these spots. The graph illustrates the relative spot volume for each spot in the treatments and data are presented as the average of five replicates with standard deviations.

Fig. 4. High-resolution protein profiles by two-dimensional gel electrophoresis gels from the rootstock bark tissue of Rangpur lime: **A**, citrus sudden death (CSD)-affected, **B**, healthy, and **C**, CSD-tolerant. Samples were analyzed by employing immobilized pH gradient strips pH 4 to 7 and 10% Tris-tricine polyacrylamide gel electrophoresis.

To validate our data, we analyzed the rootstock bark from five symptomless plants that were collected in the same plot as the affected plants (i.e., in the CSD endemic region). Although three out of five plants showed patterns typical of healthy samples (as in Fig. 2E), two plants showed a protein pattern characteristic of CSD-affected plants (as in Fig. 2D). This indicates that the alteration in the protein profile is a biochemical alteration that occurs before a canopy symptom becomes conspicuous (data not shown); such a pattern is not observed for healthy tree bark collected where CSD is not present.

The two sets of differentially expressed protein (spots numbered in Figures 3 and 5) were submitted to MALDI-TOF/TOF analysis and MS/MS spectra comparison. Proteins highlighted in Figure 3 (spots 1 to 9) were identified by MS/MS as putative miraculin-like protein (accession ABL67650), which are homologs of the Kunitz family of proteinase inhibitors. These proteins had scores up to 189, which is well above the cut-off value for identification (32). Four peptides (DYLVSAIR, ALAVVLVPGNER, TSIINEDVDLNVNR, and NELCPLDVLVQLSSDSER) were sequenced by tracking their y -series of ions, yielding an overall coverage of 28% of the amino acid sequence from protein ABL67650. In addition, the pI and MW values of the miraculin-like protein ABL67650 (pI 8.18 and MW of 23610) are in accordance with the experimental 2-DE data (pI ranging from 6.1 to 9.6 and MW around 20 kDa). Since different spots with different pI values and nearly the same MW were identified as the same protein (putative miraculin-like), this protein either undergoes PTM or the spots represent expression of a multigene family.

Spots 2 through 13 (Fig. 5), corresponding to the set of proteins with pIs ranging from 4.5 to 5.2 and MW around 30 kDa, were identified as chitinases by MS/MS (accession T10106). These proteins displayed scores up to 116, allowing a secure identification (32). The pI 5.06 and MW of 32459 of chitinase T10106 are in good agreement with experimental 2-DE data, namely, a pI ranging from 4.5 to 5.2 and an MW around 30 kDa. Figure 6 shows the PMF and the MS/MS spectrum of a singly-charged protonated molecule of m/z 1,177.6 of the identified chitinase. In this case, the peptide amino acid sequence (IGFFTTFC*GK) was

completely determined by tracking the y -series of ions. The other two peptides were correctly identified (GPIQLSWNYNYLR and EMFDDLLEYR+oxidation(M)), and the overall coverage for chitinase was equal to 11.3%. Either chitinase isoforms or chitinases with PTM were present since protein spots with different pI values were identified as the same protein.

Protein sequencing by mass spectrometry (de novo sequencing) is an emerging technology that adds a new approach to the characterization of proteins from citrus and their hybrids.

DISCUSSION

A comparison of protein profiles between healthy and CSD-affected bark allowed us to perform the first biochemical characterization of citrus responses to CSD disease. The expression of two classes of pathogenesis-related proteins (PR-proteins), chitinases, and proteinase inhibitors, is severely down-regulated in diseased rootstock bark.

To our knowledge, only a single study related to the proteomic study of woody tree bark tissue has been published (14), in addition to a few papers dealing with proteins from green and aged plant tissues (20,48). Comprehensive protein extraction from woody plant tissues is difficult because rupture of the cell wall is required, but the proteins must be intact in solution in the presence of proteases, phenolic compounds, and other secondary metabolites. Working with bark tissues may pose additional problems due to tissue age and the consequent cell rigidity caused by lignification. However, the protocol employed here was satisfactory in extracting proteins over a wide range of pI and MW and was thus proven to be useful for proteomics studies.

All citrus plants in Brazil are infected with the CTV, and the impact of such an infection at the protein level has not yet been evaluated in citrus trees. If, in fact, a new virus (CSDaV), a new strain of CTV, or both are the cause of CSD (1,25), a combination of several agents may interact to lead to CSD:CTV, CSDaV, and scion-rootstock combination. Due to the limited information about the etiology of CSD (1,11,25,38), we chose to use tree symptoms as the criterion for sample collection because it is a well-reported characteristic (1,3,11,16,25,26,38,49).

