NIFA SCRI – HLB RESEARCH AWARDS – 2015 (7 projects) and 2016 (7 projects)

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USDA National Institute of Food and Agriculture

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Current Research Information System CRIS

Retrieved 14 records

Title	Initial Award Yr	Grant Yr	Prop No	Investigator	Institution	View
EFFECTOROMICS OF THE HUANGLONGBING (HLB)-ASSOCIATED PATHOGEN	2016	2016	2015-10731	Ma, W.	UNIVERSITY OF CALIFORNIA, RIVERSIDE RIVERSIDE, CALIFORNIA	Brief Full
NIFA CENTERS OF EXCELLENCE: MULTIFUNCTIONAL SURFACE/SUB-SURFACE/SYSTEMIC THERAPEUTIC (COE:MS3T) TECHNOLOGY FOR HLB MANAGEMENT	2016	2016	2015-10490	Santra, S.	UNIVERSITY OF CENTRAL FLORIDA ORLANDO, FLORIDA	Brief Full
TARGETING MICROBES TO CONTROL HUANGLONGBING DISEASE OF CITRUS	2016	2016	2015-10479	Stelinski, K. S.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA	Brief Full
DEVELOPMENT NEW THERAPIES FOR HUANGLONGBING VIA CULTURING CA. LIBERIBACTER ASIATICUS	2016	2016	2015-10491	Gabriel, D. W.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA	Brief Full
HARNESSING NATURAL VARIATION IN TRANSMISSION OF LIBERIBACTER BY THE ASIAN CITRUS PSYLLID TO DEVELOP NOVEL HLB CONTROL STRATEGIES	2016	2016	2015-10475	Cilia, M.	NATIONAL AGRICULTURAL LIBRARY BELTSVILLE, MARYLAND	Brief Full
DESIGN AND DELIVERY OF THERAPEUTIC PROTEINS FOR HLB PROTECTION	2016	2016	2015-10483	Gupta, G.	NMC, INC. LOS ALAMOS, NEW MEXICO	Brief Full
DEVELOPMENT OF IN VITRO BIOFILM AND PLANKTONIC CULTURE OF CA. LIBERIBACTER ASIATICUS: A GAME CHANGE IN HLB RESEARCH	2016	2016	2015-10480	Gang, D. R.	WASHINGTON STATE UNIVERSITY PULLMAN, WASHINGTON	Brief Full
CHARACTERIZATION OF LIBERIBACTER POPULATIONS AND DEVELOPMENT OF FIELD DETECTION SYSTEM FOR CITRUS HUANGLONGBING	2015	2015	2014-10148	Ramadugu, C.	The Regents of University of California Riverside, CALIFORNIA	Brief Full
NON-TRANSGENIC, NEAR TERM RNA INTERFERENCE-BASED APPLICATION STRATEGIES FOR MANAGING DIAPHORINA CITRI AND CITRUS GREENING HUANGLONGBING	2015	2015	2014-10128	Falk, B.	UNIVERSITY OF CALIFORNIA, DAVIS DAVIS, CALIFORNIA	Brief Full
STEAM-GENERATED SUPPLEMENTARY HEAT THERMOTHERAPY AS AN IMMEDIATE TREATMENT FOR PROLONGING PRODUCTIVITY OF HLB-INFECTED CITRUS TREES	2015	2015	2014-10141	Ehsani, R. J.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA	Brief Full
ZINKICIDE A NANOTHERAPEUTIC FOR HLB	2015	2015	2014-10120	Johnson, EV, G.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA	Brief Full
A NOVEL ANTIMICROBIAL APPROACH TO COMBAT HUANGLONGBING DISEASE	2015	2015	2014-10146	Lorca, G. L.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA	Brief Full
DETERMINING THE ROLES OF CANDIDATE GENES IN CITRUS-HLB INTERACTIONS AND CREATING HLB-RESISTANT CITRUS CULTIVARS	2015	2015	2014-10119	Gmitter, FR, G.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA	Brief Full
DEVELOPING AN INFRASTRUCTURE AND PRODUCT TEST PIPELINE TO DELIVER NOVEL THERAPIES FOR CITRUS GREENING DISEASE	2015	2015	2014-10154	Brown, S. J.	KANSAS STATE UNIV MANHATTAN, KANSAS	Brief Full

USDA, NIFA SCRI HLB Citrus Disease Research and Extension Program
2016 Awards and Project Overview

Source: Current Research Information System

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ACCESSION NO: 1008978 **SUBFILE:** CRIS
PROJ NO: CA-R-PPA-5119-CG **AGENCY:** NIFA CALB
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2016-70016-24833 **PROPOSAL NO:** 2015-10731
START: 01 FEB 2016 **TERM:** 31 JAN 2021
GRANT AMT: $3,990,772 **GRANT YR:** 2016
AWARD TOTAL: $3,990,772
INITIAL AWARD YEAR: 2016

INVESTIGATOR: Ma, W.

PERFORMING INSTITUTION:
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EFFECTOROMICS OF THE HUANGLONGBING (HLB)-ASSOCIATED PATHOGEN

NON-TECHNICAL SUMMARY: Huanglongbing (HLB) has caused unprecedented crisis to the citrus industry worldwide. In the US, HLB is associated with the phloem-colonizing, insect-transmitted bacterium Candidatus Liberibacter asiaticus (Las). Since no cures for HLB are available and true resistance in citrus has not been found, methodologies that effectively detect Las in large-scale set-ups and the development of resistant citrus varieties are urgently needed for a long-term solution.One of the most important virulence mechanisms utilized by bacterial pathogens is manipulation of host immunity and physiology through the function of secreted effector proteins. Las possesses the Sec secretion system, through which a variety of Sec-delivered effectors (SDEs) could be secreted into the citrus phloem. Research findings from our research team and other laboratories strongly suggest that SDEs are promising detection markers for robust HLB diagnosis and excellent molecular probes to identify key components required for HLB development.In this project, we will systematically characterize SDEs produced by Las isolated from HLB-infected citrus in the three major producing states, FL, CA and TX. A "core" (likely essential) SDEs produced by all Las isolates will be identified and further used to develop antibody cocktails for HLB detection. Robust HLB detection is essential for the timely removal of HLB-infected trees in order to: 1) prevent the spread of HLB in California; 2) implement therapies and nutritional management programs to extend the productive life of trees before they decline in Texas; and 3) reinforce the replanting effort in Florida. Furthermore, the core set of SDEs will be investigated to understand the molecular basis of their virulence functions. In particular, we will identify the citrus targets of these SDEs and then generate genome-edited citrus to achieve enhanced resistance to HLB. The availability of HLB-resistant citrus would represent a major milestone on combating HLB. Therefore, the outcome of this project will benefit citrus production and profitability over the long term.

OBJECTIVES: This Standard Research and Extension Project (SREP) directly addresses critical stakeholder needs represented by two of the four priority areas identified by the Citrus Disease Sub-committee, i.e. "Development of methodologies that allow for the early detection of Las" and "Development of rootstocks resistant to, or tolerant of, Las".The goal of this project is to systematically analyze the Sec-delivered effectors (SDEs) from a variety of Las isolates in different citrus growing areas using genome sequence analysis, expression profiling, and host target characterization. Using this knowledge, we will: 1) develop antibody cocktail-based HLB diagnosis methods that directly detect Las; 2) generate HLB resistant citrus by modifying the citrus targets of SDEs using the recently developed genome-editing approach. Facilitated by vigorous extension and outreach activities, this project will benefit the development of integrative management program for HLB in a sustainable manner.The specific objectives are:1. Systematic analysis of Sec-delivered effectors from various Las isolates;2. Antibody development targeting the core SDEs for HLB detection;3. Identification of SDEs that contribute to HLB pathogenesis;4. Development of genome-edited citrus with HLB resistance;5. Sociological analysis of consumer responses to genome-edited citrus;6. Extension and outreach.

APPROACH: Objective 1. Systematic analysis of Sec-delivered effectors from various Las isolatesWe will obtain full genome sequences of Las isolated from Texas, Florida and California using next-generation sequencing including Illumina and PacBio. Potential Sec-delivered effectors (SDEs) will be bioinformatically predicted from the genomes. The expression of SDEs will be determined from HLB-infected trees in the field and using greenhouse experiments.Objective 2. Antibody development targeting the core SDEs for HLB detectionWe will screen a library of synthetic, monoclonal antibodies for those that specifically bind to individual "core" SDEs with high affinity. These antibodies will be used as a cocktail for HLB detection by direct tissue imprint assay and enzyme-linked immunosorbent assay (ELISA).Objective 3. Identification of SDEs that contribute to HLB pathogenesisWe will characterize the impact of SDEs on citrus development and immunity using transgenic citrus expressing individual "core" SDEs. The transgenic citrus plants will be examined for susceptibility to Las infection and monitored for developmental phenotypes reminiscent to HLB symptoms.Objective 4. Development of genome-edited citrus with HLB resistanceWe will: 1) identify targets of SDEs using yeast two-hybrid screening and co-immunoprecipitation followed by mass spectrometry; 2) develop genome-edited citrus plants that interrupt SDE targets using a CRISPR/Cas9 system, which enables simultaneous manipulation of multiple targets; 3) examine HLB resistance of the genome-edited citrus. Note: genome-editing does not involve introducing foreign genes into citrus; genome-edited crops are not considered "GMO" and therefore expected to be better accepted by customers.Objective 5. Sociological analysis of consumer responses to genome-edited citrusWe will investigate how the use of biotechnology and the source of genetic modification influence consumer reactions to citrus products from genome-edited plants when the motivation for the use of biotechnology is to preserve a crop and no external genes are used.Objective 6. Extension and outreachWe will collaborate with communication strategists to recommend antibody-based HLB detection methods and to introduce the concepts and uses of genome-edited citrus through workshops, field day events, grower meetings, and websites.

ACCESSION NO: 1008984 **SUBFILE:** CRIS
PROJ NO: FLAW-2015-10490 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2016-70016-24828 **PROPOSAL NO:** 2015-10490
START: 01 FEB 2016 **TERM:** 31 JAN 2018
GRANT AMT: $1,975,000 **GRANT YR:** 2016
AWARD TOTAL: $1,975,000
INITIAL AWARD YEAR: 2016

INVESTIGATOR: Santra, S.

PERFORMING INSTITUTION:
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NIFA CENTERS OF EXCELLENCE: MULTIFUNCTIONAL SURFACE/SUB-SURFACE/SYSTEMIC THERAPEUTIC (COE:MS3T) TECHNOLOGY FOR HLB MANAGEMENT

NON-TECHNICAL SUMMARY: Huanglongbing (HLB) is one of the most devastating citrus diseases, caused by the phloem-restricted bacterium 'Candidatus Liberibacter asiaticus' (CLas). The Asian Citrus Psyllid (ACP, Diaphorina citri) is an insect that carries CLas in its gut, spreading the disease from tree to tree. HLB has caused serious damage to the Florida citrus industry (with over 95% of commercial groves affected), now threatening to become endemic in Texas and increasing numbers of infected trees found in residential California threatening commercial groves. Today, citrus growers are in a critical need of robust treatment methods to save their orchards, protect their investments and continue to make profits. To meet the urgent need of growers, this multi-state, multi-institutional and trans-disciplinary NIFA-CoE project will focus on a comprehensive HLB management solution that targets both the insect and the bacteria. The long-term goal of this project is to develop an industrially-viable, multifunctional bactericidal technology (MS3T) for delivering foliar spray based products for HLB, and others citrus diseases (a path-forward to sustainable citriculture). The proposed non-phytotoxic MS3T product is formulated with natural clay based film-forming ACP repellent material, which also serves as a delivery system for two potent bactericides (surface/sub-surface restricted and systemic). The project will focus on determining how the formulations perform regarding to their stability, rainfastness, roughness, thickness, composition and residuals using state-of-the-art analytical tools for material science. The formulations will be optimized for prime effect on target HLB bacteria and ACP repellency, satisfactory ability to withstand rainfall, while minimizing toxicity to the plants or negative environmental and human health impacts. Growers will apply the MS3T products using conventional foliar spray methods and existing pesticide application equipment. MS3T has attributes to prevent ACP invasion, eradicate CLas population in ACPs and control bacteria in infected trees. Successful outcome of this project will deliver a HLB management solution to growers which will allow them to make profit from HLB-affected trees through minimizing yield loss, improving fruit quality and reducing application frequency (saving labor and materials cost).

