CRB Project Plan – Research Grant Proposal for FY2010-2011

Project Title: Identification of *Spiroplasma citri* secreted proteins as detection markers for citrus stubborn disease

CRB Project No. 5300-139
(in 16 pt. bold)

CITRUS RESEARCH BOARD

PROJECT PLAN - RESEARCH GRANT PROPOSAL FOR FY 2010-2011

Fiscal Year: 2010-2011 **Anticipated Duration of Project:** 3 years

This Project is: _____ **New or** X **Ongoing (Year** 3 **of** 3**)**

Project Leader: Wenbo Ma & Georgios Vidalakis
Name
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Cooperating Personnel: 1 SRA (60%) and 1 Postdoctoral Researcher (25%)

Project Title: Identification of *Spiroplasma citri* secreted proteins as detection markers for citrus stubborn disease

Keywords: *Spiroplasma citri*, phytopathogenic mollicutes, citrus stubborn, proteomics, detection marker

Abstract *(limit 200 words):*

The phloem-limited and insect vector-transmitted citrus Stubborn disease has resulted in constant yield and quality reductions in California citriculture. However, the currently available diagnostic methods are either labor-intensive or inaccurate due to the uneven distribution and the low titer of the pathogen in citrus. The main goal of this project is to develop an immunoassay-based diagnostic method for the Stubborn disease by detecting proteins secreted from the causal agent *Spiroplasma citri* into citrus phloem. Secreted proteins are not restricted to the infection sites and can be relatively abundant, enabling robust detection of the disease. We will employ a proteomic approach to identify secreted proteins; and suitable proteins will then be evaluated as detection markers. This novel diagnostic tool is especially important to support large-scale testing for citrus certification programs, such as the Citrus Clonal Protection Program (CCPP).

In addition to providing a robust detection tool that improves the overall scheme of citrus disease diagnostics, this research will also significantly advance our understanding on the vector-transmitted and phloem-limited bacterial diseases, including the devastating Huanglongbing (HLB). HLB and citrus Stubborn diseases share extensive similarities. The novel knowledge and detection tool developed from this project will facilitate the development of detection and control strategies for HLB.

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Problem and its Significance*:

Phloem-limited and insect vector-transmitted bacterial diseases, such as the stubborn disease and citrus greening (Huanglongbing or HLB), have presented major challenges and losses to the citrus industry worldwide. Citrus stubborn disease, caused by the bacterial pathogen *Spiroplasma citri*, has resulted in constant yield and quality reductions in California citriculture since it was first observed in 1915 (5). Spiroplasmas belong to a group of soft-skinned bacteria called mollicutes, which lack an outer cell wall and capable of infecting both animals and plants (3). Transmitted by phloem-sapping leafhoppers, *S. citri* invades the host phloem sieve tubes. The infected plants are usually severely stunted, and the fruit quality and quantity is greatly reduced. For example, the yield reduction of stubborn-infected Valencia orange trees is 44-74%, depending on the rootstock; while the yields of navel oranges can be reduced up to 100% (6, 9).

Although citrus stubborn has been a research subject for decades, the detection of the pathogen remains to be challenging nowadays. Individual symptoms can be easily confused with those caused by nutritional problems or other pathogens, such as HLB. Due to the erratic distribution and the low titer of the pathogen in plants (7, 11), PCR-based methods, which target individual *S. citri* cells, are not always accurate despite the recent developments of PCR primers based on the sequences of multicopy genes encoding membrane surface proteins (16). So far, the only definitive diagnostic methods are bioindexing and culturing in artificial medium, both of which are time-consuming and labor-intensive (5).

The main goal of this proposal is to develop a rapid, reliable and cost-efficient diagnostic tool for citrus stubborn disease by detecting the secreted proteins from *S. citri* into citrus phloem. Because the secreted proteins may not be restricted to the infection sites and can be relatively abundant, this new technique can overcome the problems of uneven distribution and the low titer of *S. citri* in plants. This novel serological assay is especially important for field surveys dealing with a large number of samples.

