CRB Project No. 5300-131

CITRUS RESEARCH BOARD

PROJECT PLAN - RESEARCH GRANT PROPOSAL FOR FY2010-2011

Fiscal Year: 2010-2011 **Anticipated Duration of Project:** 6

This Project is: New or X **Ongoing (Year 5 of 6)**

Project Leader: Hailing Jin
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Cooperating Personnel: Svetlana Folimonova, Ph.D., Research Assistant Scientist, University of Florida, Citrus Research and Education Center

Project Title: Expression profiling of *Candidatus Liberobacter*-induced small RNAs from HLB-susceptible and tolerant genotypes

Keywords: citrus greening, small RNA, tolerant genotypes, sensitive genotypes

Abstract *(limit 200 words):(clearly and succinctly state what your project is about why you are doing it and expected out come and how the industry will use these outcomes)*

We aim to utilize host rapid defense responses and identify unique host biomarkers---endogenous small RNAs for early diagnosis of Citrus greening or "Huanglongbing" (HLB) disease. HLB is one of the most devastating diseases of citrus. Early diagnosis before the appearance of the dreaded symptoms is particularly important, but somehow very difficult due to the low titer and uneven distribution of the bacterium *Candidatus* Liberibacter. Instead of focusing on the bacteria, we have taken the advantage of host rapid defense responses. Some host small RNAs are rapidly and specifically induced by pathogens, which makes them one of the most attractive markers for early diagnosis. Recent studies show that miRNAs are successfully developed into cancer diagnosis markers in medical field. With the support of Citrus Research Board, we have identified several small RNAs that are induced by HLB. We will continue to further study the specificity and regulation of these candidate HLB-induced small RNAs. In addition, to better understand the mechanism of plant defense and HLB pathogenicity, we will conduct small RNA and messenger RNA (mRNA) profiling on HLB-susceptible and HLB-tolerant natural citrus genotypes in collaboration with Dr. Svetlana Folimonova at University of Florida. We will profile endogenous small RNAs by high-throughput sequencing of small RNA libraries and mRNAs using RNA-Seq high-throughput methods. This study will help us identify small RNAs and their potential target genes that are specific to susceptible and tolerant genotypes, which will elucidate the mechanisms of HLB pathogenicity and natural defense responses from the hosts.

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Project Title: _____

Problem and its Significance*: *(include literature review)*

Citrus greening or HLB disease is considered the most destructive disease of citrus in the world. The disease is associated to species of the gram-negative bacterium *Candidatus* Liberibacter, and transmitted by vector Psyllids. Recent detection of Psyllids in California-Mexico border and the spread of HLB carrying Psyllids in Texas and Florida give a serious warning to California citrus industry and scientists. How to prevent its spread into California became the highest priority issue. The disease is widespread throughout Asia, the Indian subcontinent and neighboring islands, eastern and southern Africa, Saudi Arabia, and the islands Réunion and Mauritius (Bové, 2006). In the Americas the disease is spreading fast. In March 2004 and August 2005 the disease was detected in the State of São Paulo, Brazil (Teixeira et al. 2005), and in Florida (Knighten et al., 2005), respectively, which are two of the largest citrus producing areas in the world. Recently, HLB was also reported from Belize and Mexico (summer of 2009).