OBJECTIVES: The goal of the proposed multifunctional surface/sub-surface/systemic therapeutic (MS3T) technology is to offer a comprehensive HLB management solution that is industrially-viable, affordable to growers, sustainable and has minimal negative impact on the

environment and human health. The project is expected to deliver a line of products customized to meet the needs of citrus industries to prevent ACP invasion, eradicate Candidatus Liberibacter asiaticus (CLas) population, eventually controlling CLas titer in infected trees.The MS3T technology offers powerful attributes, which will enable growers to profit from HLB-affected trees through minimizing yield loss, improving fruit quality and reducing application frequency (saving labor cost). After consulting with growers, agrichemical industry partners, regulatory consultants and extension specialists, the Center of Excellence (CoE) team has identified the following immediate research and outreach objectives to meet the project goal.Research objectives:Objective 1. Develop non-phytotoxic MS3T formulation, characterize for residual and optimize synthesis process for achieving best efficacy at low product cost (Santra, Tetard, Labbe).1.1. MS3T material development and optimization (Santra): We will optimize MS3T composition (relative ratio of active components and inerts) to achieve optimal bactericidal (in vitro, greenhouse and field efficacy), rainfastness, phytotoxicity and ACP repellency, while minimizing negative environmental (residual), and human health (cytotoxicity) impacts (comparable to industry standards such as Kocide® 3000, Nordox 30/30 WG). This optimization process will require a team of material characterization experts and therefore will involve Co-PDs (Tetard, Labbe) with relevant expertise.1.2. MS3T material characterization (Santra, Tetard, Labbe)1.2.1. Characterization of MS3T film (Santra, Tetard): Properties of MS3T (and its components) which includes stability, rainfastness, roughness, thickness, composition and residual will be studied using a battery of material characterization techniques (SEM, SEM-EDS, AFM, Optical, Raman, FT-IR, AAS and Disulfine Blue Assay).1.2.2. Zn-chelate structure, stability and interactions (Labbe, Santra): To expedite the Clay+Zn-chelate and Zn-Chelate control (Santra) optimization, molecular modeling and simulations (computational) work will be developed. Specifically we will focus using the models to: (1) study the structure and stability in aqueous solution, (2) study the structure and stability in planta, and (3) study the interaction of Zn-chelate with lignin and cellulose. This study will help the team to understand the stability, transport and mobility of Zn-Chelate in planta.1.2.3. Antibacterial properties studies (Santra): Preliminary antibacterial properties of MS3T formulations and appropriate controls will be evaluated against several model plant pathogens, X. alfalfae (ATCC 49120); P. syringae (ATCC 19310) and C. michiganensis (ATCC 10202) using the following two assays.1.2.3.1. Bacterial growth Minimum Inhibition Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC) Assay1.2.3.2. CFU assay to determine bacterial killing1.2.4. Rainfastness study (Santra): Rainfastness of the MS3T materials will be evaluated by spray treating citrus seedlings in greenhouse conditions. Treated samples and controls will be analyzed using SEM, AAS, Raman, FT-IR techniques.1.2.5. Phytotoxicity study (Santra): Plant tissue damage (phytotoxicity) of MS3T formulations will be studied in an environmental growth chamber using tomato plants (model plant system for preliminary evaluation). We will use citrus plants only for the optimized formulations, which are ready for field trial.1.2.6. Preliminary cytotoxicity study (Santra): Two relevant eukaryotic cell lines will be used for in vitro screening of contact and inhalational toxicity. Cell viability assays will be performed as a measure of toxicity using the standard MTS assay.Objective 2. Evaluate the efficacy of MS3T formulations against ACP and CLas, and optimize the application rate and schedule for optimal disease control (De La Fuente, Lee, Chumbimuni -Torres, Labbe, Tetard, Johnson, Graham, Santra)2.1. Mode of action and systemic activity evaluation (De La Fuente, Lee, Chumbimuni -Torres, Labbe, Tetard): We will study the interaction of MS3T with plant tissues at the surface, sub-surface and systemic level. Results from this study will allow for optimization of the MS3T formulations and will provide valuable information of residual activity.2.1.1. Surface/Sub-surface probing of MS3T (actives) with FT-IR and confocal Raman imaging and spectroscopy (Tetard): We will track Zn-Chelate movement (from surface/sub-surface to phloem tissue) using a FT-IR/Confocal Raman based integrated imaging and spectroscopy technique.2.1.2. Systemic activity of Zn-chelate2.1.2.1. Effect of Zn-chelate on CLas in planta using a model vascular

channels (Santra, De La Fuente): We will study the effect of Zn-chelate (systemic component of MS3T) on the bacterial communities present in phloem, by mimicking the vascular channels using microfluidic chambers, custom microfabricated devices, where bacteria grow under constant liquid flow.2.1.2.2. Zn-chelate - systemic movement, phloem concentration and half-life (Lee, Chumbimuni-Torres, Santra, Johnson): A reliable chemical sensing tool, capable of tracking systemic activity and, estimating phloem concentration level of Zn-chelate between two successive spray applications will be developed for the assessment of spray rate and timings. Using this tool we will estimate the half-life of Zn-chelate to understand its potential fate in planta (proof-of-concept).2.1.2.3. Measuring residual Zn-chelate in plants (Santra, Labbe, Tetard, Johnson): We will develop and use a near infrared (NIR) sensor capable of detecting the presence of these materials using the IR fingerprints of the individual components to study the potential Zn-chelate and Fixed-Quat residuals in plant tissues (proof-of-concept).2.2. Preliminary greenhouse trials: application timing (Johnson, Santra, Labbe): Greenhouse trees will be used for all preliminary tests of flush related application timing, rainfastness and efficacy of MS3T formulations.2.3. Field trials (Johnson, Graham, Santra): The efficacy of the MS3T formulation will be evaluated at three timing schedules and a control: 1) every 21 days in the rainy season, every 60 days in the dry season 2) every 30 days from first spring flush to last fall flush; 3) as needed based on observation of Kaolin clay on leaves and compared to 4) untreated control. Timing may be adjusted based on Kaolin clay coverage and rain patterns.2.3.1. Field trials-efficacy of MS3T formulations against HLB (and citrus canker) and its impact on ACP: Two age classes of trees will be evaluated to determine the field efficacy of selected MS3T formulations against HLB: 3-yr-old (10-20% HLB incidence) and 5-year-old (80-90% 'Ruby Red' grapefruit blocks).2.3.2. Field trial - Efficacy of MS3T against HLB in orange: A sweet orange field trial will test the same applications as for grapefruit to determine the field efficacy of selected MS3T formulations against HLB.2.3.3 Effect of film-based treatment for pest control: Film based trials for ACP controls carried out under CRDF funding (project # 858). Control potential of ACP with Clay-modified material will be studied within the scope of this project.2.4 Effect of MS3T on Candidatus Liberibacter asiaticus in the ACP gut (Johnson): ACP adults and nymphs (if present) will be collected from treated and untreated trees in the field trial for this study. Psyllid DNA extraction and quantification of CLas by qPCR using standard techniques will be performed.

APPROACH: The project will generate samples and experimental data from research activities related to:MS3T formulations and their optimizationSamples and experimental data resulting from the comprehensive characterization of MS3T formulationSamples and experimental data resulting from the characterization of MS3T properties and residues on/in citrus plantsExperimental data from greenhouse and field efficacy studiesThe following data will be collected during the characterization of the samples:Optical, AFM, SEM, SEM-EDX, Raman, infrared and UV/Vis spectra, AAS, electrochemistry sensing,New biosensing tools will be developed for systemic study of metal-chelatesAssays: Disulfine Blue Assay, Bacterial growth Minimum Inhibition Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC) Assay, CFU assay, cytotoxicity assay, trypan blue exclusion assayAt least, three sets of data will be acquired for each set of experiments.The data will be analyzed and presented in graphic and numerical form. Graphics will be generated using Origin, PowerPoint and Adobe Creative Suite software. Images that will be generated from SEM, AFM and optical microscopy will be processed using the software available for respective instruments (Zeiss for SEM, and Witec Project Four, Nanoscope and Gwyddion for AFM).Greenhouse and field trials on grapefruit and sweet orange will be performed to determine efficacy and the most effective method and timing of application. Currently used field spray application methods will be used assess the economic sustainability of the treatment. Efficacy will be determined based on

bacterial titer, fruit production (boxes), and quality (size, shape, color, and standard juice quality characters including brix/acid ratio). Samples will also be taken from these trees to determine the systemic movement and residue of the particles using the detection techniques under development in objective 1. Using special microfluidic chamber techniques, developed to study vascular bacterial pathogens, the mode of action of MS3T against Liberibacter and/or related bacteria will be determined.All numerical data will be saved in txt files. Figures will be produced in .eps, .jpg or .tif formats. Documents and presentation will be created using Tex, Word, Portable Document Format (PDF), and PowerPoint.Throughout the project, students will be taught the ethical responsibilities towards data management and sharing. The team of PDs will regularly meet student to discuss experimental design and optimization, data acquisition, data evaluation, numerical calculations to evaluate the progress of the project based on collected data.Progress towards project objectives will be assessed annually at project meetings and as needed during the year with focus group meetings of the PD, co-PDs and students.Data management responsibility:Each co-PD will be responsible for storing the data acquired from the project, under a folder that is accessible to PD.Data developed under the proposed project will be deposited onto a data server at the University of Central Florida's NanoScience Technology Center. Access to these data will be controlled by a username/password scheme for faculty and student working on the proposed project. Physical access to the server is controlled by key entry and is limited to IT staff only. In addition to this a copy will be stored on an additional computer and a third copy will be stored on back-up storage equipment. The team will share data regularly and the data and research plan will be discussed at joint group meetings.Archiving of data and samples:The data server will have a space reserved to preserve the data produced over the duration of the proposed project. The team will maintain their access to the data during and after the completion of the proposed project without time limit. In addition, data will also be archived through backup on external hard drives to preserve data in case of loss of server issues. When students collect data at the labs of collaborators, they will be responsible for storing and archiving that data according to policies outlined above. Samples will be stored at the labs were analyses occurred according to safety regulations. All the samples will be labeled and stored.

ACCESSION NO: 1008974 **SUBFILE:** CRIS
PROJ NO: FLAW-2015-10479 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2016-70016-24782 **PROPOSAL NO:** 2015-10479
START: 01 FEB 2016 **TERM:** 31 JAN 2018
GRANT AMT: $2,800,000 **GRANT YR:** 2016
AWARD TOTAL: $2,800,000
INITIAL AWARD YEAR: 2016

INVESTIGATOR: Stelinski, K. S.

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TARGETING MICROBES TO CONTROL HUANGLONGBING DISEASE OF CITRUS

NON-TECHNICAL SUMMARY: This project proposes to eliminate huanglongbing (HLB) using a novel, gene-based bacterial therapy strategy that targets the HLB pathogen and Asian citrus psyllid symbionts. Specifically, we propose to develop morpholino-based bactericides to reduce pathogen transmission and eliminate infections in existing trees. Peptide phosphorodiamidate morpholino oligomers (PPMOs) are synthetic molecules that mimic DNA and inhibit bacterial gene expression. They have a proven track record of limiting bacterial populations in the treatment of human pathogens. Development of PPMO bactericides to manage HLB offers a significant advantage over traditional insecticide use or antibiotic therapy. These engineered molecules can be delivered specifically to target bacteria based on gene sequence, avoiding the problems of effecting non-target bacteria. Insecticidal application and replanting infected trees are currently the most effective HLB management strategies; however, these options are not economically sustainable and may not be sufficient to protect young trees from infection. Targeting specific bacteria, such as the HLB pathogen, or ACP endosymbionts needed for transmission and insect survival, are tactics that have gained much favor within the scientific community. The goal of the current project is to provide proof-of-concept data supporting the further development and use of PPMO bactericides for HLB managment in large-scale citrus project.The ultimategoal is to develop a "patentable" product that could be employed by growers within the next five years. Significant extension and socio-economic components are built into the project for training purposes to integrate the novel tools for HLB managment into current programs.