Proteins secreted to the environment or directly into the plant cells perform essential virulence functions in a broad range of bacterial and fungal pathogens (1). For example, the type III secretion system is indispensable for the pathogenicity in many gram-negative plant pathogens (2), such as *Pseudomonas syringae* (bacterial blights, specks and spots in over 100 plant species), *Xanthomonas campestris* (bacterial spot in tomato and pepper), *Ralstonia solanacearum* (wilt disease on many crops), and *Erwinia carotovora* (potato soft rot and blackleg). The type I secretion system is important for *Xylella fastidiosa*, a xylem-restricted pathogen to cause the Pierce's disease of grapevines as well as a series of scorching diseases of ornamental trees and plants (12). Although the function of *S. citri* secretion systems in pathogenesis is yet unknown, evidence indicated that secreted proteins are key virulence factors in other mollicutes. In *Mycoplasma*, mollicutes infecting animals, secreted proteins are fundamental in pathogenesis (14). Phytoplasmas is a group of phytopathogenic mollicutes that is closely related to Spiroplasmas. Similar to Spiroplasmas, Phytoplasmas cause insect-transmitted diseases in plants and reside in phloem (3). The Aster Yellows phytoplasma strain secretes approximately 56 proteins to host phloem. Some of these proteins significantly contribute to disease development (4).

In order to use *S. citri* secreted proteins as detection markers for the Stubborn disease, we will employ a proteomic approach to identify the proteins secreted from *S. citri* to citrus phloem. Proteins secreted by the pathogen upon induction with phloem extract will be identified using electrophoresis followed by Mass Spectrometry analysis. Our laboratories have been using a similar approach to study the secreted proteins in other plant-associated bacteria including *Pseudomonas syringae* and *Sinorhizobium fredii*. The smaller genome size of *S. citri* (~ 2 Mb) (13) compared to other eubacteria (an average of 5 Mb) will make this approach even more promising and feasible. These secreted proteins will be evaluated for their suitability as detection markers according to the following criteria: 1) high abundance and dispersal in phloem; 2) wide distribution in

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representative *S. citri* isolates. One or two suitable protein markers will be used to generate polyclonal antibodies for an immunoassay-based detection of citrus stubborn disease, which is a sensitive, simple and economic method for field surveys.

Since secreted proteins play an essential role in pathogen infection and disease development, the identification of secreted proteins from *S. citri* will also greatly contribute to our understanding of how phloem-limited, insect vector-transmitted bacterial pathogens cause widespread and severe diseases in citrus. This knowledge is urgently required to provide key information for the development of sustainable control strategies, which are yet lacking. The extensive similarity of the host responses, disease symptoms and pathogen life styles between Stubborn and HLB (Fig. 1) lead to the hypothesis that similar virulence factors might be used by these different pathogens to cause diseases. Therefore, the protein markers and detection tools developed from the proposed research will greatly facilitate the development of detection strategies for HLB, which is uncultivable and thus very difficult to work with, at this time. Moreover, since HLB research is not possible at this point in California due to the selective agent status, the proposed project is California's best option to study virulence proteins and secretion systems that might be involved in the pathogenicity of HLB or of other ecologically related pathogens such as *Phytoplasma* spp.

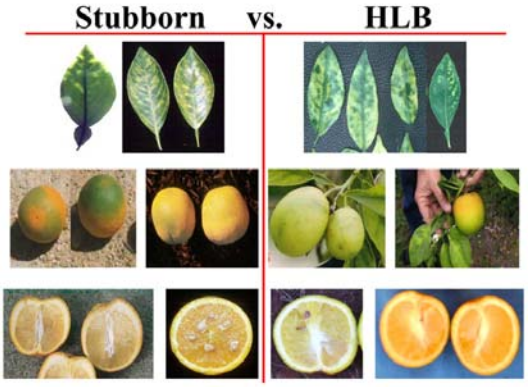

Figure 1. Similar symptoms in foliage, external and internal fruit, and seeds of citrus trees infected with the Stubborn or Huanglongbing (HLB) diseases.

