Novel Diagnosis for Citrus Stubborn Disease by Detection of a *Spiroplasma citri*-Secreted Protein

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ABSTRACT

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Citrus stubborn disease (CSD), first identified in California, is a widespread bacterial disease found in most arid citrus-producing regions in the United States and the Mediterranean Region. The disease is caused by *Spiroplasma citri*, an insect-transmitted and phloem-colonizing bacterium. CSD causes significant tree damage resulting in loss of fruit production and quality. Detection of CSD is challenging due to low and fluctuating titer and sporadic distribution of the pathogen in infected trees. In this study, we report the development of a novel diagnostic method for CSD using an *S. citri*-secreted protein as the detection marker. Microbial pathogens secrete a variety of proteins during infection that can potentially disperse systemically in infected plants with the vascular flow. Therefore, their distribution may not be restricted to the pathogen infection sites and could be used as a biological marker for infection. Using mass spectrometry analysis, we identified a unique secreted protein from *S. citri* that is highly expressed in the presence of citrus phloem extract. ScCCPP1, an antibody generated against this protein, was able to distinguish *S. citri*-infected citrus and periwinkle from healthy plants. In addition, the antiserum could be used to detect CSD using a simple direct tissue print assay without the need for sample processing or specialized lab equipment and may be suitable for field surveys. This study provides proof of a novel concept of using pathogen-secreted protein as a marker for diagnosis of a citrus bacterial disease and can probably be applied to other plant diseases.

Additional keywords: bacterial pathogens, effectors.

Citrus stubborn disease (CSD) is a prevalent disease of citrus grown in arid or semiarid climates. Since its first report in 1915 in California, CSD has been found in most citrus-producing areas, including California, Arizona, North Africa, the Mediterranean Basin, and the Middle East (5,7,30). Although comprehensive studies on the impact of CSD on commercial production of citrus is limited, it is generally accepted that the damage depends on the severity of infection (42,54). Reduction in orange cumulative yield due to CSD has been reported at 19 to 100%, with reduction in fruit weight and size at 6 to 30 and 12%, respectively. In addition, entire groves have been destroyed as a CSD management strategy in central California; therefore, economic damages due to CSD can be substantial (12,34,42,55).

Symptoms of CSD can vary with season, citrus cultivar, and disease severity. In general, CSD-affected trees are stunted and often flattened across the top. Stems have shortened internodes with smaller leaves that are usually mottled. Most fruit drop while very small. The few fruit that could reach maturity are misshapen and abnormally matured with aborted seed (7,10,54). These disease symptoms can be easily confused with those associated with nutritional deficiencies or other bacterial diseases such as huanglongbing (HLB) (also known as citrus greening) (7,8,11, 13,45). Therefore, it is important to develop robust disease detection for CSD.

The CSD causative agent *Spiroplasma citri* belongs to a group of cell-wall-less bacteria that are collectively called mollicutes. Mollicutes, including phytoplasmas and spiroplasmas, are pathogens that cause many important diseases in plants and animals (28). In addition to *S. citri*, *S. kunkuii* is a major pathogen of maize and *S. phoeniceum* is responsible for periwinkle yellows. *S. citri* is injected into the phloem sieve tubes by phloem-feeding leafhoppers that are infected by the bacterium (33). The main economic hosts of *S. citri* include the major commercial citrus species, including grapefruit, lemon, mandarin, orange, and sour orange (21). *S. citri* has also been detected from carrot in Washington (37) and California (43), with symptoms of general stunting, rosette formation, and yellow-purple discoloration on leaf margins (37); and isolated from horseradish with brittle root disease with symptoms of foliar chlorosis, stunting, and discoloration in the phloem ring of affected roots (24). This incidence reflects the adaptation of *S. citri* to new hosts.

Although CSD has been a research subject for decades, robust diagnosis of the disease remains challenging. In recent years, polymerase chain reaction (PCR)-based detections have been developed using primers amplifying the highly abundant spiralin gene (25,47) or multicopy genes encoding membrane proteins (56). However, PCR-based methods, which require the presence of pathogen cells or DNA in the plant sample tested, may result in erratic detection because *S. citri* has highly uneven distribution in infected trees, especially when titer is low (7,30,55). Other more definitive diagnostic methods include bioindexing and culturing in artificial media, both of which are time consuming and labor intensive (7). Although sensitivity of *S. citri* to antibiotics in vitro and remission of disease symptoms in planta have been demonstrated similar to other phloem-colonizing and insect-transmitted bacterial pathogens, there is no practical cure for CSD once a tree becomes infected. Therefore, the control of the disease is heavily dependent on preventative and roughing measures which, in turn, are based on accurate and early detection (4,6,9,19,32,38,54). As such, new diagnostic methods that can robustly detect CSD are

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figures 1 and 3 appear in color online.

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urgently needed for field surveys and quarantine practices to ensure clean source trees for the industry.