OBJECTIVES: The primary goal of the proposed research is to develop a novel technology, Morpholino (PPMO)-EGS, which utilizes RNA-based bactericidal agents for management of CLas and ACP. PPMO-EGS technology will be used to inactivate gene expression in the bacterial plant pathogen, Candidatus Liberibacter asiaticus (CLas) and symbionts in the Asian citrus psyllid (ACP). Research directed toward identification of antimicrobial or insecticidal PPMOs will occur during the first phase of the project (years 1-2).The PPMO biochemistry will be carried out at Yale University, but will be evaluated by the PD and Co-PD to test the efficacy of the PPMO-EGS technology in this biological system (bacteria and psyllid). As mentioned earlier, we have developed a system that can grow CLas under a short term culture period which is long enough to evaluate, antibacterial efficacy experiments using the AA medium plus the PPMO product with CLas bacteria isolated from positive citrus plant sap solutions. We have

successfully cultured two of the endosymbiotic bacteria isolated from ACP, and have sequences to target a known Wolbcachia. Efficacy will be evaluate PPMO in cultures, and in live ACP.Following critical assessment of results, several PPMO candidates will be selected for further analysis in larger-scale laboratory and glasshouse efficacy experiments using citrus seedlings and ACP. Comparative analysis of candidates via microinjection, bacteria cultures, and feeding assays will be conducted to analyze the effects on Las, psyllid fitness and transmission capacity.Objective 1: Development of morpholino (PPMO)-EGS Technology to disrupt CLas in Citrus plants. CLas gene expression in plants can be specifically inhibited by developing a cell-penetrating peptide-morpholino oligonucleotide conjugate (PPMO), resulting in a bactericidal effect.Hypothesis 1: CLas-infected citrus trees can be "cured" of infection.Objective 2: Development of morpholino (PPMO)-EGS Technology targeting CLas and bacterial symbionts in ACP. CLas and symbiont gene expression can be specifically inhibited in the psyllid by developing a cell-specific PPMO, resulting in a bactericidal effect. Gene-specific antimicrobials can be delivered using methods developed for RNAi-based technologies to reduce CLas infection and transmission in field settings.Hypothesis 2.1 ACP can be "cured" of infection, effectively eliminating pathogen transmission. Hypothesis 2.2 Removal of ACP symbionts will reduce psyllid fitness, eliminating the population of vectors (insecticidal approach).Socio-economic modeling and outreach: Our proposed economic approach is to perform a benefit cost analysis under the standard assumption that growers' objective is to maximize profit (Nicholson 2002). Therefore, we aim to identify and quantify costs and benefits for Florida citrus growers to manage HLB using a gene-based bactericide. HLB is endemic in Florida, where the average percentage of infected acres (trees) in a citrus operation is 90% (80%) (Singerman 2015). The costs and benefits from using a morpholino-based bactericide will be compared to those arising from a baseline alternative defined by standard current practices, and from a third alternative consisting of replanting trees using Citrus Health Management Areas (CHMA). In this way, we will be able to establish which of the alternatives outweighs the others in each case from an economic standpoint. The expected economic benefit from the use of the bactericide to citrus growers is increased profitability as the result of increased yields due to HLB mitigation. During the two-year timespan of this project, efforts will be focused on surveying growers to establish the costs of citrus production under a baseline. We will share our findings with citrus growers throughout the duration of the project by publishing extension articles that will become available in the project's website. We will also present our findings at different industry meetings.The proposed extension approach is to initiate communication with stakeholders regarding synthetic bactericide/biopesticide information and benefits, and education of public and commodity industries through existing university extension. The outreach objectives for this proposal include determining the most effective means to educate urban and grower communities about benefits of harnessing Morpholino-EGS technology as bactericidal agents to reduce Las and ACP. A key component of this project will be the dissemination of the research findings to the Florida and Texas citrus industries where this research will be conducted. Information will also be disseminated to California citrus growers, who are now looking for new tools for ACP and HLB management as the pest and disease spread throughout that state. We will target each of these state citrus industries with our outreach activities since the entire U.S. industry can potentially benefit from the research findings.During this initial phase of the research program, a series of focus groups and one-on-one interviews will be conducted with growers to better understand their concerns about, and knowledge of, non-insecticide food production techniques. In addition, we will ask the industry to participate in an online survey through the Citrus Research and Education Center (CREC) website. The survey will ask growers about their current knowledge of and feelings for management of ACP and HLB. We will also ask growers about their current practices with regard vector and disease management within each state. At the conclusion of the study, those growers who responded to the original study will be contacted to complete a follow up survey.Trade magazine articles are planned during the course of the project

to discuss the development of PPMOs as novel bactericidal management tools. We will also gather quarterly at events organized by IFAS extension throughout the season with a member or members of the project team to discuss the progress of the project. Evaluation of research progress will be discussed and grower input regarding adoption of our new technologies will be assessed at these meetings. Throughout the year, at regular intervals, we will post updates about our research progress on the CREC extension website (www.crec.ifas.ufl.edu).

APPROACH: Hypothesis 1: CLas-infected citrus trees can be "cured" of infection. Gene targets will be examined and used to develop suitable PPMO products. DNA can be treated with a suitable restriction enzyme, after an examination of the sequence at either ends of the gyrA gene, and isolation of the relevant gyrA fragment pursued. The fragment is cloned into a plasmid in which it will be under the control of a T7 RNA polymerase promoter, transcribed and the RNA used in tests in vitro with RNase P and an EGS designed suitable to show that the EGS technology will work. The region of the conserved sequence of the gene can be easily utilized for any further work, Southern and Northern blots, on the gyrA mRNA isolated from the bacterium after exposure to the appropriate EGS to demonstrate the effectiveness of the technology in terms of cleaving the targeted RNA.The last step of synthesis of the conjugate will be joining the CPP with the MO to produce a PPMO construct. Some minor adjustments inthe conditions of synthesis have already yielded higher yields, e.g., raising the temperature of the overnight incubation (20 hr) to 60oC, and calibrating with accuracy the concentrations of different chemicals in the reaction. For example, the concentration of various chemicals used in the linking reaction has to be normalized to the amount of MO in the reaction. The concentration of N, N diiso-propylethylamine was calculated accurately (4.5 equivalents per equivalent of the peptide) in lieu of the prescription to use two drops from a syringe. Aside from delivery of the PPMO products into trees and ACP, the laboratory at Yale will provide sufficient material to carry out all the experiments mentioned here.The amount of PPMO, several grams, needed to do a realistic test of this agent with respect to the infection of full sized citrus plants infected with CLas would stretch the capabilities of any research laboratory. However, quick assays of the effect of the PPMO on the specific mRNA from CLas in our AA medium bioassay, along with already developed bioassays for evaluating the movement and efficacy to eliminate CLas from infected citrus seedlings can be done (Hunter USDA). The medium bioassay is conducted in 96 well plates and for those assays synthesis of conjugates can be easily produced. The PPMO (5 to15 µM final concentration) can be added to 50 µl of sap and the number of bacteria assayed after an amount of time to be determined by the growth characteristics of CLas under these conditions. Validation of PPMO efficacy in Planta. Citrus cuttings or one year-old single-stem Valencia trees (C. sinensis) that are five to eight month post-grafting, and not treated with systemic insecticide will be used for plant assays. rees will be placed in a growth room with CLas-infested ACP for a period of one month until trees are uniformly infected. A subsample of psyllids will be collected following plant inoculations to confirm the CLas infestation rate of the insects. The trees will then be removed and treated with insecticide to eliminate all developmental stages ACP. The trees will be transferred to a greenhouse and maintained in a greenhouse for four months to allow for systemic infection.Prior to treatment, four leaves will be removed from each tree, two from each side of the apex of the tree and two from each side of the base of the canopy, for initial titer (T0) using quantitative real-time polymerase chain reaction (qPCR) assays. Trees will then be treated with candidate PPMOs by either foliar sprays or by root infusion. For root infusions, one lateral root from each plant will be carefully separated, but kept attached to the main root system. This intact root will be used after gently cleaning the

lateral root of medium, cutting the root tip, and placing the cut tip into a 50 ml tube containing the sample material. After 24 hours the material will be removed from the tube and replaced by distilled water for the duration of the assay. Seven days post-treatment, four additional leaves will be removed from similar locations as the T0 samples and used to monitor movement of the antimicrobial materials. Data collected will include leaf location for T0 and T1 (right/top, right/bottom, left/top or left/bottom, unless it is determined that this does not provide valuable information). The assay will consist of 25 trees per assay with twenty infected/treated and five healthy/treated. For each assay there also will be five healthy untreated and five infected untreated. Similar assays and monitoring will be completed using foliar spray applications of PPMOs.Detection of CLas will be done using quantitative real-time PCR assays,performed in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using a TaqMan qPCR assay as described by Li et al. (2006) for detection of CLas.Hypothesis 2.1 ACP can be "cured" of infection, effectively eliminating pathogen transmission. Injection and feeding bioassays with psyllids will be conducted by Pelz-Stelinski (Univ. Fla.) to evaluate the efficacy of PPMOs for inhibiting infection/transmission by ACP, and to destroy ACP symbionts, thereby killing ACP at all life stages. Recent investigations suggest that elimination of the ACP symbiont, Candidatus Proftella armatura, should reduce or eliminate CLas transmission.Acquisition assays will be conducted by injecting 20 adult ACP with the PPMO treatment, and caging on Las-infected 'Valencia' sweet orange plants in mesh enclosures for 2 or 4 week acquisition access periods (AAPs). After each AAP, psyllids will be collected and preserved in 80% ethanol at -20°C for subsequent detection of Las by quantitative real-time PCR (qPCR) following the procedures described below and by Pelz-Stelinski et al. (2010).To evaluate inoculation efficiency following exposure to PPMO's, 50 newly-emerged adult psyllids reared on infected plants will be injected with PPMO treatments and released onto uninfected plant leaves. Successful inoculation of leaves by treated ACP will be compared to inoculation by ACP not exposed to CLas-targeting PPMOs. This experiment will be replicated five times per treatment. After a two-week inoculation access period (IAP), psyllids and leaves will be removed and tested for CLas with qPCR as described by Pelz-Stelinski et al. (2010) and Coy et al. (2014).Hypothesis 2.2 Removal of ACP symbionts will reduce psyllid fitness, eliminating the population of vectors (insecticidal approach). The effect of symbiont-targeting PPMOs on psyllid life history will be evaluated in a series of fitness studies that will assess fecundity, survival, and development. To assess the fecundity of PPMO-exposed insects, individual pairs of 3-day-old male and female psyllids will be held on in Petri dishes for acquisition of symbionts using an artificial feeding method or by direct microinjection of symbiont cultures. Control insects will be fed a symbiont-free (filter-sterilized) sugar solution or injected with saline buffer. Insect pairs will then be transferred to pathogen-free plants with flushing plant tissue for mating and oviposition. The number of eggs produced per pair will be counted and removed every five days under a stereomicroscope. Each treatment will be replicated 15 times per psyllid pair.Development time of psyllid nymphs will be assessed by releasing 200 PPMO-exposed or non-exposed pathogen-free citrus plants. After five days, adults will be removed and the number of eggs per plant counted. Immature and adult progeny will be counted at 3 d intervals until adult emergence. Symbiont infections in parent and adult psyllid offspring will be determined using the qPCR assay described below. Survival of symbiont exposed or unexposed psyllids on healthy citrus plants will be compared by releasing 20 newly emerged adult insects of each gender onto plants.

ACCESSION NO: 1009017 **SUBFILE:** CRIS
PROJ NO: FLAW-2015-10491 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2016-70016-24844 **PROPOSAL NO:** 2015-10491
START: 01 FEB 2016 **TERM:** 31 JAN 2020
GRANT AMT: $3,999,508 **GRANT YR:** 2016
AWARD TOTAL: $3,999,508
INITIAL AWARD YEAR: 2016

INVESTIGATOR: Gabriel, D. W.