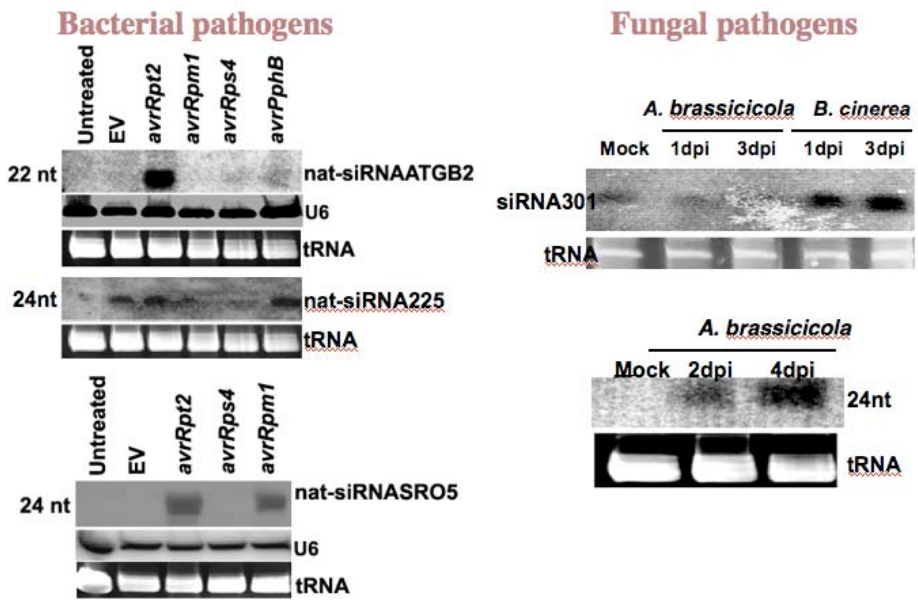

Figure 1. Small RNAs induced by both bacterial and fungal pathogens. Examples of host endogenous small RNAs that are induced by bacteria *Pst DC3000* carrying different avr genes at 15 hours post inoculation (hpi) or fungal pathgens *Botrytis* and *Alternaria brassicicola* at 1 and 3 or 2 and 4 days post inoculation (dpi). Small RNAs were extracted from 4 week old plants under different pathogen treatments and resolved on a 17 % denaturing PAGE gel for Northern analysis.

Up to now, no cure has been found against the HLB disease and control is mainly achieved by removal of infected trees and chemical treatment against the insect vector. There is no known resistance in *Citrus* spp., though some species expressed a greater tolerance in several studies (Halbert & Manjunath, 2004). All citrus growing regions are threatened by the disease and many regions outside the United States have already been destroyed. Research on HLB has been hampered by the difficulty in culturing the causal bacterium in artificial media. It has been difficult to detect and identify the pathogen, because of low concentration and uneven distribution in host plants and vector psyllids. To prevent its spread to other states, including California, accurate, rapid and robust

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detection methodologies are highly desirable for early diagnosis and effective management of HLB.

Pathogen infection can induce a series of defense responses in host plants, including the generation of reactive oxygen species, thickening of the cell walls, induction of salicylic acid and sometimes induction of host cell death (Dangl & Jones 2001, Parker *et al.* 2001., Martin *et al.* 2003). Many expression-profiling studies using microarray chips have demonstrated that these responses are mediated by activation or suppression of a large array of host genes. However, how gene expression is regulated is still largely unknown.

Our recent studies have discovered that some small RNAs are induced by various pathogens more rapidly and specifically than mRNA transcripts and may play an important role in regulating gene expression in defense responses. Figure 1 displays a few such examples. Figure 1A, B and C show three different small RNAs that are induced by the same bacteria *Pseudomonas syringae* carrying different avirulent proteins. Figure 1D shows another small RNA that is induced by fungal pathogen *Alternaria brassicicola*. These results demonstrate the high specificity of the small RNA induction. The small RNA in Figure 1A was extensively examined, and it can be detected as early as 4 hours post bacteria infection (Figure 2), which indicates that the small RNA induction is very rapid.

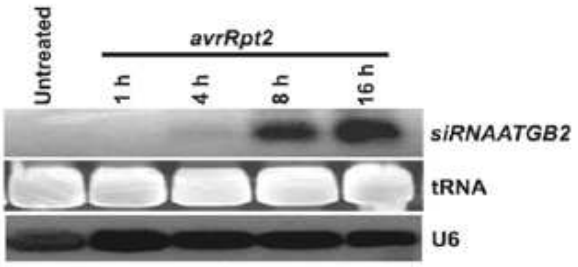

Figure 2. Bacterial pathogen Pst (avrRpt2) induces the siRNAATGB2 as early as 4 hours post infection. The small RNA northern was performed as described in Figure 1. The oligo probe that complementary to siRNA sequence was used.

The high specificity and rapid induction nature of these small RNAs makes them to be one of the most attractive markers for early detection and diagnosis of pathogen infection.

Objectives*: (*succinctly state each objective and milestone, ie the time expected to successfully complete an achieve each objective)*

With the support of CRB, we have identified several small RNAs, including both microRNAs and endogenous small interfering RNAs that are induced by HLB (Data not shown because we are in the process of applying for patent). We are currently conducting further analysis on the specificity and expression patterns of these small RNAs.

In addition, we would like to expend the small RNA expression profiling study to different genotypes that show strong differences in plant defense responses. Folimonova et al. have examined the pathogenicity and distribution of *Candidatus* Liberibacter in different citrus varieties under greenhouse conditions (Folimonova et al., 2009). Although *Candidatus* Liberibacter was able to multiply in all of the plants, a wide range of responses was observed among different hosts. Based on the symptoms developed and the ability of plants to continue growth the different genotypes were

grouped into four categories. Two groups are of particular interest because they represent the weak and strong defense responses from the host. The first group is **sensitive**, which exhibited severe chlorosis on leaves, greatly reduced growth, and eventual death (for example, sweet oranges, grapefruit, and some mandarins - Nules Clementine and Minneola tangelo); whereas the second group exhibited very minimal or no symptoms, which was named **tolerant** (for example, Eureka lemon, Persian lime, Carrizo). We propose to use high-throughput deep-sequencing approach to profile the small RNA and mRNA populations of these two distinct varieties. Both Psyllid-infected HLB citrus trees and grafting-inoculated citrus trees will be included.