Serological detections using antibodies targeting pathogen-specific proteins support economical and accurate diagnosis. Several antibodies were generated using *S. citri* cell cultures as the antigens (18,23,49); however, a detection kit developed using some of these antibodies failed to detect some California isolates that are commonly used as positive controls (47), suggesting that these antibodies are not suitable for accurate diagnosis. Here, we set out to identify pathogen proteins that can be used as markers for such serological detection. We are particularly interested in proteins that are secreted from *S. citri* into the phloem because these secreted proteins could be dispersed throughout the infected trees along with the vascular flow and, thereby, are not restricted to the infection sites. As such, they could be used as promising detection markers by overcoming uneven distribution of the pathogen cells in infected trees.

Many bacterial pathogens rely on the secreted proteins (effectors) to cause disease on plant and animal hosts (22). Some effectors are injected directly into host cells through specialized protein secretion apparatus, such as the type II and III secretion systems (1). These specialized protein delivery systems are not present in *S. citri* (48). This is not surprising because, as an intracellular pathogen injected into phloem by insect vectors, *S. citri* proteins secreted through the general Sec secretion system are readily located inside the plant tissues. Similarly, specialized protein delivery systems are also absent in other intracellular pathogens, including '*Candidatus* Liberibacter', the bacterium associated with HLB (20,40).

Systemic movement of the secreted proteins by insect-transmitted and phloem-colonizing bacteria has been reported in phytoplasmas, the closest relatives of spiroplasmas. The aster yellows phytoplasma secretes 56 proteins through the Sec secretion system into the phloem (3). Two of these effectors significantly contribute to plant defense suppression and disease symptom development (41,53). Remarkably, although phytoplasma cells are restricted to the phloem sieves, these effectors can systemically move along the phloem and be transported to shoot and root meristem. Moreover, some phytoplasma effectors can also be uploaded from phloem to the neighboring tissues and manipulate physiological processes in these plant cells (31,41). Thus, effectors produced by phloem-colonizing bacterial pathogens can have profound impacts on plant development and systemic resistance.

In order to explore the concept of using secreted proteins of bacterial pathogens as detection markers for diagnosis, we identified a unique Sec-secreted protein of *S. citri* by mass spectrometry. An antibody, called ScCCPP1, was generated against this secreted protein and used to detect specific signals from *S. citri*-infected citrus trees using a simple direct tissue print assay. The signals were mainly from the phloem-rich tissues, consistent with the primary location of the pathogen (26). Importantly, ScCCPP1-based detection gave comparable, and in some cases, more reliable and consistent results than the PCR-based assays and was also superior to an antibody generated against the spiralin protein, a major *S. citri* cell membrane component. These data demonstrate that ScCCPP1 antibody can detect CSD in the absence of *S. citri* cells and, therefore, is an excellent marker for disease diagnosis.

MATERIALS AND METHODS

Plant materials. Sweet orange (*Citrus sinensis* L. Osb.) and pummelo (*C. maxima* Burm.) that were graft inoculated with *S. citri* strains C189, S600, and S616 were used as *S. citri* source plants and maintained at the Rubidoux quarantine greenhouse of the Citrus Clonal Protection Program (CCPP) at the University of California (UC) Riverside (32 to 38°C during the day and >27°C at night). Inoculum from these *S. citri* source plants was used to graft inoculate sweet orange seedlings that were subsequently used in the present study. Inoculated and healthy sweet orange plants were then maintained under standard greenhouse conditions at the UC Riverside Agricultural Operations greenhouse at temperatures of 18 to 35°C.

Periwinkle (*Catharanthus roseus*) 'Jaio Dark Red' plants were propagated by seed and mechanically inoculated with *S. citri* strain C189 axenic culture on the stems of 3-month-old seedlings using a biojector gun (http://www.bioject.com/) as described below. Both the inoculated periwinkle plants and the noninoculated controls were maintained at the UC Riverside Agricultural Operations greenhouse.

Finally, plant materials from naturally infected sweet orange trees planted in 1983 in field 12B of the UC Riverside Agricultural Operations were also used in this study in October 2012 and April 2013.

Bacterial cultivation. *S. citri* stain C189 was isolated from a CSD-symptomatic citrus tree of the CCPP disease bank. Phloem-rich tissues (leaf midveins and petioles) were excised, surface sterilized, and diced with a sterile razor blade before being suspended in 10 ml of LD8 broth medium (36). The suspension was passed through a 0.45-μm filter, then incubated at 30°C. Presence of *S. citri* was confirmed after 3 and up to 14 days by examining 10 to 20 μl of the culture under a dark-field microscope (×1,000) (Leica DMLB) for the presence of motile, helical spiroplasma cells.