Fig. 5. Image enlargement of a specific region of the two-dimensional gel electrophoresis gels/immobilized pH gradient 4 to 7 from Figure 4, for citrus sudden death (CSD)-affected, healthy, and CSD-tolerant rootstock bark. The region has a pI from 4.5 to 5.2 and a molecular weight around 30 kDa, and 6 of 13 spots are down-regulated in CSD-affected plants. The graph illustrates the relative spot volume for each spot in the treatments. The relative volume data are presented as the average of five replicates with standard deviations.

It is clear that there are consistent modifications in protein profiles of healthy and diseased plants. Such alterations follow a distinct change in the structure of secondary phloem in the bark of Rangpur lime rootstock (38). Measuring spot volume is a useful way to compare spots from replicates since natural variation may occur among plants. Analysis of protein profiles allowed us to detect protein expression alterations more easily in rootstock barks than in scion barks.

Cooper et al. (10) identified a protein spot that corresponded to the coat protein of tobacco mosaic virus; it was present only in 2-DE gels from infected *Nicotiana tabacum*, indicating a high accumulation of viral particles in that host (10). In the case of CSD, no differential spot for infected bark was detected in the MW and pI range corresponding to coat proteins of CTV or CSDaV. This indicates the low titer achieved by these viruses in the CSD-affected citrus (25).

Plant protease inhibitors are PR-proteins belonging to family 6. These proteins are both developmentally regulated and induced in response to insect and pathogen attack. Such proteins are thought to be involved in protein turnover (house-keeping function), blockage of the activity of digestive enzymes, and many are active against insect herbivores and pathogens. Such proteins are usually encoded by multigene families (40). In citrus, cDNAs of putative protease inhibitors have been described for *C. paradisi* flavedo (43). In *C. sinensis*, upon leafhopper herbivory, putative protease inhibitors as well as chitinase genes were up-regulated (29). In *C. jambhiri*, two cDNAs encoding miraculin-like proteins were detected in fruits but not in leaves (47).

Plant chitinases are well-known PR-proteins, belonging to PR-protein families PR-3, PR-4, PR-8, and PR-11 (22); together with β -glucanases, they are induced upon pathogen attack (45). PR-proteins of several classes have been identified by MS in tomato-*Fusarium oxysporum* interaction (36), tomato fruits infected with TMV (7), and rice leaves infected with *Magnaporthe grisea* (23).

Chitinases are markers of disease resistance and systemic acquired resistance (39) due to their ability to break down fungal cell walls by hydrolysis of *N*-acetyl- β -D-glucosamine 1,4- β -linkages (42). Some chitinases are also able to carry out hydrolysis of 1,4- β -linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycans, through action as lysozymes (4) or through their ability to deacetylate chitins, thereby acting as chitosanases (27,31). Moreover, as part of the multicomplex defense reaction of plants, the roles of chitinases are not restricted to preventing pathogen growth since they are constitutively expressed in several tissues, such as healthy floral organs. However, not all proteins ascribed as chitinases exhibit chitinase/chitinolytic activity (22).

Several citrus chitinases have been cloned (35) or purified (28,31). In grapefruit flavedo (*C. paradisi*), both chitinases and β -1,3-glucanases were present as acidic and basic forms (28). In grapefruits, UV irradiation, and wounding induced expression of chitinases; whereas the level of β -1,3-glucanase remained unchanged (34). While the expression of chitinase was demonstrated at the mRNA level for citrus bark chitinases (30), our results are the first to report expression of chitinases at the protein level in the bark of Rangpur lime rootstock and sweet orange scion. Furthermore, to our knowledge, expression of proteinase inhibitor proteins in such tissues has not yet been reported in citrus. Citrus trees are perennial woody plants that have to handle pathogen and insect attacks over their entire life span. The constitutive expression of a defense mechanism in plants represents a way to overcome such attacks; thus, some PR-proteins may be constitutively expressed, such as chitinases and β -1,3-glucanases (15). Both classes of proteins identified in this study (Figs. 3 and 5) are among the most abundant in citrus bark.