With the matching funds from UC Discovery, we have started a genome sequencing project that is closely related to this project. We will fully sequence the genomes of two *S. citri* strains and determine their secreted protein profiles using bioinformatics prediction (as described later in the proposal). With the recent publication of the genome sequence of HLB (8), we can compare these genome sequences to identify homologous secreted proteins from both bacteria. These proteins may play important roles in the adaptation to citrus phloems.

Objectives*:

This project aims to develop a quick and robust diagnostic tool for the citrus Stubborn disease by targeting an abundant secreted product of the causal agent – *S. citri*. Such serological technique will overcome the problems of uneven distribution and low titer of the pathogen in citrus; and therefore will greatly benefit the citrus industry as an accurate and economical diagnostic tool for field surveys. It will also be very useful for the clean citrus stock program of the Citrus Clonal Protection Program (CCPP). Novel knowledge obtained from the proposed research will offer important insights into the molecular mechanisms underlying phloem-limited and insect vector-transmitted bacterial diseases in citrus.

The specific objectives are:

1st and 2nd year:

1) Develop a reliable virulence assay for *S. citri*

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2) Determine the induction condition of *S. citri* by citrus phloem extract for secreted protein extraction
3) Identify *S. citri* secreted proteins using Mass Spectrometry
4) Compare the secreted protein profiles of several representative *S. citri* strains to identify common ones for the development as detection markers

3rd year

5) Evaluate and select one or two secreted proteins to generate polyclonal antibody
6) Develop an ELISA - based detection tool for citrus stubborn disease

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Project's Benefit to the Industry*:

The development of a rapid and robust diagnostic technique for the Stubborn disease will increase the capacity of CCPP to test source plants of citrus germplasm from its own foundation material as well as from other registered budwood sources. The fundamental control method for the Stubborn disease, like most of the graft transmissible diseases of citrus, is to use disease-tested propagative material. Modern diagnostic techniques that improve the overall scheme of diagnostics necessary for the safe release of new varieties to the industry are essential for a sustainable and viable California citriculture. Furthermore, a better understanding of interactions between citrus-host and pathogen-secreted proteins that lead to citrus Stubborn disease will be extremely important for the development of early disease diagnosis and control approaches for other ecologically related graft transmissible phloem-limited citrus pathogens.

Research Collaboration* (*be specific*):

The research activities of Dr. Vidalakis and the functions of CCPP in the Department of Plant Pathology & Microbiology at UCR continue to trigger the interest of young scientists for collaborations. Wenbo Ma has extensive experience on the identification and functional analysis of virulence proteins, especially the secreted effector proteins from bacterial pathogens. The Vidalakis lab is focused on developing new diagnostic tools for citrus disease. This collaboration takes full advantage of the research experience and fulfills the interests and mission of both groups. Wenbo Ma's research team will get the opportunity to directly contribute to California citrus industry; and Georgios Vidalakis and CCPP will develop novel diagnostic techniques that help to provide clean citrus propagative material to growers, breeders, and researchers around the globe.

During a visit in Dr. B. Dawson's laboratory in the Citrus Research and Education Center in Lake Alfred, University of Florida in the summer of 2010, we were introduced to a cytopathological study related to the development of the HLB disease in citrus phloem. In this study, the correlation between ultrastructural changes, the presence of the causal agent, and the symptom produced on the infected plants was examined. Large numbers of bacterial cells were found in phloem sieve tubes in pre-symptomatic young flushes. In contrast, HLB bacteria were not observed in the highly symptomatic leaf samples. Based on the similarities between HLB and Stubborn,citrus pathosystem. This analysis will help to validate our hypothesis that the secreted proteins and not necessarily the present of the pathogen per se are associated with the symptom development. Therefore, the use of antibodies generated against pathogen secreted proteins is a valid tool for diagnosis.