Periwinkle inoculation. We used a biojector 2000 injection system gun to inoculate periwinkle plants with *S. citri* strain C189. Each plant was injected three times with 200 μl of bacterial inoculum with the concentration of *S. citri* at $\approx 10^9$ CFU/ml. To prepare the inoculum, *S. citri* cells were collected from 240 ml of 3-day-old culture grown in LD8 medium by centrifugation at 9,000 × *g* for 30 min. The precipitated cell pellet was resuspended in 15 ml of fresh LD8 medium, which was then loaded in a biojector for inoculation. Plants inoculated with clean LD8 medium (mock infected) were used as negative controls. DNA and proteins were extracted from newly emerged tissues away from the inoculation sites at 0 and 8 days postinoculation (dpi), and thereafter at 14-day intervals for a total of 110 days.

Secreted protein extraction. Proteins secreted from the *S. citri* cells in liquid culture in the presence of citrus phloem extracts were analyzed using mass spectrometry. Phloem extracts were made by cutting off a long branch from a healthy sweet orange seedling. Five grams of the phloem-rich outer layer (i.e., bark) was separated from the branch and ground in a mortar and pestle in liquid nitrogen. The tissue powder was suspended into 3 ml of distilled water and incubated on ice for 1 h. The supernatant of the phloem extract was then filter sterilized and stored in –80°C. *S. citri* cells were collected from 3 ml of 1-week-old culture grown in C-3G medium (39) and suspended in 500 μl of 0.3 M sucrose supplemented with 250 μl of the phloem extract at 30°C without shaking for 24 h. Secreted proteins induced in the presence of citrus phloem extracts were identified by comparing the relative abundance in the induced versus noninduced samples. Three independent biological replicates were analyzed.

Supernatant of the induced cell culture was collected by centrifugation at 10,000 rpm for 5 min. The cells were further eliminated from the supernatant portion by repeating the centrifugation. Proteins in the cell-free supernatant were precipitated by ice-cold trichloroacetic acid (TCA)-acetone (50) using a modified protocol. Briefly, the supernatant was mixed with nine volumes of acetone containing 10% TCA and 20 mM dithiothreitol (DTT) at –20°C overnight. Proteins were precipitated by centrifugation at 6,800 × *g* for 5 min at 4°C. The pellet was washed twice with ice-cold acetone containing 20 mM DTT, air dried for 5 min, then dissolved in 50 mM NH_4HCO_3 solution.

Proteomic analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel slices containing candidate-secreted proteins from the phloem extract-induced *S. citri* cells

were analyzed by mass spectrometry for protein identities. Relative abundance of individual proteins was estimated based on the number of peptides from each protein that were detected. The identities of the secreted proteins were determined using a Bruker UltraReflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Karlsruhe, Germany) operated in reflection mode. The proteins were digested with porcine trypsin, then solubilized in 2 µl of 0.5% trifluoroacetic acid. Tryptic peptides were subjected to mass spectrometry analysis. Peptide mass fingerprints were analyzed and searched against the theoretical spectra of *S. citri* strains GII3-3X (17) and the unassembled shotgun library sequencing (W. Ma, *unpublished data*) of another *S. citri* strain S616. Protein identification was performed using the standard pattern-matching approach.

Antibody evaluation. Polyclonal antibody ScCCPP1 was generated against the protein CAK98563 at Genemed Synthesis, Inc. (San Antonio, TX). The antibody was evaluated using healthy, graft-inoculated, and naturally CSD-infected field citrus, as well as periwinkle plants mechanically inoculated with *S. citri* axenic culture by Western blots or immunoblots with direct tissue print.

For Western blot, phloem-rich tissues (bark and midribs) were excised from stems and leaves of surface-sterilized citrus or periwinkle and ground in liquid nitrogen. The tissue powder was suspended in 2× Laemmli buffer (35) and the total proteins were separated by 12% SDS-PAGE. For immunoblots with direct tissue print, 6 to 11 young branches were collected around the canopy of individual citrus trees. Each branch was cut with a sterile razor blade and the fresh cross sections were immediately pressed on a nitrocellulose membrane (Plant Print Diagnostics S.L., Spain). The presence of *S. citri*, as indicated by the presence of the CAK98563 protein, was detected using the ScCCPP1 antibody as the primary antibody and a horseradish peroxidase-conjugated goat-anti-rabbit immunoglobulin G (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as the secondary antibody. The dilution concentrations of primary and secondary antibodies in Western blots and imprint assays were 1:500 and 1:2,000, and 1:3,000 and 1:30,000, respectively. The binding of the antibodies with their target proteins were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) following the manufacturer's instructions.

Quantitative qPCR for *S. citri* detection. Total nucleic acid was extracted from citrus and periwinkle using an optimized procedure for citrus phloem tissues modified from a previously reported protocol (44). The system utilized Cryo-station and Geno Grinder 2010 (SPEX SamplePrep, NJ), the MagMAX Express-96 (Life Technologies, Carlsbad, CA), and the MagMAX-96 Viral RNA Isolation Kit (Life Technologies). Briefly, 250 mg of plant tissue (midveins or midribs) was placed in a 2-ml Eppendorf tube and submerged in liquid N_2 for 30 s. Two 5/32-in. stainless-steel grinding balls were added in each tube along with 600 µl of guanidine extraction buffer and then placed in the cryo-blocks of Geno Grinder 2010, where the tissue was ground for 20 to 30 s at 1,750 rpm. The crude homogenate was then centrifuged at 4°C for 30 min on a bench-top centrifuge at 14,000 rpm. The supernatant was subjected to total nucleic acid extraction, as described by Osman et al (44). Total nucleic acid was suspended in 50 µl of elution buffer.