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DEVELOPMENT NEW THERAPIES FOR HUANGLONGBING VIA CULTURING CA. LIBERIBACTER ASIATICUS

NON-TECHNICAL SUMMARY: Much progress has been achieved in the control of newly emerging plant diseases by first understanding the various strategies used by the pathogens to cause disease and overcome native plant defenses, and then by interfering with key elements of their life cycles, infection strategies, pathogenicity determinants and/or counter-defenses. Genomic DNA sequencing allows the identification of all of the suspected genes in a pathogen's offensive and defensive arsenals. Functional genomics typically confirms suspected pathogen mechanisms for both offense and defense, and provides a firm basis for testing of disease control strategies in infected plants. With newly emerging pathogens, this often involves identification of key new pathogenicity genes, functional confirmation, and hypothesis testing to develop control measures. When a pathogen is uncultured, Koch's postulates cannot be completed, functional genomics becomes severely impacted, and hypothesis testing is extremely difficult and limited. This has been the case with Huanglongbing (HLB) and its associated agent, Ca. Liberibacter asiaticus (Las). A priority for functional studies is to culture Las, in order to leverage the abundant available genomic information and use it towards developing disease control and/or management methods. Limited progress has been made in attempting to culture Las. Only 2 labs (Davis and De La Fuente) have published substantial lab results indicating partial progress towards the goal. Lack of success has likely been due to multiple reasons, including: 1) missing required metabolites in media tested; 2) missing genes or pathways required for free-living growth in the specific Las strain used; 3) unrecognized toxic substances in some media used; 4) activation of phage lytic cycles genes in the Las genome, 5) a substantially weak Las outer membrane barrier, and 6) missing host gene regulation functions following separation of Las from an intracellular host environment. Any combination of these factors could prevent growth, even when a perfect combination is hit with a particular media formulation. What is perhaps most frustrating is that Liberibacter crescens (Lcr) can be readily cultured, and yet it has only a 20% larger genome than Las (1.5 Mbp for Lcr vs 1.26 Mbp for Las). Lcr is limited in that it may no longer be pathogenic or capable of growth in any host, despite dedicated efforts by several labs to inoculate it into different plants and insects. Koch's postulates have yet to be completed for Las or Lcr. Lcr has nevertheless been used (Gabriel lab) to develop a functional genomics toolkit for 1) Las gene expression; 2) characterization of Las phage gene promoters and key regulators, and 3) early and late gene reporter constructs capable of high throughput screening for small molecules that may affect phage gene expression, either directly or indirectly. The Lcr

functional genomics system allowed identification of the Las prophage peroxidase as a likely critical lysogenic conversion gene that is needed by Las to both suppress citrus defense (peroxide) and citrus defense signaling (Gabriel lab). Las peroxidase is thus a potential target for Las control in citrus. Published comparative gene expression analyses (Gabriel lab) have shown that high levels of phage late gene expression, including lytic genes, occurs in Las-infected periwinkle, less in citrus, and almost none in psyllids. Since phage lytic replication does not occur in psyllids, indicating that all phage genes are under stringent repression in psyllids, but less so in citrus. The Las late gene reporter construct was used in the Lcr system to allow identification of a 27 kDa protein in psyllid extracts that directly binds to the late gene promoter and behaves as a strong repressor (Gabriel lab, unpublished). Since all Florida Las strains examined to date carry similar phage, it may be possible to artificially de-repress the phage lytic cycle and/or individual lytic genes in Las infected psyllids and citrus trees in the field and thereby cure both. This repressor and the promoter it binds to are thus two additional potential targets for Las control in psyllids, and expression of this protein is proposed here to help enable culturing of Las. More recently an additional key Las phage regulatory gene was identified by analysis of Las gene expression constructs in Lcr (Gabriel lab), helping to explain why Las phage particles readily form in periwinkle, but not in citrus, and providing another potential control target. Although Lcr has been genetically manipulated and is tractable for transformation, including knockout and knock-in mutations, Las has yet to be transformed so that not even a single genetically marked Las strain has been produced. Although antibodies have been produced that might be used to trap and concentrate Las (for observational, transformation, and culturing purposes), the specificity and threshold level of detection of the antibodies was not reported. This proposal is to: 1) coordinate simultaneous parallel culturing efforts in 4 labs (Duan, Davis, Killiny and De La Fuente) using a variety of complementary approaches and real-time data and deliverable sharing of what works and what does not, and using different Las sources (insect vs. plant); 2) to leverage what has been learned from functional genomic analyses of Lcr and the Las phage in continuing efforts to use Lcr as a culturable proxy (Gabriel) to engineer Lcr to be more Las-like in terms of its outer membrane to allow a more straightforward approach to obtaining antibodies with high specificity and affinity (Ma), and 3) to develop a phage vector (Jones & Gabriel) and/or conjugation system (Gabriel) so that Las can be genetically marked and engineered to enable growth on artificial media.

OBJECTIVES: This is a Standard Research and Extension Project (SREP) focused on culturing Ca. Liberibacterasiaticus (Las), with direct applications expected in Huanglongbing (HLB) control and detection/diagnostics. Despite the enormous efforts that have gone into obtaining 5 complete genomic DNAsequences of the 3 different species of HLB bacteria, not one of the causal species has beencultured, and potential molecular targets for control cannot be functionally validated. Mostculturing efforts have focused on nutritional supplements and various media formulations in anattempt to supply presumably missing nutrients, but only to a single Las genotype or clonal group.This may be the wrong approach. One Liberibacter species, L. crescens (Lcr), was readily culturedmany years ago using very standard bacterial techniques. Its genome is 20% larger than all othersequenced Liberibacters, and has all genes needed for culture. It is also missing specific phage lyticgenes that can kill their bacterial hosts under stress conditions and are found in most Las strains andmay limit culture. Unfortunately, the single extant Lcr strain also appears to have lostpathogenicity and so is not useful for citrus or general plant pathogenicity assays. Continuedattempts to culture a single Las genotype or clone may be fruitless, and therefore multiple parallelapproaches are proposed here, including attempts to culture a phageless Las strain, a new citrusspecies from Colombia that may be related to Lcr, microfluidic chambers, improved chemicalscreens and those involving improved monoclonal antibodies, and both chemical and phagetherapies for HLB.Objective 1: Concerted, parallel and coordinated efforts towards obtaining viable Las and/or Lca cultures (Castañeda, Davis, De La

Fuente, Duan and Killiny). a. Psyllid hemolymph and citrus phloem sap composition b. Enhanced growth observed using the TPIMS chemical libraryObjective 2: Define the influence of physical environment in Las culturability (De La Fuente & Ma). a. Cell surface antigens suitable for antibody generation b. Outer membrane proteins as targeted c. Generation of antibodies d. Antibody evaluations e. Microfluidic chambersObjective 3: Define the role of chemical signaling and co?factors in culturability of Las and/or Lca (Davis, De La Fuente, Gabriel & Killiny)Objective 4: Develop genetic tools that enable delivery of (missing) candidate growth factor genes identified and identified and partially characterized into Las (Gabriel & Jones) a. Screen multiple sources of Liberibacter spp. for phage particles and for Lcr?infecting phages b. Develop assays for detecting phage binding to and infection of Las c. Develop Lcr/Las-cross-reacting phages as a transduction system for genetic modification of Las d. Use elements of the SC1 and SC2 prophages of Las to develop a cosmid vector/phage transfection systemObjective 5: Outreach (Alabi, Roberts, Vidalakis)

APPROACH: Objective 1: Concerted, parallel and coordinated efforts towards obtaining viable Las and/or Lca cultures (Castañeda, Davis, De La Fuente, Duan and Killiny). Published empirical approaches to Las culture and use of diverse nutritional resources will be continued (Davis, De La Fuente). These will be expanded in two additional labs (Duan, Killiny), using microarrays, combinatorial libraries, detailed phloem sap analyses from healthy versus infected citrus, fresh versus spent media, psyllid hemolymph, and honeydew. Data will be shared amongst all laboratories so that continual improvements in media formulations can be made. Inoculum derived from both psyllid (Davis, Killiny and Duan labs) and citrus, including phageless Japanese isolates (De La Fuente) will be tested. Phage lytic cycle inhibitors from psyllids will also be provided (Gabriel).Lca is tentatively expected to be imported on bud-sticks from infected material in Colombia; materials will be collected in Colombia (refer attached letter, Castañeda) and imported by Gabriel. Following grafting to clean citrus, material will be monitored for Lca and curated separately from Las infected materials in the Plant Containment Facility. If Lca can be maintained in citrus, Davis will travel to Gainesville to assist in attempts to culture from this material.Objective 2: Define the influence of physical environment in Las culturability (De La Fuente & Ma). All attempts to culture Las to date have used standard batch systems such as agar plates, test tubes, and flasks. Bacteria can behave much differently under different pressures than when grown in batch cultures. Las grows actively in phloem cells where nutrients are being transported by liquid flow, and pressures are high and fluctuating. The microfuidic chamber currently used in the De La Fuente lab allows experimental variation in growth pressures, temperatures, gas exchange and flow rates. Antibodies are needed in order to coat the microfluidic chambers and both increase initial titer of the inoculum and to observe Las behavior in these chambers. The Ma lab will generate high affinity monoclonal antibodies (MAbs) against two classes of cell surface antigens. Firstly, the variable O-antigen epitopes in the outer membrane structural lipopolysaccharide (LPS) will be used to develop species-specific antibodies. LPS will be extracted from Lcr, and high affinity, Liberibacter-specific antibodies will be screened using a library of synthetic, monoclonal antibodies generated by Dr. Xin Ge's laboratory at U.C., Riverside. Secondly, antigenic outer membrane proteins, such as abundant adhesins, will be identified for antibody development using the same library. MAbs will also be useful to develop non-PCR detection methods, such as ELISAObjective 3: Define the role of chemical signaling and co?factors in culturability of Las and/or Lca (Davis, De La Fuente, Gabriel & Killiny) One of the few examples of successful culturing of Las (Davis) was achieved when Las was co-cultured with another bacterium. Attempts at co-cultivation with Lcr will be made to identify unknown signals using a diffusion 'sandwich' approach in agar plates with

macerated suspensions of infected and non-infected citrus and psyllids. In addition, filtrates from secreted compounds obtained from a metagenomics library; and addition of quorum sensing molecules (Killiny) and siderophores will be evaluated. Direct cultures of Las with Lcr engineered with conditional inhibitors (sucrose sensitivity or temperature sensitive lethal conditional mutations will be attempted. Microfluidic chambers are ideal for direct injection and testing of identified signals or co-factors for Las response (De La Fuente).Objective 4: Develop genetic tools that enable delivery of (missing) candidate growth factor genes identified and identified and partially characterized into Las (Gabriel & Jones) At least one likely necessary growth factor gene of Lcr has been identified (Gabriel lab) that is completely missing from the Las genome. Lcr has proven to be genetically tractable for gene knockouts and gene additions, and two compatible DNA shuttle vectors capable of conjugational transfer and phage packaging (cosmid) and transfection have been proven for use in Lcr (Gabriel lab). The standard functional tools developed for Lcr have not been applied to Las because such techniques are not readily applied to uncultured cells, primarily because of low cell density. Conjugation or phage transfection could overcome these issues, and both will be attempted. If co-cultures are successful (Objective 3), conjugational transfer of a vector carrying the growth factor gene plus marker could be likely achieved using an Lcr strain engineered to die if chemically or genetically induced (CRISPR-Cas9; Yang lab). T4-like phages from S. meliloti have been characterized and some, but not all key Las phage structural phage elements identified (Jones lab). Gabriel & Jones will attempt to create an in vitro Las phage packaging system for genetic manipulation of Las. If successful, such a phage system may be further developed (Gabriel & Yang) into a novel therapeutic agent for targeted killing of Las in infected tissue. Once cultured, Las would need to be reintroduced into psyllids, likely by injection (Killiny, Duan labs), and subsequently into citrus.Objective 5: Outreach (Alabi, Roberts, Vidalakis) The ability to successfully culture Las in vitro will be a huge milestone for the scientific community working on the citrus-HLB-ACP pathosystem. However, it is uncertain if growers and other stakeholders in the citrus industry will fully appreciate the importance of such a breakthrough. Hence, outreach activities will need to be developed and implemented to educate members of the public on the importance/ relevance of the project right from its inception. Even if not considering the different degrees of HLB epidemic in the three leading citrus-producing states, the proposed outreach plan will be implemented such that there is a cohesive message on the promise and outcomes of the project. At the project inception, the focus of the outreach activities will be to educate growers, industry stakeholders and members of the public on the importance of being able to culture Las in vitro. Subsequent activities will then focus on providing periodic project updates and breakthroughs to the stakeholders. Finally, news of successful culturing of Las will be announced via face-to-face, print and electronic media materials to be developed jointly by Vidalakis (California), Roberts (Florida) and Alabi (Texas) in consultation with the PD and other co-PIs. Specific outreach activities planned for the project include:

ACCESSION NO: 1009024 **SUBFILE:** CRIS
PROJ NO: MD.W-2015-10475 **AGENCY:** NIFA MD.W
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2016-70016-24779 **PROPOSAL NO:** 2015-10475
START: 01 FEB 2016 **TERM:** 31 JAN 2019
GRANT AMT: $1,951,763 **GRANT YR:** 2016
AWARD TOTAL: $1,951,763
INITIAL AWARD YEAR: 2016

INVESTIGATOR: Cilia, M.