In poplar, chitinases and the Kunitz trypsin inhibitors were among the most abundant wound-induced ESTs (8), indicating up-regulation of the defense reaction. In *Prunus armeniaca*, the

Fig. 6. Matrix-assisted laser desorption and ionization coupled with tandem time of flight mass spectrometry (MALDI-TOF-TOF) analysis of spot 12 (Figure 5, healthy sample). **A**, Peptide mass fingerprint (PMF), **B**, tandem MS (MS/MS) spectrum of tryptic peptide $[M+H]^+$; m/z 1,177.6 (indicated by an arrow in **A**), with the deduced amino acid sequence. C* represents the carbamidomethylcysteine.

Plum pox virus (PPV) represses the expression of a putative class III chitinase at the mRNA level in infected plants of the partially resistant genotype Goldrich, while it is expressed in the susceptible cv. Screara (13). In CSD-affected plants, down-regulation of such defense responses was clearly shown. However, we were unable to find any previous study addressing chitinase suppression at the protein level in a plant-pathogen interaction in woody perennial plants.

Protein identification by MS/MS, along with previous reports (31,28), led us to assume that citrus bark has a large number of chitinase isoforms since PR-proteins might be expressed as isoforms (15). Eight acidic isoforms (31) and seven basic chitinases (27) were purified from cell cultures of *C. sinensis* cv. Valencia. The reported acidic chitinase isoforms with MW between 26 and 37.4 kDa and pIs from 4.5 to 5.4 (31) are consistent with our data (Fig. 3), and they may represent PTM, as has been previously proposed (31). Previous studies utilized cell cultures of *Citrus* spp. (27,31) or grapefruit flavedo (28). 2-DE enabled us to demonstrate expression of chitinases and proteinase-inhibitors in citrus bark but also revealed several isoforms in the bark tissue of Rangpur lime rootstock and 'Valencia' scion adult trees.

Because CSD is a disease with unknown etiology, probably viral (1,25,38), the pattern of chitinase and proteinase inhibitor suppression in CSD-affected plants is remarkable. Our data show that, in CSD-affected bark, defense reactions (i.e., expression of chitinases and miraculin-like proteins) have been suppressed. Such an effect was not observed in tolerant scion-rootstock combinations, even though such plants were growing in the presence of pathogen pressure (Figs. 2 to 5); nor ever in healthy plants from outside the epidemic.

This is the first report of a proteomic approach to studying citrus trees, and the results are remarkable since the observation of chitinase as well as the suppression of a miraculin-like protein/Kunitz trypsin inhibitor highlights the suppression of PR-proteins in citrus plants with CSD. Once these PR-proteins are down-regulated in CSD-affected plants, it is possible that these plants become more susceptible to infection by secondary pathogens such as fungi since profuse root rot is observed in affected plants. The roles of such proteins in citrus physiology have not yet been assessed.

Down-regulation of the identified proteins was stronger in Rangpur lime bark than in Sweet orange bark, indicating that this is a more sensitive region than the scion bark, a further indication of the incompatibility of Rangpur lime with scion (38) in the presence of the pathogen. Furthermore, a difference in down-regulation between rootstock and scion indicate this deregulation to be a specific response of citrus to CSD and does not represent a declining response due to root rot caused by CSD. The applicability of such results can be seen by the analysis of five symptomless bark samples collected in an affected grove. The protein profile of two samples was identical to that of CSD-affected plants, while the three remaining were healthy-like profiles. This is also an indication that biochemical alterations precede the yellowing of bark, as is the case for the anatomical alterations (38).

Understanding the molecular events that lead to diseases such as CSD is essential since the citrus industry is based on the low genetic variability of clonally multiplied scion citrus and the restricted availability of rootstock. Losing the predominant rootstock Rangpur lime to disease is a key concern for both the growers and the citrus industry in Brazil. Down-regulated proteins can be useful as negative markers to identify CSD affected trees. Our next steps will be aimed toward the evaluation of mRNA expression levels and enzyme activities of such groups of PR-proteins at different levels of CSD symptoms and relate this study to the occurrence of CSDaV in affected plants.

ACKNOWLEDGMENTS

We thank the Brazilian Synchrotron Light Laboratory (LNLS) for the use of Mass Spectrometry Laboratory under proposal MAS-3213 and A. R. A. Nogueira (CPPSE - Embrapa) for the use of the Freezing Mill. We thank A. Sanches for helping collecting samples in the field, M. de A. Santos for technical assistance, and D. C. Teixeira and E. Martins for RAPD protocol. M. D. Cantú acknowledges financial support and fellowship from FAPESP.

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