A quantitative PCR (qPCR) assay using a fluorogenic (TaqMan) probe was developed to improve the detection of *S. citri*. Two primers, CCPPscitriJFD-F (5′-ATTGCAGCACCTGC AACTGTAG-3′) and CCPPscitriJFD-R (5′-TGTTTTTACAA CTCCTTGCACTGC-3′), and a probe, CCPPscitriJFD-P (5′-AC AGCGTTAGAAGCTAAT-3′), were designed to amplify the spiralin gene of *S. citri* (GenBank accession numbers AF012877, AM285305, AM157770, U13996, U13994, U13995, U13998, FJ755921, U13997, HM641856, HM641861, HM641857, HM641859, HM641854, HM641860, HM641855, and HM641858) using Beacon Designer (Premier Biosoft). The probe was labeled with FAM dye on the 5′ end and with minor groove binder nonfluorescent quencher on the 3′ end (Applied Biosystems).

Each TaqMan qPCR reaction consisted of 300 nM forward primer, 600 nM reverse primer, 200 nM probe, 2 µl of DNA extract from plant tissue, or 1 µl of *S. citri* cell culture in a total volume of 20 µl of 1× iTaq universal probe supermix (Bio-Rad). The PCR reactions were performed on a CFX96 Real-Time PCR System (Bio-Rad). Control samples in each run included DNA extracted from CSD-infected and healthy plants and *S. citri* culture.

In order to estimate the *S. citri* DNA concentration in unknown samples and determine the detection threshold, a standard curve of TaqMan qPCR was generated following a protocol by Applied Biosystems. For this purpose, a partial spiralin gene was cloned into the pGEMT-Easy vector (Promega Corp.) and serial dilutions of the recombinant plasmid DNA were used to generate the standard curve.

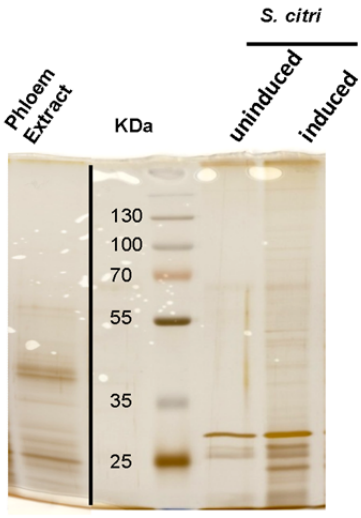

Fig. 1. Protein secretion of *Spiroplasma citri* was induced by citrus phloem extract. *S. citri* cells cultivated in artificial medium were suspended in 0.3 M sucrose solution and incubated in the presence or absence of phloem extracts. Secreted proteins in the supernatant portion of the cell suspension were precipitated and separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel was stained with silver nitrate for visualization.

RESULTS

Identification of secreted proteins from *S. citri*. *S. citri* can be cultivated in artificial media (36,39), which allowed us to experimentally analyze the secreted proteins using a proteomic approach. The cells were induced in 0.3 M sucrose solution because it did not interfere with the subsequent proteomic analysis and also mimicked the major solute composition in the phloem. We further added a crude phloem extract from sweet orange seedlings in order to mimic the environmental condition of the habitat of *S. citri*. Indeed, in the presence of phloem extract, more secreted proteins were observed from *S. citri* compared with the noninduced cells (Fig. 1), indicating that *S. citri* secreted specific proteins during infection.

According to mass spectrometry results from three independently prepared samples, we identified candidate *S. citri*-secreted proteins with relatively greater abundance, especially in the presence of phloem extracts (Table 1). These proteins were further analyzed for their identity.

Identification of Sec-secreted proteins. Similar to other insect-transmitted bacterial pathogens, *S. citri* does not have specialized protein delivery systems (48). Therefore, we assumed that most of the secreted proteins are secreted in a Sec-secretion system-dependent manner. Sec-secreted proteins contain a con-

served N-terminal signal peptide that can be readily predicted using established bioinformatics tools, such as SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP-4.0/) (46) and SOSUIsignal (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/) (29). This peptide cannot be detected from the candidate sequences identified from our proteomic analysis because the Sec-secreted proteins are cleaved at a peptidase recognition site following the signal peptide immediately after secretion (51).