PERFORMING INSTITUTION:
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ABRAHAM LINCOLN BUILDING 10301 BALTIMORE AVENUE
BELTSVILLE, MARYLAND 20705

HARNESSING NATURAL VARIATION IN TRANSMISSION OF LIBERIBACTER BY THE ASIAN CITRUS PSYLLID TO DEVELOP NOVEL HLB CONTROL STRATEGIES

NON-TECHNICAL SUMMARY: Huanlongbing (HLB) is the most serious disease of citrus. Candidatus Liberibacter asiaticus (CLas), the pathogen associated with HLB, is spread around the grove by the Asian citrus psyllid, a sap-sucking insect vector. Controlling the insect spread of CLas represents the most promising way to control the disease. However, there is a paucity of information on how CLas is spread by the psyllid and currently no tools for a grower to use to detect CLas in the insect. Current detection methods in insects and in plants rely on qPCR and microscopy, and both methods require a laboratory, a skilled researcher, expensive equipment and costly consumables. Our team will pursue four research objectives and one educational objective to better understand how the psyllid transmits CLas in an effort to develop new transmission blocking tools. Drawing upon a new cutting edge scientific field called synthetic biology, we will also develop a low-tech biosensor that a grower can use to detect CLas in insects in the field, one of the goals of the SCRI program. Undergraduate researchers will be involved in all aspects of the synthetic biology project. Although the research plans within each objective are synergistic, the success of one objective does not depend on the others. We realize our research plans are ambitious but our team is up to the challenge.

OBJECTIVES: Huanglongbing (HLB) is a tritrophic disease complex involving citrus host trees, the Asian citrus psyllid (ACP) insect and a phloem-restricted, bacterial pathogen Candidatus Liberibacter asiaticus (CLas). HLB is considered to be the most devastating of all citrus diseases, and there is currently no adequate control strategy. In Florida, an estimated 40-70% of all citrus trees are infected, and HLB effects include production declines (10-20% per year), diminished fruit quality and increased production costs. Some growers have already been forced into bankruptcy. California and Texas have the ACP and isolated reports of HLB, where the spread of HLB is imminent without discovery and implementation of new management practices. Control of ACP-mediated CLas transmission represents a promising, new avenue for HLB control, but there is a paucity of tools available for growers to control HLB from this angle.Our team will pursue four research objectives and one educational objective. Although the research plans within each objective are synergistic, the success of one objective does not depend on the others. We will:1. Discover genetic populations of ACP that

segregate for CLas acquisition and transmission competency.2. Functionally characterize the ACP endosymbiont toxin diaphorin and establish whether the relationship between the ACP endosymbiont Profftella and the ACP is a viable target for ACP control.3. Functionally characterize ACP proteins involved in CLas transmission.4. Develop an ACP-CLas yeast biosensor that can be used by growers5. Enhance cross-disciplinary undergraduate education and research at Cornell through participation in the International Genetically Engineered Machines (iGEM) Competition.The overall, long-term goal of our project is to develop new tools that can be used by citrus growers for CLas-infected ACP monitoring and management, as detailed in Objectives 3 and 4. Two short-term goals of our project that we will achieve during the lifetime of our grant include: to characterize populations of the ACP that vary in their ability to transmit CLas and to produce a yeast biosensor that can readily detect CLas-infected ACP. The ACP populations will be immediately useful for challenging citrus germplasm against HLB and ACP inoculation and feeding. A long-term goal is the distribution of the biosensor technology to growers. A second long-term goal of our proposal is to leverage the knowledge of the molecular basis of CLas transmission by the ACP to produce a genetically modified citrus that will prevent the spread of CLas within a grove. All research will be performed with constant engagement of the stakeholders to provide support and guidance on technology advancement through to commercialization.

APPROACH: Objective 1: Colonies of potentially good or poor vectors will be established using an individual adult female ACP paired with a single male collected from various locations. Insects will be determined to be CLas-free to start and CLas transmission efficiencies will be calculated using transmission assays. Efficient and poor transmitting lines will be crossed and their progeny randomly mated to develop populations segregating in CLas transmission ability.Objective 2: Mass spectrometry-based proteomics will be used to identify proteins interacting with the toxin diaphorin. Potential receptors will be functionally validated surface plasmon resonance and other methods. CTV technology will be used for silencing the diaphorin receptor in the ACP.Objective 3: The CTV technology has been established by the Dawson lab previously. Construction of CTV vectors that target ACP proteins involved in valine catabolism, immune system regulation and bacterial entry is currently in progress in the Cilia and Dawson labs, greenhouse tests to follow.Objective 4: Ongoing research in the Cornish?Laboratory has demonstrated the ability to take?advantage of an endogenous G protein-coupled?receptor (GPCR) signaling pathway present in Saccharomyces cerevisiae to drive a lycopene?biosynthetic pathway and directed evolution. These methods may be implemented to engineer?a GPCR to bind a specific peptide biomarker of CLas in infected ACP. These methods will be used to engineer yeast to turn red when mixed with insects or plant tissue harboring CLas, enabling a rapid and clear determination of CLas status by a grower in the field.Objective 5: iGEM undergraduate research teams will be formed at Cornell and Columbia Universities and will assist with achieving objective 4.

ACCESSION NO: 1008989 **SUBFILE:** CRIS
PROJ NO: NM.W-2015-10483 **AGENCY:** NIFA NM.W
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2016-70016-24781 **PROPOSAL NO:** 2015-10483
START: 01 FEB 2016 **TERM:** 31 JAN 2018
GRANT AMT: $3,320,000 **GRANT YR:** 2016
AWARD TOTAL: $3,320,000
INITIAL AWARD YEAR: 2016

INVESTIGATOR: Gupta, G.

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DESIGN AND DELIVERY OF THERAPEUTIC PROTEINS FOR HLB PROTECTION

NON-TECHNICAL SUMMARY: So far there is no cure for HLB nor is there any HLB-resistant citrus. Therefore, protection measures are urgently needed to save the infected trees and to protect the healthy ones. In this CAP, we will perform systems level studies to understand the battle between Liberibacter and citrus, i.e., Liberibacter attempting to propagate its lifecycle while citrus is trying to clear Liberibacter by mounting innate immune defense. We will use this knowledge to make the innate immune system of citrus stronger to win the battle against Liberibacter. This will be achieved by designing two classes of citrus or citrus-like proteins, i.e., one that directly targets and clears Liberibacter from the phloem and the other that prevents detrimental Liberibacter proteins from disrupting innate immune defense in citrus. We propose to develop delivery strategies for short-term (6 months), intermediate-term (1-2 years), and long-term protection (3-5 years) protection. With these robust and cost-effective tools, the growers will be able to save the infected trees and protect the healthy ones.

OBJECTIVES: GoalsThis CAP focuses on engineering novel innate immunity in citrus via the design and delivery of therapeutic proteins that facilitate rapid clearance of Liberibacter and inhibition of key steps in the development of Huanglongbing (HLB). Unlike small molecule antibiotics and RNAi, the proposed therapy is based on endogenous proteins derived from the citrus innate immune repertoire. The main goals are to develop: (i) a novel strategy for designing therapeutic proteins based on genome-wide studies on Liberibacter-citrus interactions and (ii) transgenic and non-transgenic delivery methods that offer short-, intermediate-, and long-term solutions for Huanglongbing (HLB) protection. This CAP specifically addresses the areas 1 and 4 of the call (page 6 in the call):Bacterial therapy systems that either kill or suppress Candidatus Liberibacter asiaticus (Liberibacter)Development of citrus scions and rootstocks resistant to, or tolerant of, Liberibacter that are suitable for a wide range of growing environments.ObjectivesObjective 1. Identify (a) Liberibacter genes/proteins and (b) citrus genes/proteins as potential targets for therapies to protect against HLB.Objective 2. Design and express HLB-protective proteins that either clear Liberibacter or block disease development.Objective 3. Deliver HLB-protective proteins in planta for short-, intermediate-, and long-term protection.

APPROACH: Objective 1 (Target Identification)(Team: Mikeal Roose, UC Riverside; Rakesh Kaundal, UC Riverside; Goutam Gupta, NMC/NMC; Geoffrey Waldo, NMC/NMC; Hau Ngyuen, NMC)For Liberibacter killer proteins, we will first identify the molecular entities on the Liberibacter membrane that can be targeted by specific citrus proteins. Two high priority targets are the outer-membrane proteins (OMPs) and the conserved lipopolysaccharide (LPS) core, which, as we have shown (see below), can be recognized respectively by citrus proteases and LPS-binding proteins (LBP). For the chimeras, lysis domains will be citrus linear or disulfide-bridgedantimicrobial peptides (AMP), which bind and rupture gram-negative bacterial membranes. We will perform structural and computational analyses to prioritize Liberibacter recognition and lysis domains. For the protein inhibitors of HLB, we will first identify the critical protein-protein interactions in Liberibacter-citrus interactions, for example the ones in the signaling pathways involving pathogen-associated molecular pattern triggered (PTI) and effector triggered signaling. PTI and ETI signaling constitute an important part of plant innate immune defense. Specific pathogen proteins (virulence factors and effectors) tend to disrupt one or more steps in the PTI/ETI signaling pathway, and inhibiting this disruption will be an effective strategy for HLB protection.Specific Tasks will include:Task 1a: (i) To analyze Liberibacter genomes to identify the conserved outer-membrane proteins (OMP) and lipopolysaccharides (LPS); (ii) To screen citrus genomes to identify citrus proteases that cleave Liberibacter OMP and LBP that bind LPS; (iii) To analyze citrus genomes to select citrus AMPs that lyse gram-negative bacteria such as Liberibacter.Task 1b: (i) To measure and analyze dual Liberibacter-citrus transcriptome at different stages of infection (early to late) to predict candidate interactions between Liberibacter effectors and citrus innate immune defense proteins; (ii) To validate the predicted protein-protein interaction pairs by a three-body split-GFP reporter assay.Objective 2 (Design and expression of therapeutic proteins for HLB Protection)(Team: Goutam Gupta, NMC/NMC; Hau Nguyen, NMC/NMC; Geoffrey Waldo, NMC/NMC)We will use structure-based algorithms to design Liberibacter killers and HLB-blockers and will express the therapeutic proteins in tobacco BY-2 cell lines.Specific Tasks will include:Task 2a: To design protein chimeras with recognition (e.g., protease and LBP) and lysis (AMP) domains to rapidly clear Liberibacter. We have already designed three chimeras: (i) tobacco Thionin-D4E1 chimera (tobacco Thionin is 70% identical in sequence to citrus Thionin; D4E1 is a citrus-friendly synthetic AMP); (ii) citrus Thionin-LBP1 chimera; and (iii) citrus Thionin-LBP2 (LBP1 and LBP2 are two citrus LBPs). Note that Thionins have both recognition and lysis functions.Task 2b. To design citrus protein mimics that bind and sequester Liberibacter effectors and inhibit their detrimental interaction with citrus proteins involved in plant innate immune defense.Task 2c. To use two-body split-GFP system to monitor expression therapeutic proteins in tobacco BY-2 cell expression system.Objective 3 (Delivery and efficacy testing of the therapeutic proteins)(Team: Ed Stover, USDA-ARS, Ft. Pierce, FL; William Belknap, Beltsville, MD; James Thomson, USDA-ARS, Albany, CA; Siddarame Gowda; University of Florida, Lake Alfred)We will develop short-, intermediate-, and long-term strategies for in planta delivery of the therapeutic proteins.Specific Tasks will include:Task 3a: To design lipid-based nanocapsules coated with citrus tristeza virus (CTV) capsid protein to deliver the therapeutic proteins; To test efficacy by determining Liberibacter level in greenhouse infected trees by qPCR at 0.5, 1, 2, 4, 8, and 12 months after initial delivery of the therapeutic proteins. Tree growth, health, and HLB symptoms will be monitored at 1, 2, 4, 8, and 12 months after the delivery of the therapeutic proteins.Task 3b: (i) To use CTV as a vector to express the therapeutic proteins for protection against HLB; (ii) To graft transmit this vector to express HLB therapeutic proteins in different infected citrus cultivars and monitor the efficacy as described in Task 3a.Task 3c: (i) To develop

tools for more efficient and robust transgene expression (i.e., improved transcriptional control elements for optimal phloem-specific expression, CRISPR-like systems to repress, edit, or overexpress endogenous genes); (ii) To construct transgenic citrus lines expressing already designed Tobacco Thionin-D4E1, Citrus Thionin-LBP1, and Citrus Thionin-LBP2 chimera and test their efficacy in HLB protection. Note that we have already demonstrated that transgenic citrus (Carrizo) expressing Tobacco Thionin-D4E1 is protective against HLB. The other two (i.e., Citrus Thionin-LBP1, and Citrus Thionin-LBP2) chimeras have been designed to show higher efficacy than the Tobacco Thionin-D4E1 chimera.