Plans and Procedures* *(include site location and description in discussion of experimental design):*

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1) Development of a virulence assay for *S. citri* (Vidalakis lab, 1st and 2nd year)

Pure culture of *S. citri* is obtained from stubborn-infected citrus leaves by culturing the mid-veins extract in mycoplasma medium containing fetal serum albumin (9). Bacterial inoculum will be placed into young citrus seedlings through single vertical cuts using a razor blade. Systemic infection can be confirmed four weeks post inoculation by isolating *S. citri* DNA from the inoculated citrus seedlings. Using this protocol, we reported the successful mechanical transmission of *S. citri* from pure culture into pineapple sweet orange (*Citrus sinensis* var Pineapple) last year. However, this result could not be replicated recently despite the positive PCR confirmations indicating that systemic infection was established four weeks post inoculation in the previous trials. While we are working on different experimental procedures to replicate our original mechanical transmission experiment, samples from *S. citri*-infected trees from greenhouse and field will be used to test the antibodies as detection markers.

2) Identification of proteins secreted by *S. citri* (Ma lab, 1st and 2nd year)

In order to identify secreted proteins of *S. citri* which will be used as detection markers, we performed proteomic analyses on the proteins secreted from *S. citri* cells to liquid artificial media. This objective has been completed.

During the past two years, we have optimized the bacterial growth conditions, induction time, induction temperature and protein precipitation method to achieve minimum interference in protein analysis and best protein induction efficiency. After numerous trials, an optimized experimental protocol has been developed. In brief, single *S. citri* clones are grown in C-3G medium for a week before the cells are collected and resuspended in 0.3M sucrose in the presence or absence of citrus phloem extracts at room temperature for 24 hours. Total proteins from the cell-free supernatant are then precipitated using the pyrogallol red-molybdate-methanol (PRMM) protocol (Fig. 2).

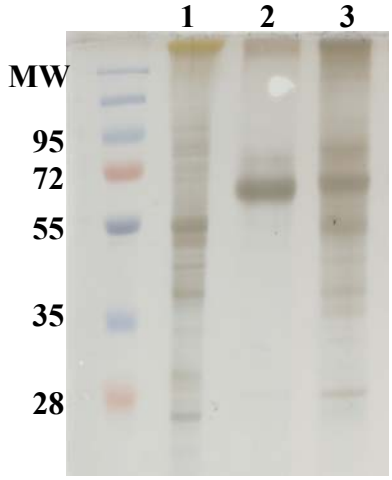

Figure 2. Comparison of the secreted protein profiles of *S. citri* in the absence (uninduced) and presence (induced) of citrus phloem extracts.
Lane 1: phloem extracts only;
Lane 2: supernatant from uninduced *S. citri* cultures;
Lane 3: supernatant from induced *S. citri* cultures

The secreted protein profiles from *S. citri* cell cultures with or without the induction of citrus phloem extracts were then determined by Mass Spectrometry. Three independently prepared protein samples were analyzed and several potential secreted proteins highly induced in the presence of citrus phloem extracts were identified. To facilitate with the analysis on the Mass Spectrometry data, we obtained an additional funding from UC Discovery Grant to fully sequence the genome of *S. citri* strain S616 that we are working on using the next generation Illumina sequencing. We have obtained approximately 4800Mb of sequence data, which equals to approximately 2000 times coverage of the genome. Using these sequencing data, we were able to match most of the peptides from the Mass Spectrometry analysis to the *S. citri* genome sequences and obtained the

complete gene sequences for most of them. The deduced amino acid sequences of many proteins identified from Mass Spectrometry contain potential N-terminal secretion signals (predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/) and Pred-Lipo (http://bioinformatics.biol.uoa.gr/PRED-LIPO)), indicating that they are secreted in a Sec-dependent manner.

3) Validation and development of detection markers (Ma and Vidalakis lab, 2nd and 3rd year)

After the secreted proteins are identified, they are screened for their suitability as good detection markers. For use as detection markers, we chose the proteins with higher abundance and stability indicated by the larger number of peptides identified in the Mass Spectrometry in all three independent experiments. Using these criteria, we chose two proteins CAK99824 and CAK98563 for further analysis. By comparing the genome sequences of *S. citri* strain S616 from our own sequencing and the available genome sequence of another *S. citri* strain GII3 (7), we confirmed that the selected protein candidates are present in both genomes with almost identical DNA sequences. This result indicates that they are conserved genes in *S. citri*. In addition, none of these proteins has close homologs in other organisms according to a search in Genbank database, suggesting that they are also unique for *S. citri*. These characters make them excellent detection markers for citrus stubborn disease.