To determine potential Sec-dependent secretion of the candidate proteins, we examined their complete sequence for the presence or absence of Sec signal peptide at the N termini. The corresponding gene sequences encoding the candidate-secreted proteins were extracted from the genome sequences of *S. citri* strains GII3-3X (17) and S616 (W. Ma, *unpublished data*). The deduced amino acid sequences were analyzed using the program SignalP 4.0. The presence of a putative Sec signal peptide was further confirmed using another program, SOSUIsignal. The sequence analysis confirmed that all six of the *S. citri* proteins with relatively high abundance in the presence of citrus phloem extract carried an N-terminal Sec signal peptide (Table 1). Furthermore, these six proteins also have the conserved peptidase recognition site in their N-termini following the Sec signal peptide, suggesting a high likelihood that they would be secreted in a Sec-dependent manner.

Detection marker development. To select the candidate-secreted proteins as detection markers, we chose proteins that can fulfill the following criteria: (i) relatively higher abundance and stability indicated by larger number of peptides detected in the mass spectrometry analysis, (ii) unique sequence for spiroplasmas or *S. citri* with no homologous genes in plants or other organisms, (iii) conserved in the *S. citri* strains (GII3-3X and S616) whose partial sequences are available, and (iv) absence of transmembrane domains. Some Sec-secreted proteins contain transmembrane domains and are anchored to the cell membrane after being secreted from the cell (3). These proteins are not suitable for detection under our working hypothesis. Based on these criteria, we focused on the protein CAK98563 for further development as a CSD detection marker.

To generate antibodies against CAK98563, we cloned the gene excluding the predicted N-terminal Sec signal peptide into an *Escherichia coli* expression vector pET28a (Novagen). However, the recombinant protein was insoluble when overexpressed in *E. coli*; therefore, a different strategy was used. We directly generated a polyclonal antibody against two synthesized peptides within the CAK98563 sequence excluding the predicted signal peptide. This antibody, called ScCCPP1, was able to detect CAK98563 from *E. coli* expressing the recombinant protein or from the cell culture of *S. citri* (Fig. 2A). Importantly, Western blots developed with this antibody confirmed that CAK98563 was induced by the phloem extract and secreted to the medium by *S. citri* growing in liquid culture (Fig. 2A).

In addition to ScCCPP1, we generated a polyclonal antibody against spiralin, one of the most abundant cell-membrane proteins of *S. citri* (25). Similar to ScCCPP1, the anti-spiralin antibody also strongly detected the spiralin proteins from *E. coli* expressing the recombinant protein and from the cell culture of *S. citri* (Fig. 2B). In contrast to CAK98563, spiralin was not induced by the phloem extract, consistent with its function as a major component of the cell membrane. The anti-spiralin antibody, which only detected the *S. citri* cells, was used as a comparison with ScCCPP1, which was hypothesized to detect CSD in the absence of *S. citri* cells.

Evaluation of ScCCPP1 for CSD detection. Direct tissue print immunoblot is an effective, antibody-based detection method for field surveys because of the use of simple experimental equipment and sample preparation (14–16,27). We imprinted young branches and petioles on nitrocellulose membrane and incubated them with either ScCCPP1 or anti-spiralin antibody. ScCCPP1 gave clear positive signals from citrus graft inoculated with both S616 and C189 *S. citri* strains, whereas the healthy tissues didn't show any signal (Fig. 3A). In addition, no signal was detected with the anti-spiralin antibody for all the samples, including the healthy and the *S. citri*-graft-inoculated trees (Fig. 3A). The failure of detection by anti-spiralin antibody was likely due to the low titers or the absence of *S. citri* cells in these particular samples. Nonetheless, ScCCPP1 successfully detected CSD-infected trees using the same samples.

We next evaluated ScCCPP1 for CSD detection using both graft-inoculated and naturally infected trees from the field using direct tissue prints. First, we examined healthy samples from three different citrus species—navel orange (*Citrus sinensis* L. Osb), sour orange (*C. aurantium* L.), and mandarin (*C. reticulata* Blanco)—and samples graft infected with *S. citri* strains S616, S600, and C189 (Fig. 3B). ScCCPP1 could clearly distinguish uninfected trees from the infected trees, which showed positive signals from multiple stem prints. Furthermore, the detected signals were located in the phloem area of the stems (notice the clear area of the pith in the center of the stem print). This was consistent with the primary localization of *S. citri* as a phloem-

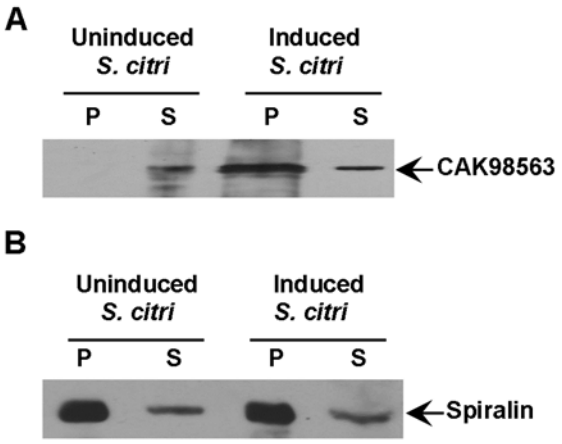

Fig. 2. Antibodies raised against *Spiroplasma citri* proteins CAK98563 and spiralin could detect the corresponding proteins in *S. citri* cell culture. **A,** CAK98563 was present in the supernatant (S) portion of the cell suspension and highly induced by the phloem extract. **B,** Spiralin was mainly present in the cell pellet (P) and its abundance was unchanged in the presence of phloem extract. The relatively small amount of spiralin that was also detected in the supernatant (S) portion was likely due to cell debris from broken cells after centrifugations.