This study not only leads us to the discovery of HLB-induced small RNAs for early diagnosis marker development, but also helps us understand the pathogenisity of citrus greening, and helps us identify important components in natural defense of HLB disease.

Project's Benefit to the Industry*: *(How will the industry utilize your research results or product)*

To develop a rapid and robust diagnostic technique for HLB 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 HLB disease, like most of the graft transmissible diseases of citrus, is the use of disease tested propagative material. Modern diagnostic techniques that improve the overall scheme of diagnostics necessary for the safe release of new varieties are essential for a sustainable and viable California citrus industry. The HLB testing of budwood source trees has become even more important after the recent decision of the California nurseries to establish a mandatory testing program that will include HLB testing of their tree sources. In addition, any HLB diagnostics developed from this project can also be used by the newly established CRB diagnostic labs enchasing their operations and capacity.

Furthermore, the comparison analysis between sensitive and tolerant genotypes will help identify important components in natural host defense against HLB, which have the potential for developing natural HLB-tolerant or resistant cultivars.

Research Collaboration* (*be specific*):

Collaboration has been established between my lab and Dr. Svetlana Folimonova at University of Florida during this second phase of this project. She will provide the HLB-infected and uninfected tolerant and susceptible citrus plants for our expression profiling.

The first phase of the project was in collaboration with Dr. Kim Bowman at USDA, ARS, USHRL, Florida. Dr. Bowman has generated grafting-transmmitted HLB plants and control plants and collected the plant tissue at various infection stages.

We will also collected citrus tissue from various pathogen infected trees, including CTV, Stubborn and/or other citrus pathogens (Dr. Vidalakis's lab).

Plans and Procedures* *(use this section to describe your experimental design site location(s)and elaborate on objectives and milestones)*

Year 10-11:

1. Continue the validation and in-depth analysis of small RNAs identified from grafting-inoculated HLB trees in Jin's lab.

Compare the small RNA signals between HLB and Spiroplasma (CRB Project 5210-07I Vidalakis & Hailing) infected citruses by Northern blot analysis. Similar material was also obtained from Dr. Ray Yokomi lab from USDA.

Both miRNAs and siRNAs will be validated. In addition to 33 conserved miRNAs, we also identified 11 new citrus miRNAs (data not shown). There are 2 miRNAs showed promising induction in HLB-infected plants.

In addition, table 1 is the short list of candidate siRNAs that we are in the process of validation. The ones that are highlighted have targets that are biologically interesting in plant response to pathogen challenges.

Table 1. Potential siRNA Markers that show differential expression patterns in the HLB and control samples.

ID	Locus ID	Arab.protein.database	Un.5 weeks	HLB.5 weeks	HLB/Un
EY651181	AT4G11810.1	SPX (SYG1/Pho81/XPR1) domain-containing protein | c	2	37	**18.5**
EY690904		none	34	402	**11.8**
EY704378	AT1G70620	cyclin-related | chr1:- FORWARD	644	6831	**10.6**
CV718260		none	19	178	**9.4**
CV712967		none	3	23	**7.7**
EY682140	AT5G58140	NON PHOTOTROPIC HYPOCOTYL 1-LIKE); kinase | c	3	21	**7.0**
DY266446		none	6	39	**6.5**
DY301111	AT3G01930	nodulin family protein | chr3:319296-321495 REVERSE	7	42	**6.0**
EY684810	AT4G27520	plastocyanin-like domain-containing protein | chr5:- RE	13	76	**5.8**
EY760154	AT1G63320	pentatricopeptide (PPR) repeat-containing protein | chr1	96	491	**5.1**

ID	Locus ID	Arab.protein.database	Un. 9 weeks	HLB 9 weeks	HLB/Un
EY796661	AT2G30600	BTB/POZ domain-containing protein | chr2:- FORWAI	2	119	59.5
CV715942	AT1G28290	ARABINOGALACTAN-PROTEIN 31) | chr1:9889318-9	7	373	53.3
CV712967		none	3	33	11.0
EY690904		none	38	367	9.7
DN621447	AT5G54540	similar to unknown protein [Arabidopsis thaliana] (TAI	5	39	7.8
EY704378	AT1G70620	cyclin-related | chr1:- FORWARD	671	4655	6.9
CV717895	AT1G12700	helicase domain-containing protein / pentatricopeptide	5	34	6.8
EY890843	AT1G62500	protease inhibitor/seed storage/lipid transfer protein (L	3	20	6.7
CV718260		none	21	130	6.2
Contig19543	AT5G13460	IQD11 | IQD11 (IQ-domain 11); calmodulin binding | ch	4	24	6.0
EY851887	AT3G63280	ATNEK4 | ATNEK4 | chr3:- FORWARD	60	349	5.8
CX052561	AT4G38180	FRS5 | FRS5 (FAR1-RELATED SEQUENCE 5); zinc	15	86	5.7
Contig20419	ATCG00480	ATPB | chloroplast-encoded gene for beta subunit of A	6	34	5.7
EY663138	AT3G14470	disease resistance protein (NBS-LRR class), putative	3	17	5.7
Contig17493	AT1G30330	ARF6 | ARF6 (AUXIN RESPONSE FACTOR 6) | chr1:	4	21	5.3

2. Dr. Svetlana Folimonova at University of Florida will prepare and collect the material for our analysis.

At least one susceptible and one tolerant genotype will be prepared for HLB-infection. Two time points will be used.