To generate polyclonal antibody, we have cloned these genes (CAK99824 and CAK98563) into the *E. coli* expression vector pET28a. We have also cloned the spiralin gene into the same vector and planned to generate anti-spiralin antibody, which will be used as a control. After over-expression in *E. coli*, the recombinant proteins were purified using nickel column and sent for polyclonal antibody generation (Robert Sargeant, Ramona, CA). As planned, we will start receiving anti-sera in early August, 2010. These anti-sera will be first tested using bacterial cultures and then evaluated on their usage for the detection of *S. citri* from plant tissues and plant sap using immunoassays.

Literature:

The applicants have conducted a thorough literature search on this subject. All the cited references have been carefully reviewed.

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Other Funding Sources for this Project (current and/or pending)*:

We have successfully obtained matching funds from the UC Discovery and the funding period is from October, 2009 to September, 2012. The UC Discovery funds allow us to expand our research plans and determine the genome sequences of two *S. citri* strains. So far, we have sequenced the strain S616 and the sequencing data have already significantly facilitated our Mass Spectrometry analysis. The genome sequence will also allow us to identify secreted proteins using bioinformatic predictions.

Indirect support to the proposed research activities is provided by the faculty, personnel and infrastructure of the Department of Plant Pathology and Microbiology at UC Riverside and the CCPP. We are committed to this project because we believe this will make significant contributions to the industry as well as the scientific community.

Technology Transfer* (*be specific):*

At the third year of this project, we expect to have one or two peer-reviewed publications submitted or published this year. The developed marker and diagnostic tool will be permanently available to CCPP and the citrus industry for disease detection.

Budget Justification:

A. Senior/Key Person

Dr. Wenbo Ma, Principal Investigator. Dr. Ma will be responsible for coordinating the project. A staff

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research associate (SRA), Ms. Robyn Morgan, will conduct experiments including proteomic analysis, sequence analysis, gene cloning and protein secretion experiments. Funds are requested to support Ms. Morgan in the amount of $38,400 (including salary and benefits) at 60% time.

Dr. Georgios Vidalakis, Co-Principal Investigator. Dr. Vidalakis will be responsible for supervising his Assistant Specialist, Dr. Mohammed Afunian, for citrus infection assays, generation of antibodies and evaluation of detection markers. Funds are requested for Dr. Afunian in the amount of $16,200 (including salary and benefits) at 25% time.

All salaries are in accordance with approved University scales and include a 3% escalation each year. Standard fringe benefits rates are 32.65% for Dr. Afunian and 36.09% for Ms. Morgan.

B. Travel

Funds are requested in the amount of $1,000 each year for domestic traveling including attending related scientific conferences, such as the annual APS (American Phytophathological Society) meetings and the Citrus Research Board meetings.

C. Other Direct Costs:

Materials & Supplies

Funds are requested in the amount of $3,000 in year 2010-2011 for protein and DNA extraction and detection. Consumable materials include: DNA extraction reagents and kits, oligonucleotides synthesis, Taq polymerase, restriction and modification enzymes, agarose, polyacrylamide gels, nylon membranes, Southern and western blot reagents, bacteria growth media and other general laboratory supplies.

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Project Budget

Department Account Number: *(if applicable)*_____

	Year: **2010-2011**	Year: **2011-2012**	Year: **2012-2013**
Salaries and Benefits:			
Postdocs/Research Assistants	$ 16,200		
SRA's	$ 38,400		
Lab/Field Assistance			
Benefits			
Supplies and Expenses:	$ 3,000		
Equipment:			
Operating Expenses and Travel:	$ 1,000		
Lindcove Recharges:			
Lindcove Packline:			
Other: _____			
_____			
ANNUAL TOTAL:	$ 58,600		

Specifics regarding contract (i.e., "split" funding to more than one PI):

Signatures

Project Leader: _____ **Date:**_____

_____ **Date:**_____

_____ **Date:**_____

Dept. Chair: _____ **Date:**_____

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