TABLE 1. Candidate-secreted proteins of *Spiroplasma citri* that were identified by mass spectrometry in three biological replicates

Accession number	Location	Signal peptide prediction[a]	Predicted cleavage site	Number of peptide hits in mass spectrometry		
CAK99824	Chromosome	0.843	23	1	12	13
CAK99227	Chromosome	0.998	29	5	1	8
CAK99727	Chromosome	0.906	30	3	1	1
CAL00019	Chromosome	0.75	27	2	3	3
CAK98956	Chromosome	0.99	29	3	1	1
CAK98563	Chromosome	0.777	23	1	5	5

[a] Signal peptides were predicted by SignalP 4.0 and then further confirmed by SOSUIsignal.

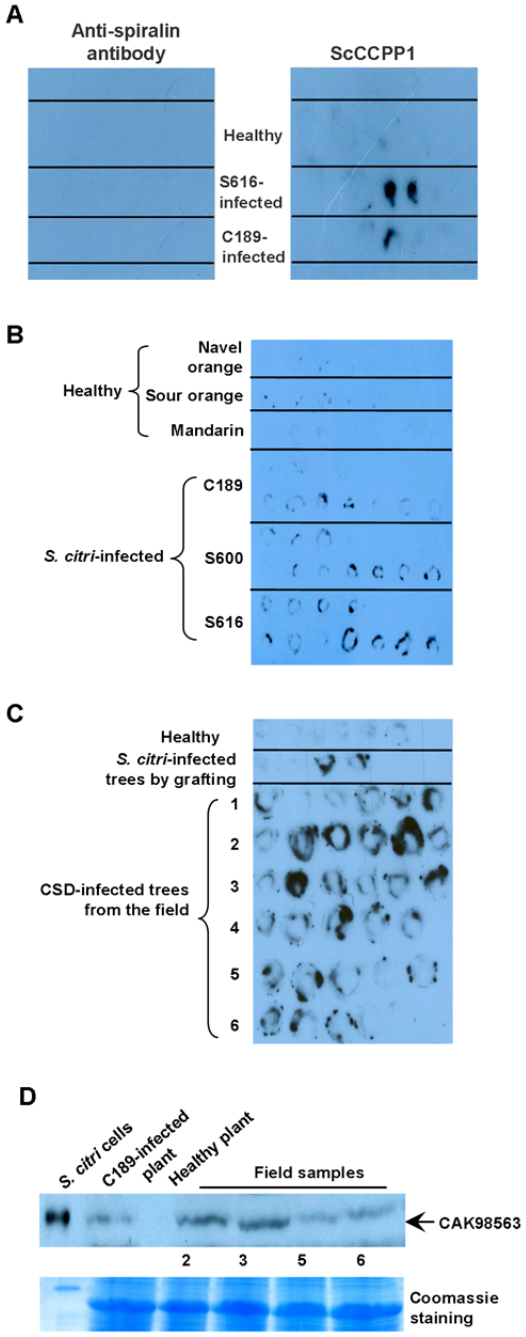

Fig. 3. ScCCPP1 antibody successfully and specifically detects *Spiroplasma citri* using direct tissue prints of leaf petioles and stems. **A,** Three stems and two leaf petioles from sweet orange (*Citrus sinensis* L. Osb.) seedlings graft inoculated with two *S. citri* strains (S616 and C189), respectively were printed on nitrocellulose membranes. The membranes were incubated with antibodies generated against CAK98563 (ScCCPP1) or spiralin. Anti-spiralin antibody failed to give positive signals from the same samples, suggesting that these samples do not contain pathogen cells or low titers. **B and C,** Stem imprints showing positive signals from the naturally infected citrus stubborn disease (CSD) field sweet orange trees. Up to 11 stems from each of the healthy or *S. citri* graft-inoculated trees (B) or CSD naturally infected field trees (C) were printed on membranes, which were then incubated with the ScCCPP1 antibody. Four of the six field trees were also examined using Western blots (D). Positive signals present exclusively in the regions corresponding to the phloem-rich tissues (barks) of a branch, where the bacterium and, presumably, the CAK98563 protein should be located. Also note that not all tested branches exhibited positive signals, indicating the sporadic distribution of the disease. Samples were collected in October 2012 or April 2013. **D,** Western blots detecting the CAK98563 protein from proteins extracted either from a citrus seedling that was grafting-inoculated with *S. citri* (lane 2) or from four CSD naturally infected adult trees from the field (lanes 4 to 7). Proteins extracted from *S. citri* cells (lane 1) were used as a positive control. Arrowhead indicates the position of CAK98563 based on its predicted molecular weight.

limited pathogen and its secreted proteins. We next examined six adult trees that were known to be naturally infected with CSD from the field using the imprint assay (Fig. 3C). Again, all six naturally infected trees showed positive signals from the phloem area of the stem prints using ScCCPP1.