ACCESSION NO: 1009006 **SUBFILE:** CRIS
PROJ NO: WNP06833 **AGENCY:** NIFA WN.P
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2016-70016-24824 **PROPOSAL NO:** 2015-10480
START: 01 FEB 2016 **TERM:** 31 JAN 2018
GRANT AMT: $2,115,000 **GRANT YR:** 2016
AWARD TOTAL: $2,115,000
INITIAL AWARD YEAR: 2016

INVESTIGATOR: Gang, D. R.

PERFORMING INSTITUTION:
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DEVELOPMENT OF IN VITRO BIOFILM AND PLANKTONIC CULTURE OF CA. LIBERIBACTER ASIATICUS: A GAME CHANGE IN HLB RESEARCH

NON-TECHNICAL SUMMARY: The lack of ability to culture "Candidatus Liberibacter asiaticus" (CLas) has precluded the use of genetics and gene transformation approaches to identify gene functions involved in CLas viability or virulence. Those are critical tools that will ultimately be needed in development of a fully integrated strategy to control CLas and eventually develop the capability to stop HLB spread by the Asian Citrus Psyllid (ACP) and the resulting economic devastation to citrus agriculture. This project aims to develop means to culture CLas in vitro using two novel, parallel, complementary and integrated strategies that also integrate proteomics, metabolomics and community sequencing approaches. This will ultimately lead to successful culture of CLas under both planktonic growth conditions and as biofilms.

OBJECTIVES: The first major goal of this project is to develop a system to culture, in vitro, "Candidatus Liberibacter asiaticus" (CLas), the causative agent of Citrus Greening Disease, or Huanglongbin (HLB). A second major goal is to then make that system readily available and adopted by the national stakeholders, particularly citrus breeders and other researchers, thereby enabling genetics-based research that will have an enhanced capability to identify means to control the transmission of CLas. A third goal, which will be a natural offshoot of the other goals, is to develop rapid and easy methods to isolate and cultivate new strains of CLas from groves across the nation as well as adapting or mutating strains of CLas within regions of outbreaks or reintroductions that may occur even if means to control CLas are indeed developed. Culturing of new strains like that will be critical long term strategies to control HLB in the face of a changing, adapting pathogen. In order to accomplish those goals, four major objectives have been developed, as outlined directly below. The PD/CoPDs involved as major contributors to efforts to accomplish each objective are indicated in parentheses. The major hypotheses underlying each objective are also listed.Objective 1 (Beyenal, Gang, Killiny): Establish a system for in vitro culture of CLas biofilms.Hypothesis: CLas is so successful as a pathogen in citrus trees and citrus psyllids, and yet is so difficult to culture, because it grows primarily within biofilms in the psyllid and in the citrus tree. Corollaries: CLas may not be the only microbe in these biofilms and identification of the other biofilm community members may be required in order to culture CLas-containing biofilms. Synergetic interactions among the microbes in biofilms support CLas' survival.Objective 2 (Omsland, Gang, Killiny): Establish a system for host cell-free culture of CLas.Hypothesis: CLas is a parasitic bacterium with limited metabolic

capacity that depends on scavenging of nutrients from infected tissue or surrounding cells in a biofilm for replication. By matching pathogen nutrient requirements to the organism's natural metabolic capacity, replication of CLas in pure culture will be achieved.Objective 3 (Gang, Futch, Killiny, Brown, Beyenal and Omsland): Provide SOPs and CLas culture tools to the research community.Hypothesis: The products of this project, a CLas culturing system and information about CLas growth in vivo, will be extremely beneficial to anyone interested in HLB, and thus there will be a demand for access to such information and technology. Targeted extension activities, in addition to standard research publications, will more rapidly lead to adoption of the to-be-developed technology once it is indeed available.The original proposal outlined 3 major research objectives to be accomplished within a 5-year timeframe, plus an additional objective related to extension and outreach. One of those objectives was eliminated at the request of NIFA. For one of the major goals of the project, development of planktonic growth conditions and a planktonic culture system ofCLas, we are limited by the biology of the system, where some things just take a long time to occur within the biological system. Thus, the timeframe originally proposed, where 2 - 3 years to establish conditions for host cell-free metabolic activity (i.e., not necessarily cell division) ofCLas and about 5 years to develop a reliable culture system forCLas in the absence of host cells, is not likely to change much, but we are likely to be well on the way to meeting that goal by the end of the second year of the project. Indeed, we should have a good idea at that point (two year mark) how likely the project is to be a success. Thus, a two year proof of concept effort will be undertaken, which will allow enough research to provide proof of concept that the overall project is likely to be a success. If such is demonstrated, then an additional ~3 years of funding will be requested in a follow-on application.?

APPROACH: Methods applied to accomplish each Objective are outlined below:Objective 1 (biofilm culture method): Proof of CLas existence in biofilms in psyllids has been obtained, suggesting that identification of conditions for culture of CLas under biofilm growth are within reach with the right approach. Using methods well established by Co-PI Beyenal, CLas bacteria will be isolated from biofilms growing in insect and potentially plant tissues, and will be the subject of a battery of experiments designed to identify conditions required for their growth in in vitro biofilms using an approach that enables separation of individual microbial community members (even species found only within biofilms) and analysis of their contribution to biofilm establishment, growth, protection and metabolism. This, in combination with detailed proteomic and metabolomic analysis of the test cultures, will identify those metabolic components required for biofilm growth in vitro.Objective 2 (host cell-free and planktonic culture): Establishment of planktonic growth will be achieved by first determining responses of CLas to nutrients for which the pathogen is a predicted auxotroph. By matching pathogen nutrient requirements to the organism's natural metabolic capacity, replication of CLas in an experimentally amenable culture system will be achieved. A major step in establishment of in vitro planktonic growth will be establishment of a cell culture model for CLas infection, which will offer i) a model to analyze interactions between CLas and insect vector tissue, and ii) a cultivation system to generate enough bacteria for physiological analysis (essential to the process of designing a host cell free cultivation system).Objective 3 (extension and outreach): The project website, scientific and extension publications and conference talks/posters, as well as cold-calls to potentially interested parties, will raise awareness of the new technology and enable the rapid dissemination of methods and developed cultures.

USDA, NIFA SCRI HLB Citrus Disease Research and Extension Program
2015 Awards Project Overview and First Year Progress

Source: Current Research Information System

http://cris.nifa.usda.gov/cgi-bin/starfinder/0?path=fastlink1.txt&id=anon&pass=&search=(GC=CDRE)&format=WEBTITLESGIY .

ACCESSION NO: 1005572 **SUBFILE:** CRIS
PROJ NO: CALW-2014-10148 **AGENCY:** NIFA CALW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-22992 **PROPOSAL NO:** 2014-10148
START: 01 FEB 2015 **TERM:** 31 JAN 2020
GRANT AMT: $1,683,429 **GRANT YR:** 2015
AWARD TOTAL: $1,683,429
INITIAL AWARD YEAR: 2015

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CHARACTERIZATION OF LIBERIBACTER POPULATIONS AND DEVELOPMENT OF FIELD DETECTION SYSTEM FOR CITRUS HUANGLONGBING

NON-TECHNICAL SUMMARY: Citrus is a very important fruit crop in the US. Huanglongbing (HLB) is a serious disease that has destroyed about half of Florida citrus and is now threatening the citrus industries in Texas and California. The pathogen is a bacterium known as Liberibacter and the disease is transmitted by an insect vector, Asian citrus psyllid. There is no known cure for HLB. Early detection and proper management done in a timely fashion are essential for disease management. Three species of the HLB pathogen are known. We have recently discovered a fourth species from Colombia. Proper identification of all forms of the pathogen is crucial for disease management. We propose to characterize pathogen variants and generate information that will be used for development of a testing system capable of detecting all species. Since the pathogen can be detected in the insect vectors several years before it is detectable in plants, we will focus mainly on testing the insect vectors of HLB. There is a need to trap the psyllids efficiently so that reliable testing can be conducted. The SmartTraps that we propose to develop will chiefly trap the insect vector of HLB and will preserve the psyllids such that they can be used efficiently for pathogen detection tests. We will use the genetic information developed for pathogen variants and design assays to detect all forms of the pathogen. In addition we will develop detection technologies using a very simple SmartDART unit that can be operated by growers and used for testing. Expensive equipment or scientific training will not be needed to utilize this system. The most important aspect of our project is extension. We will train a total of 30 growers in two states, California and Texas, in using these new technologies. SmartDART units, SmartTraps, and reagent kits will be provided to the participants. We believe that if the growers are capable of detecting HLB the surveillance system

will be stronger and disease management becomes easier. Our goal is to engage a large number of growers and extension personnel in early detection of HLB so that the disease can be better managed in areas where it is already present and prevent and suppress the disease in other areas.

OBJECTIVES: Overall goal is development of methodologies for early detection of all disease-associated forms of Liberibacter (in psyllids), monitoring of the pathogen using field based diagnostic tools and development of integrated management strategies. HLB has resulted in significant losses to citrus industries worldwide. Reliable identification of all disease-associated Liberibacter pathogens may be crucial for taking necessary steps for disease management. Development of information required for designing generic testing capabilities for all Liberibacters may be an integral part of disease management. Secondly, if we make these technologies available to the interested parties including citrus growers and extension personnel, it will facilitate large-scale testing in future and will make the surveillance system stronger. We have four major goals: a) to develop sequence information on diverse Liberibacters; b) to develop methodologies for field detection of all Liberibacters associated with huanglongbing (HLB) in psyllids and plants; c) to develop improved methodologies collection and storage of psyllid vectors, and, d) to make the technologyavailable to citrus growers and extension workers.The four objectives are outlined below:1. Since Liberibacter variants are known to exist and we propose to develop methods to detect all HLB-associated Liberibacters, we will develop genomic sequence information for Liberibacter variants currently known to exist in California, Texas, and other regions (objective 1). Our focus will be on detection of the pathogen in psyllids and plants in regions where HLB is not yet widespread (California and Texas).2. We will thenuse this information to design inexpensive, sensitive, field deployable kits using Loop-mediated amplification (LAMP) technology, and a field adaptable SmartDART™ detection device (objective 2).The methodology will be adapted to detect all disease-associated Liberibacter pathogens. As LAMP method is user-friendly, it can be utilized by interested non-scientific personnel.3. Since the project is focused on detection of the pathogen in psyllid vectors, it is essential to have efficient vector trapping and storage. Towards this goal, we will improve psyllid capture using a 'SmartTrap' that traps only psyllids and stores them at ambient temperatures (objective 3).4. We believe that if these technologies are made available to a wider audience, it will strengthen the surveillance systems and help in disease mitigation. Enabling citrus growers and nurserymen to use the technologies will result in grower empowerment and facilitate management decisions. We will provide selected growers in California and Texas with tools and reagents to trap psyllids and test for Liberibacters. Participating labs will collect data and samples from growers for confirmatory analysis. With grower participation we will develop a system that helps in containment and management of HLB (objective 4).