3. Small RNAs will be extracted and small RNA libraries will be prepared for deep-sequencing.

Year 11-12:

1. Deep sequencing of small RNA libraries by Jin's lab using the Illumina sequencer at UCR core facility.

2. Extract mRNA, construct mRNA RNA-seq libraries. RNA-seq libraries will be sequenced using pair-end analysis by Illumina sequencer at UCR core facility.

3. Bioinformatics analysis of deep sequencing data using our developed tools in Jin's lab. We will include all the citrus sequences that released up to date by the time of sequencing analysis. We will also take the bacterium *Candidatus* Liberibacter asiaticus genome sequence that published into consideration.

Hopefully, more citrus sequences could be released in the next year or two, so that we can fully analyzing the endogenous siRNAs without facing the limitation of lacking of intergenic sequences.

4. Comparative analysis between the datasets generated from small RNA libraries and RNA-seq results. Potential small RNA targets will be identified.

Other Funding Sources for this Project *(current, pending, potential; can this project be used as matching funds for other funding sources)*

We are planning to apply for the UC Discovery in Nov. 2010.

Technology Transfer* *(include any potential intellectual property issues; steps necessary for grower utilization extension/communication component):*

We are finishing up writing a manuscript of miRNA discovery in citrus and hopefully could be submitted by the end of this year. At least one more peer review publication will be produced, while the developed test will be permanently available to the CCPP and citrus industry's laboratories for disease testing. Acquired sequence data that are related to citrus genes will be posted in the appropriate online databases for public access.

Budget Justification:

Personnel

Dr. Hailing Jin, Principal Investigator: (no funds requested), will lead the project and be responsible for the research.

*Use as much space as necessary; attach additional pages as needed – budget info. and signatures will appear on the final page.

Dr. Chellappan Padmanabhan, Assistant Specialist: Funds are requested to support one assistant specialist in Dr. Jin's laboratory, Dr. Chellappan at $33,861 per year at 80% time, based upon an annual salary of $41,496 for two years. He will be responsible for small RNA isolation, small RNA library construction and responsive small RNA validation.

Junior Specialist (TBN): Funds are requested to support one Junior Specialist in the amount of $13,738 at 40% time, and is based upon an annual salary of $33,672. The Junior Specialist will be in charge of the data analysis and result validation.

All salaries are in accordance with approved University salary scales and include a 2% merit increase each year.

Fringe Benefits
Fringe benefit rates are as follows: Dr. Chellappan Padmanabhan at 30.60% (actual) and Junior Specialist at 42% (standard).

Travel
$1,000 per year is required each year for the PIs and research personnel to travel to related meetings (TBA).

Materials & Supplies
$30,000 are requested for the cost of deep-sequencing, RNA-seq, small RNA extraction, LNA oligos, RNA oligos and DNA oligos, DNA sequencing, radioisotopes, membranes, chemicals, media, disposable plastics and molecular biology reagents.

Publication
$1,000 per year is required for for publishing the results of this project.

Lab/Field Assistance
$15,000 is required for Dr. Folimonova lab to prepare, infect and collect the plant material.

CRB Project Plan – Research Grant Proposal for FY2010-2011
Project Title: _____

Project Budget

Department Account Number: *(if applicable)*_____

	Year: **2010-2011**	Year: **2011-2012**	Year: **2012-2013**
Salaries and Benefits:			
Postdocs/Research Assistants	$ 33,861	$ 34,538	
SRA's	$ 13,738	$ 14,013	
Lab/Field Assistance			
(Dr. Folimonova lab for material preparation)	**$ 15,000**		
Benefits	$ 15,422	$ 16,454	
Supplies and Expenses:	$ 30,000	$ 30,000	
Equipment:			
Operating Expenses and Travel:	$ 1,000	$ 1,000	
Lindcove Recharges:			
Lindcove Packline:			
Other: Publication costs	$ 1,000	$ 1,000	
ANNUAL TOTAL:	$ 110,021	$ 97,005	

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

Fund of $15,000 in Year 2010-2011 will be provided to the collaborator Dr. Foliminova's lab for their material preparation and collection.

Signatures

Project Leader: _____ **Date:** 7/29/10

Dept. Chair: _____ **Date:** 7/29/10

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