We further confirmed the detection of CAK98563 in *S. citri*-infected citrus by Western blots. Total proteins were extracted from the phloem-rich tissues of healthy or infected trees, including a graft-inoculated citrus with *S. citri* strain C189 and four naturally infected trees (Fig. 3C, numbers 2, 3, 5, and 6) that tested CSD positive using the direct tissue print assay (Fig. 3C). For all the samples from CSD-infected trees, we could detect a specific signal at ≈43 kDa in Western blots, which is the expected size of CAK98563 (Fig. 3D). A signal with the same size was also detected in the cell culture of *S. citri* strain C189 but it was absent in the healthy tree, further confirming that CAK98563 was, indeed, present in the CSD-infected trees.

We next compared the ScCCPP1-based imprint method with a newly developed TaqMan qPCR using the same citrus samples, including three graft-inoculated citrus plants and nine field trees. Six of these nine field trees (samples 4 to 9) are known to be naturally infected with CSD (i.e., they have typical CSD infection symptoms and tested positive for CSD at least once previously). Our results showed that all graft-inoculated citrus plants tested positive by both methods, as expected (Table 2). Furthermore, all six naturally infected trees from the field (samples 4 to 9) tested positive in the imprint assay; however, one of these trees tested negative by qPCR, demonstrating that the imprint assay, in this case, has a lower false-negative rate than qPCR. Samples 1 to 3 are adult trees from the field, and it was not known whether or not they were CSD infected. Sample 1 tested positive in both assays, suggesting that this tree was also naturally infected with CSD. Sample 3 tested negative in both assays; therefore, it is likely uninfected. Sample 2 tested negative by qPCR but positive by the imprint assay. We will monitor the potential disease symptoms and retest this tree at a later time to determine whether it is, indeed, CSD infected.

Evaluation of ScCCPP1 to detect *S. citri* infection in periwinkle. In order to confirm the detection of *S. citri* by ScCCPP1 in periwinkle, mechanical inoculation of periwinkle with *S. citri* strain C189 was monitored for the presence of CAK98563 in a time course experiment. Samples were collected after biojector inoculation at 8 and 20 dpi from newly emerged tissues, away from the inoculation sites, and DNA was extracted and tested by qPCR. Of the three plants that were inoculated, two (namely, E1 and E2) tested positive by qPCR at 8 dpi, indicating that the pathogen was established in the plants (Fig. 4A). Furthermore, E1 still tested positive by qPCR at 20 dpi, suggesting that this plant was successfully infected by *S. citri*. On the other hand, plant E3

TABLE 2. Comparison of quantitative polymerase chain reaction (qPCR) and direct tissue print results for citrus field samples[a]

Samples	qPCR	Imprint
Negative control	–	–
Positive control A (C189)	+	+
Positive control B (S600)	+	+
Positive control C (S616)	+	+
Field sample 1	+	+
Field sample 2	–	+
Field sample 3	–	–
Field sample 4	+	+
Field sample 5	–	+
Field sample 6	+	+
Field sample 7	+	+
Field sample 8	+	+
Field sample 9	+	+

[a] Citrus seedlings graft inoculated with three *Spiroplasma citri* strains were used as positive controls.

tested negative in both time points. Importantly, using ScCCPP1, both E1 and E2 but not E3 tested positive at 8 weeks post-inoculation (Fig. 4B). Furthermore, E1 seemed to show a stronger signal than E2, consistent with the more persistent infection of *S. citri* developed in E1 (Fig. 4A).

DISCUSSION

The citrus industry is facing major threats from severe invasive diseases caused by bacterial pathogens such as HLB and citrus variegated chlorosis, as well as emerging endemic bacterial diseases such as CSD (52). These diseases are especially difficult to manage because the causal pathogens are transmitted by prolific insect vectors and reside in the plant vascular systems. Because there is no cure for these diseases once trees are infected, early detection becomes extremely important for disease management. However, detection of these pathogens is further complicated due to the uneven distribution or low titers in infected trees coupled with large seasonal variation. The sporadic distribution of the pathogens is specifically challenging for diagnosis of trees. In fact, pathogen cells frequently cannot be detected even in symptomatic branches or leaves. Moreover, nucleic-acid-based assays require complex sample preparations, which make them costly and time consuming, especially when multiple samples have to be collected and tested from one tree to compensate for erratic pathogen distribution and titer (4,6,45,55).