APPROACH: Objective one: Develop genomic sequence information of Liberibacter variants found in California, Texas and other citrus-growing regions1.1 Develop universal Liberibacter detection system. Using alignments of available genomic sequences of Rhizobiaceae members, several targets will be selected and several universal Liberibacter detection systems will be designed and used to screen existing collection of psyllid DNAs to find out sequence variants.1.2. Selection of samples: We will analyze the Liberibacter strain from California (samples from CRF in Davis), include samples from Texas, citrus relative samples showing HLB symptoms from FL, Central and South American countries; primarily using single psyllid extractions.1.3. 16S metagenome analysis. We will conduct this using services of Mr. DNA Company. Analysis of the 16S metagenome data will be helpful in understanding the nature of bacteria present in the sample. The methodology helped us to confirm a new species of Liberibacter in Colombia.1.4. 384 primer array. We have designed and used this array in our Riverside labs. Will use selected samples to do the array and study populations.1.5. Agilent Sureselect hybridization. We have already designed this panel. We will use this approach for

select samples only.1.6. PACBIO and Illumina NextGen sequencing and Bioinformatic analysis. Isolates carefully selected by several of the above methods will be used for full genome sequencing. This aspect will be conducted at the core facilities of UCR and UC Irvine/Univ. of Florida.1.7. Selection of sequences conserved in Liberibacters. We will refine our universal Liberibacter detection system as needed when new sequences become available. Objective two: Develop a LAMP-based field testing system capable of detecting all HLB-associated Liberibacter populations from both psyllids and plants2.1. Develop LAMP capable of detecting all relevant Liberibacters: Identify suitable regions conserved in Liberibacters, absent in other Rhizobiaceae, design LAMP primers (Jenkins et al. 2011) and screen against multiple plant/psyllid extractions collected from various hosts and geographical locations (extractions available in the collection of the Riverside labs). We will design assimilating probes for promising primer sets and multiplex with internal controls.2.2. Standardize and optimize LAMP assays using a qPCR machine: Development of LAMP primer sets and assimilating probes for all HLB-associated Liberibacters will be done in year 1.2.3. Prepare and evaluate a lyophilized, ready-to-use, diagnostic kit for HLB: We will develop formulations which can be freeze-dried, to achieve stability at room temperature over long durations of time (> 6 months). Coordinated by Kubota, and Jenkins at the Univ. of Hawaii, Manoa.2.4. Develop Assimilating Probe-based detection systems for Liberibacter and psyllids: To enable use of CLas- and ACP-specific internal control probes in duplex assays for validation.2.5. Develop an assay for detection of Liberibacters in plants: Psyllid assay is fairly easy to handle without cross-contaminations. LAMP works well with purified plant DNAs (Keremane et al., 2014). Will develop a crude extraction method for plant samples. Instead of the COX (multicopy), we will use single copy gene as internal control (Ramadugu et al. 2013).2.6. Customization of Smart-DARTTM software tools/ interface: Existing Smart-DARTTM software has automated classification algorithms to reliably identify and report outcomes of tests. As new tests are developed with internal controls, we will customize the user interface for citrus industry stakeholders to simplify and standardize interpretation of results (i.e. sample is infected or not, or test results are inconclusive/invalid based on the presence of inhibitors or inappropriate use of sample or sample preparation).Objective three: Improve methods for psyllid collection, shipping and preservation3.1. Purchase two new 3-D printers to make variations to the shape and design of SmartTraps. Modify previous designs by adding texture, more appropriate lighting (different colors and LED lights. Placement, color and deployment of light are key elements of research in the second year.3.2 Compare SmartTraps on citrus with those on separate poles. Improve lighting parameters. Provide additional light at dawn and dusk only.3.3. Collect psyllids and the low level of non-psyllids from traps. Psyllids will be sent to Riverside lab for analysis. Document non-psyllid samples and preserve in DPI. Objective 3 will be conducted in FL by Smith and Halbert with help from Jenkins for improving the LED aspect. Objective four: Promote the use of LAMP assay and train growers, nurserymen, pest control agents, extension workers and other stakeholders for widespread testing of Liberibacters as a component of an overall integrated pest management (IPM) program.4.1 Train citrus growers (selected through advisory Board; 10 in year 1, 30 in years 3, 4 and 5; total no. is 30) in using SmartTrap, SmartDART TM and LAMP, sampling procedures, discuss anticipated project outcomes and expectations. Participants will collect psyllids from SmartTraps at biweekly intervals, conduct SmartDARTTM testing, initially for CLas and then for all Liberibacters (as soon a method is available; see objective 1). Each participant will periodically test pooled (1-10) psyllids for CLas using SmartDART TM and the remaining cohorts of trapped psyllids and crude extracts will be provided to Alabi in Texas and Keremane in Riverside for test validation. All data will be maintained in databases along with details of extension activities and test results. HLB finds in new locations will be subjected to standard reporting requirements with the local, state and federal regulatory agencies while maintaining grower confidentiality. As modifications and/or updates become available, we will provide participants with the improved units. Similarly,

updated kits and protocols will be provided when the universal Liberibacter detection kit becomes available. All reagent kits will be prepared at Riverside and shipped to individual participants in coordination with project collaborators. We will work on room temperature stable reagent kits (objective 2), and the improved kits will be supplied to all participants when they become available. Detection systems for plant samples using Smart-DART™ will also be provided when they become available (objective 2).

PROGRESS: 2015/02 TO 2016/01
Target Audience:Target audience: For the current reporting period (Feb 1, 2015 to Jan 31, 2016), the target audience include scientists (plant pathologists, entomologists, extension scientists), home owners, citrus growers, pest control agents, bioengineers, California Department of Food and Agriculture employees, APHIS personnel, project collaborators, project advisory board committee, students and technicians. Efforts: Science-based knowledge imparted to participating growers, students and extension personnel through informal educational programs. Hands-on training was provided to certain growers and pest control agents in conducting LAMP assays and subsequent detection of the HLB pathogen using SmartDart platform. Changes/Problems:In California, the second find of HLB has caused a lot of alarm among citrus growers. Several small growers approached us and are interested in using our technologies for pathogen testing. In order to accommodate this need expressed by the industry, we tried to develop cheaper technologies. We have experimented with LAMP methods and detection devices and we have now developed two promising methods that can be used instead of SmartDart (after proper validation). This will reduce the initial expenditure from $3000 (for SmartDart) to $300 (for calcein or visual PCR methods). SmartDart method is still better since it will be more sensitive than the cheaper technologies. We hope that in addition to SmartDart, we can provide materials required for the newly developed methods to interested small growers. The SmartDart units did not perform well initially since there was discrepancy among the units. With the help of our Hawaiian collaborators, we have now improved the hardware and software of the SmartDart units and enforced rigorous quality control for all the units that are used in the project. The SmartTraps are better than the standard yellow sticky traps since they capture and preserve the psyllids. Unlike psyllids captured in sticky traps, the ones from SmartTraps can be tested for the pathogen DNA. However, the current models of SmartTraps are not catching sufficient number of psyllids. Hence we are now printing many more traps using the 3D printers and the 3D technology and we ae currently evaluating the various models. This caused delay in supplying the traps to the growers. We should be able to provide the technology that is currently available to the participants in 2016. What opportunities for training and professional development has the project provided? For growers, the capability to conduct their own testing is very empowering. In the process, they understand the science behind the deadly disease and the feeling that they can keep track of the disease status in their groves - to some extent. The knowledge gained will make them feel responsible and also make them accountable to try and maintain the grove in a good condition. A group of growers in Los Angeles area of California have contacted us for training and we are in constant communication with this group. They are small growers and want to get technical support for their whole community and have the capability to conduct HLB testing. Our inspiration to develop cheaper methods of detection was because of such grower interactions. If we can provide tools to all of them without too much expenditure, that would serve the purpose of our project. We are training a middle school student to conduct a science project using LAMP technology and SmartDart unit. The student developed enormous interest in pursuing science and has learnt many scientific concepts because of her science project. The project is conducted in Universities, State departments and USDA labs in four different states. Our part time technicians and have the opportunity to visualize science in action. Periodically we have growers walk into the facility and discuss their concerns regarding management of HLB with the scientists. Working on a problem that is relevant in everyday life of a citrus grower is

very powerful in influencing the thinking of someone who is new to research. It makes them understand that research can be very meaningful for solving real-life agricultural problems. How have the results been disseminated to communities of interest? We have published the LAMP technology in Crop science Journal. As an open source article this publication has been accessed by several researchers as shown by statistics. The detection technologies included in the project are based on LAMP (loop-mediated amplification) methodology. Interested scientists and growers can easily access the research article and understand the methodology. Keremane presented results of our psyllid testing projects to scientists, researchers, students, industry personnel and citrus growers in American Phytopathological Society meeting held in Pasadena, CA and also in the HLB meeting conducted in Davis, CA. Interesting discussions followed these presentations and several useful contacts were made. We are constantly in touch with many local growers in California (through Ramadugu and Keremane) and in Texas (through Alabi). We had two team meetings with the participating scientists of this project in this year. The first meeting was held in Orlando during the HLB International Conference in February 2015. This was a planning session and many of the participating scientists were involved in discussions about the project. A second team meeting was held in Pasadena, CA in August of 2015. All the participating scientists in this project who attended American Phytopathology Society meeting in Pasadena were involved in this discussion. Many issues and concerns were raised and we have been addressing these through e mail communications and phone meetings. In addition, project personnel travelled from Riverside, CA to Gainesville, FL in November of 2015 to discuss about SmartTrap experiments. Personnel also travelled from Riverside, CA to McAllen, Texas for discussions with the scientists, for training the scientists as well as some growers in Texas. Project personnel travelled to Davis, CA to present the project work to fellow scientists and local growers. Presentations in Davis and Pasadena (Keremane). The team from Hawaii visited Riverside two times to validate the new Smart Dart units that were purchased from Diagenetix and to conduct discussions on design of inexpensive detection units for calcein based methods. We are constantly reevaluating our methods and develop technologies to improve our current protocols and devices. The interactions between scientists from Riverside and Hawaii groups have been very successful in achieving these goals. We are constantly discussing with our advisory committee consisting of Dr. Richard Lee (retired Research Leader, National Clonal Germplasm Repository for Citrus and Dates, Riverside, CA), Dr. MaryLou Polek (Past Vice-president of research from Citrus Research Board, CA) and Mr. Alan Washburn (Pest Control Advisor, Southern California). Their input and broad perspective of the needs of the citrus industry are useful in guiding our project goals. What do you plan to do during the next reporting period to accomplish the goals?In the next reporting period, we will conduct sequencing of five isolates of Liberibacters. At present our list includes: Liberibacter isolates from San Gabriel HLB find site in California, Liberibacter variants from citrus relative genera, Aegle and Afraegle, Ca. Liberibacter caribbeanus from Colombia, Las isolates from Colombia/Venezuela. The sequence variations between these isolates will be useful in devising methods that can detect all Liberibacter variants associated with citrus. We will try to make the LAMP assay more user friendly by lyophilization of the mastermix. At present these trials are being conducted in Riverside and Hawaii. Easy storage of the reaction mix will be convenient for storage of the reagents required for the detection methods. We will improve the calcein-based methods that require an investment of about $300 for the initial set up. The lower prices will encourage more growers to adopt these technologies. The detection device that we have already designed needs to be further validated. As needed, we will improve the detection devices to make them safe, user-friendly and affordable. The visual PCR method is very promising as it is simple to use and fairly sensitive in detecting Liberibacters. We will evaluate this method further in the lab and also under field conditions. We will provide training to growers that includes visual PCR and calcein-based methods in addition to SmartDart devices. We will evaluate the programmable lighting that our team has designed. If there is improvement in psyllid capture, we will design

appropriate lighting set up that can be easily incorporated into the best models of SmartTraps. Using available technologies, we will conduct training programs in California and also in Texas and increase grower participation. Certain growers in Florida are interested in participation since they would like to have the ability to monitor their nurseries for presence of psyllids that may have Liberibacters. Because of this recent development, we will provide our detection units and reaction reagents to the interested parties in Florida. We will conduct more trap evaluation experiments in backyard situations. Most growers conduct sprays as soon as psyllids arrive and it is difficult to complete the trials when pesticides are used. Conducting some of the trap trials in organic groves and backyards may provide better data for analysis and evaluation.