In this article, we report the novel concept of using pathogen-secreted proteins as detection targets and the development of a novel antibody-based diagnostic method that can detect CSD using a preparation-free direct tissue print assay. CAK98563 was found to be a protein secreted by *S. citri* cells which is unique to *S. citri* yet present in the four different *S. citri* strains (C189, S600, S616, and GII3-3X) tested. *S. citri* secreted CAK98563 abundantly, especially in the presence of citrus phloem extract; hence, an antibody was subsequently produced against CAK98563. By detecting this secreted protein as a marker, CSD-infected trees were successfully diagnosed in a highly reliable and specific manner in direct tissue prints on nitrocellulose membrane.

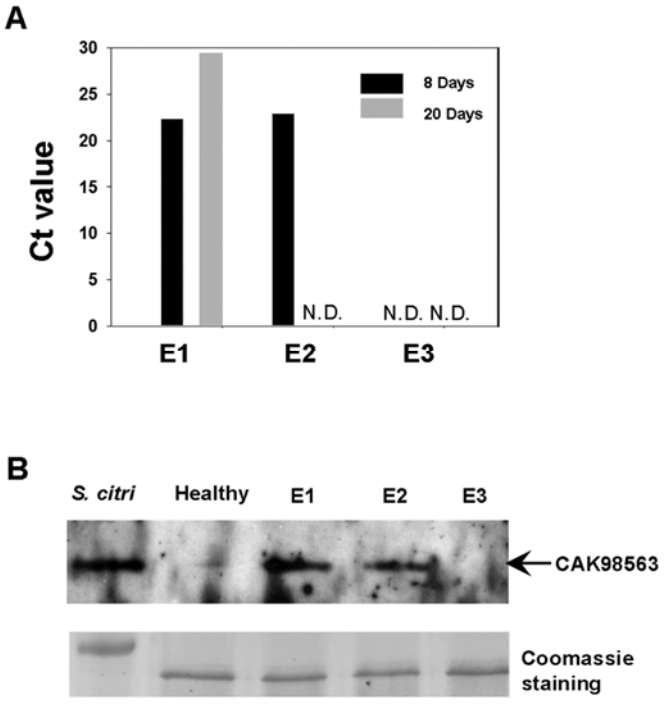

Fig. 4. ScCCPP1 detected *Spiroplasma citri* infection in periwinkle plants that were mechanically inoculated with *S. citri* strain C189. Periwinkle seedlings were inoculated with *S. citri* cell suspensions on the stem. Data include three replicates (E1, E2, and E3). **A,** Newly emergent leaf tissues were collected 8 and 20 days postinoculation for quantitative polymerase chain reaction analysis using primers amplifying the spiralin gene of *S. citri*. Ct = cycle threshold. **B,** Western blots using ScCCPP1 antibody to detect the CAK98563 proteins were performed 8 weeks postinoculation using tissues away from the inoculation sites from the same three replicates as in A.

The imprint assay developed using ScCCPP1 antibody generated against CAK98563 is simple and essentially sample-preparation-free. Antibody-based diagnosis is widely used due to its high specificity, robustness, low cost, and adaptability for large-scale field surveys with minimum laboratory equipment (2,16). Therefore, we believe that the use of ScCCPP1 is the first documentation of serological diagnosis for CSD that is independent of the presence of the *S. citri* in the tested sample. This should reduce the sample number per tree to be tested and may be suitable for large-scale field surveys. For such application, however, additional validation and specificity confirmation of the method is required. For example, ScCCPP1 testing of large numbers of field samples from several additional citrus species at different tree ages and developmental stages, co-infected with other bacterial and viral pathogens of citrus, at different times of the year and in different geographic locations will generate critical data for the suitability of the method for field deployment.

Visual inspections for symptom identification are critical elements of the currently available HLB management systems (4,6). However, in California or other areas where CSD is endemic, the introduction of HLB will take place in addition to *S. citri* infection. Given symptom similarities of CSD and HLB, the introduction of HLB may be readily confused or ignored as CSD if the only means of large-scale field surveys is visual inspection (11,45). In addition, visual misidentification of CDS as HLB may trigger unnecessary regulatory actions or management practices. A large-scale field-applicable serological method such as the ScCCPP1 will be of great importance for the differentiation of CSD and HLB infected trees.

Compared with PCR-based assays, ScCCPP1-based direct tissue print detection can overcome uneven distribution or low titers of *S. citri* in the infected plants, thereby reducing false-negative diagnosis (55,56). This challenge was clearly demonstrated from this study, where qPCR results were inconsistent with imprint data in detecting CSD-infected field trees (Table 2). From the direct tissue print assays using ScCCPP1, we observed positive signals from the phloem-rich tissues without the actual presence of pathogen cells. These data suggest that the detection marker was dispersed in the phloem, potentially moving along with the transportation flow. Therefore, this study set up a novel concept of using secreted proteins as markers to detect diseases caused by pathogens that reside in plant vascular tissues. Systemic movement of bacterial-secreted proteins has been reported in phytoplasmas (3,31,41), which are also insect-transmitted and phloem-colonizing plant pathogens. Therefore, use of pathogen-secreted proteins as detection markets can likely be adapted for other ecologically similar pathosystems, such as the devastating citrus HLB.

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