IMPACT: 2015/02 TO 2016/01
What was accomplished under these goals? Objective 1. The only way to save the citrus industry from citrus HLB is by prevention, exclusion and suppression of both the vector and the disease. Early on-site detection using 'easy to use', simple and reliable technologies would facilitate such an effort. In CA, there have been two reports of HLB positive citrus so far. Genomic sequence analyses reported by Keremane in collaboration with other scientists have shown sequence differences in the genomes. Two separate introductions are likely and the HLB find in 2012 probably did not spread to other regions; the disease containment efforts appear to be working. Information about strain differences will be useful in assessment of disease management strategies and understanding HLB epidemiology. We are conducting molecular characterization of Liberibacters of other HLB positive plant samples from San Gabriel HLB find site to test if all the San Gabriel isolates are genetically similar. We are characterizing Liberibacter isolates from citrus relative genera - Aegle and Afraegle from FL to understand sequence variability. From Colombia, South America, we have described a new species of citrus associated Liberibacter, Ca. Liberibacter caribbeanus. We have generated 250 Kb sequence of this strain by using the PAC BIO sequencing platform and using Illumina platform we aim to get the complete genome sequence. We have identified Las in another part of Colombia bordering Venezuela (first report of Las from Colombia). We will obtain citrus DNA from Venezuela and characterize the Liberibacter isolates. Objective 2. Using available genomic sequences of Liberibacters we have developed qPCR primers for universal amplification of all citrus Liberibacters. These primers have been validated with standard DNA preparations; evaluation with psyllid and plant samples is on-going. We will then design LAMP primers and develop assays for detection. We are improving the LAMP reaction mix by lyophilization of the mix to enable easy storage at room temperature. SmartDart unit works well to conduct field testing. It is suitable for large groves due to increased sensitivity and rapid assay time. Considering the current HLB situation in CA, there is an urgent need to develop less expensive technology so that a larger number of people can do testing. We are now developing a calcein-based fluorescence method to detect Las from psyllids. In this test, HLB positive samples will show fluorescence while negative samples will not. Once the method is developed, it would cost only $300 to obtain the necessary reagents and equipment to do testing (as compared to $3000 for SmartDart and the associated software). A low-cost, end-point version of the SmartDart system was developed with temperature controlled heating block and integral UV LEDs for visualizing LAMP reactions conducted with calcein indicator. As an alternate method affordable for small growers and home owners, we have developed a "visual PCR assay" that can be conducted in about an hour under field conditions. The test requires initial investment of about $250 only. Samples are scored after the simple test as positive or negative, visually, based on color. The calcein method and visual PCR will not be as sensitive as SmartDart. But, because of the decreased costs, many more growers/homeowners will be able to utilize the technology for HLB testing. Even though, our initial objective was to develop an universal detection system, we have focused presently on Las alone for field detection since there is an urgent need to conduct large scale testing in CA. We have made several improvements to SmartDart software and hardware. Objective 3. Standard sticky traps

are useful for monitoring psyllid populations but not for HLB testing. To enable capture of psyllids that can be tested for Liberibacters, we developed 'SmartTraps'. The advantages are twofold: unlike sticky traps, psyllids captured in the preservative used in the SmartTraps will be testable for the presence of the HLB pathogen. Secondly, use of a trap that can preserve psyllids well will enable a 'dynamic' method of testing. For example, if psyllids are captured using aspirators, the testing method is 'static' and reflects the situation on a single day. With the traps that can preserve psyllid DNA, samples collected after two weeks will reflect the situation in the two week period. The Las titer in psyllids changes dramatically over time and hence dynamic sampling is better. When manual collections are conducted, most locations are visited once or twice a year; a SmartTrap placed in a certain location can be used to collect samples throughout the year. To improve psyllid capture in SmartTraps, we have adjusted many parameters including color of the plastic used to print the trap, external and internal shape of the barrel, pore size, presence of awnings, smooth versus textured surfaces, addition of a slippery 'Fluon' coating on the interior, etc. Psyllid behavior is taken into consideration for the designs. Different combinations of these variables are incorporated into each trial model and the psyllid capturing ability is evaluated in closed chambers and field situation. Improvements made resulted in avoiding larger insects and in preventing rain water from entering the trap. Presence of stem like structures and rough texture on the exterior was more attractive to the psyllid than the original smooth surfaces. Addition of the 'Fluon' coating has prevented escape of the captured psyllids from the traps. A combination of alterations are now being evaluated. Addition of a light source to the existing traps may be useful in increasing the trapping efficiency of the SmartTraps. We have designed a Bluetooth programmable lighting system with multiple wavelength high intensity LEDs to test different lighting conditions (UV, blue, green, yellow, amber and red). We will alter the light intensity and test various combinations of lights. Rechargeable batteries and solar panel capability are integrated into the system. Depending on the preliminary results, we will incorporate the most effective type of lighting into the trap design. Objective 4. The key to finding HLB quickly is - a) testing psyllids and b) large scale testing of psyllids. Towards this goal, we require traps capable of capturing psyllids and preserving them so that they are suitable for testing and secondly, we need testing systems that are easy to use, economical and efficient. At this time we have made significant improvements in both these areas. In CA, we have worked in cooperation with home owners, organic growers, extension agents, Pest Control Advisor (PCA; Alan Washburn), researchers and owners of small citrus groves. Evaluation studies were conducted in a citrus park, psyllid rearing and bio-control facilities. We discussed the trap evaluation project with entomologists, CDFA personnel and APHIS employees. Provided training to pest control agents and small growers in conducting LAMP assays using Smart Dart. In TX, we have trained scientists and some growers. Technical problems with the units were observed by Diagenetix Inc. personnel and corrected later. We revisited the hardware, software and reagent components of the detection technology in a systematic fashion and brought about several improvements. Strict quality control is now in place. Two alternate technologies - a) calcein based LAMP and b) visual PCR were pursued. We designed detection components for the calcein method and constructed 50 units at a cost of about $150 per unit. The calcein based method is still under development. The visual PCR method is at present very promising. After a few more validations in the lab setting, we will be able to launch this method as an alternate, more affordable technique. We will distribute the SmartTraps, SmartDart units and visual PCR kits to growers this spring as soon as the psyllid populations build up.

PUBLICATIONS (not previously reported): 2015/02 TO 2016/01

1. Type: Journal Articles Status: Published Year Published: 2015 Citation: Keremane, M. L., C. Ramadugu, E. Rodriguez, R. Kubota, S. Shibata, D. G. Hall, M. L. Roose, D. M. Jenkins, and R. F. Lee. 2015. A rapid field detection system for citrus huanglongbing associated 'Candidatus

Liberibacter asiaticus' from the psyllid vector, Diaphorina citri Kuwayama and its implications in disease management. Crop Protection 68:41-48.
2. Type: Journal Articles Status: Published Year Published: 2015 Citation: Kubota, R. and D. M. Jenkins. 2015. Real-time multiplex applications of Loop Mediated AMPlification by Assimilating Probes. International Journal of Molecular Sciences. 16(3), 4786-4799.
3. Type: Journal Articles Status: Published Year Published: 2015 Citation: Jenkins, D. M., J. Jones, and R. Kubota. Evaluation of portable DNA-based technologies for identification of Ralstonia solanacearum race 3 biovar 2 in the field. 2014. Biological Engineering Transactions 7(2):83-96.
4. Type: Conference Papers and Presentations Status: Published Year Published: 2016 Citation: Eric Rohrig, Susan Halbert, Amy Howe, Tony Dickens and Walter Winn. 2016. Using 3D printing technology to make a better Asian citrus psyllid trap. Florida Dept of Agriculture and Consumer services ? Division of Plant Industry. Presented on January 27, 2016 at the Citrus Show, held in Fort Pierce, Florida.
5. Type: Conference Papers and Presentations Status: Published Year Published: 2015 Citation: M. L. Keremane, C. Ramadugu, A. Castaneda, J. E. Diaz, E. A. Pe?aranda, J. Chen, Y. P. Duan, S. E. Halbert, R. F. Lee. 2015. Report of Candidatus Liberibacter caribbeanus, a new citrus- and psyllid-associated Liberibacter from Colombia, South America Presented at the Annual meeting of American Phytopathological Society. Pasadena, CA. Aug 2015.
6. Type: Conference Papers and Presentations Status: Published Year Published: 2015 Citation: Manjunath L. Keremane, Chandrika Ramadugu, Adriana Casta?eda, Jorge E.A. Diaz, Emilio A. Pe?aranda, Richard F. Lee. 2015. Candidatus Liberibacter caribbeanus, a new citrus associated Liberibacter from Colombia, South America. Presented at the International Research on Citrus HLB, February 2015, Orlando, FL.
7. Type: Conference Papers and Presentations Status: Published Year Published: 2015 Citation: . Halbert, S.E., Keremane, M., Ramadugu, C., Dawson, W.O., Lee, J. A., Keesling, J.E., Singer, B.H., Lee, R.F. 2015. Surprising results and implications of the Florida psyllid testing project. Presented at the International Research on Citrus HLB, February 2015, Orlando, FL.
8. Type: Conference Papers and Presentations Status: Published Year Published: 2015 Citation: Manjunath Keremane, 2015. Early detection of HLB-associated Liberibacters through monitoring Asian citrus psyllids. Presented at the California Asian Citrus Psyllid and Huanglongbing Research and Extension Summit. Sep 9, 10, 2015. UC Davis.

ACCESSION NO: 1005650 **SUBFILE:** CRIS
PROJ NO: CA-D-PPA-2283-CG **AGENCY:** NIFA CALB
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23011 **PROPOSAL NO:** 2014-10128
START: 01 MAR 2015 **TERM:** 28 FEB 2018
GRANT AMT: $4,579,067 **GRANT YR:** 2015
AWARD TOTAL: $4,579,067
INITIAL AWARD YEAR: 2015

INVESTIGATOR: Falk, B.

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NON-TRANSGENIC, NEAR TERM RNA INTERFERENCE-BASED APPLICATION STRATEGIES FOR MANAGING DIAPHORINA CITRI AND CITRUS GREENING HUANGLONGBING

NON-TECHNICAL SUMMARY: Effective techniques to reduce the rate of Huanglongbing (HLB) spread are key to slowing its incidence, especially for new citrus plantings. RNA-interference (RNAi) is a natural regulatory and anti-viral response in eukaryotes and can be manipulated to target mRNA/gene expression, including to control insects. Our on-going collaboration has found that RNAi inducers, expressed in citrus trees using the Citrus tristeza virus (CTV) vector, reduce the survival of adult Diaphorina citri moving onto the trees, and greatly reduce their reproduction and acquisition of Candidatus Liberibacter asiaticus by psyllid progeny. Our goal is to further improve RNAi activity such that it can be used to help manage D. citri and HLB, allow reductions in pesticide use and lower grower costs for U.S. citrus. A second strategy for applying RNAi towards D. citri takes advantage of insect viruses that could be modified to deliver interfering RNAs directly to D. citri. This approach offers a non-modified plant alternative that could complement the use of CTV to express RNAi inducing sequences against psyllids and help manage HLB spread. Here we expand collaborative efforts in RNAi research with mathematical modeling, economic analysis, and extension/outreach to quickly move this technology to the growers' fields

OBJECTIVES: Objective 1). Optimize the Florida CTV to deliver efficacious D. citri-interfering RNAs in Florida non-transgenic citrus. Objective 2). Develop a CTV vector for applications in California citrus. Objective 3). Develop D. citri-infecting viruses for non-plant-based induction of RNAi effects in psyllids. Objective 4). Model/test RNAi systems under greenhouse and/or field conditions. Objective 5). Evaluate the economic impact of using RNAi technologies in citrus for controlling D. citri and HLB. Objective 6). Develop effective Extension Outreach programs for RNAi-based strategies.

APPROACH: Objective 1-The 35S binary plasmid constructs engineered to harbor the CTV vector, plus the different RNAi-inducer sequences, will be used to agro-inoculate Nicotiana benthaminia from which virions are extracted & used to inoculate small citrus trees. Inoculum from these trees are used to graft-inoculate test trees.Objective 2-The Ng lab has been developing the cloned infectious cDNA of a CTV isolate from California by using the

approaches performed by the Dawson lab, culling from past experience constructing the infectious clone of Lettuce chlorosis virus, another member of the family Closteroviridae. The overarching guideline that we adhered to when choosing candidate CTV isolates for cDNA cloning is that they have to be mild or asymptomatic CTV isolates, and that they must be prevalent in California. We have identified several isolates that met those criteria: 1) CCTEA-5 and a few others that share a T30 genotype, 2) RB25, a Poncirus trifoliata-resistant breaking albeit mild isolate, and 3) CCTEA 96339, an isolate with a T36 genotype. Objective 3- This work is being led by the Falk lab. Because small RNAs are hallmarks of antiviral responses in plants & insects, & with new sequencing platforms, small RNAs can be sequenced directly. We have & continue to sequence siRNAs from various populations of D. citri, and then use bioinformatics to identify D. citri-infecting viruses. So far RNAs have been extracted from 33 different D. citri populations collected from nine locations around the world, including U.S. populations from Florida, Texas, California & Hawaii. Four were used for small RNA deep sequencing & two for transcriptome sequencing on HiSeq and MiSeq platforms, respectively. Bioinformatics analyses has so far allowed us to identify three different types of virus sequences among both types of libraries (probably more virus sequences are present but not yet confirmed). D. citri from Taiwan, China, Hawaii & some from Florida were found to be infected with DcRV. We also found evidence for Densovirus sequences in all D. citri populations examined, but we are not yet sure if these data represent a replicating virus or possibly integrated Densovirus sequences. Densoviruses are widespread viruses with relatively simple ssDNA genomes. Furthermore, a Densovirus has been used recently to induce RNAi effects in mosquitoes & we are further investigating this for D. citri. Objective 4- The CRDF, which is coordinating the design and implementation of field tests, has enlisted the assistance of the Keesling lab to assist in the designs. The Keesling lab has already developed a mathematical model of CLas spread based on the lifecycle of D. citri populations. Their original model was designed as a proof of concept to predict the rapid asymptomatic spread of CLas through a grove. After a tree is infected, it is some time before symptoms of HLB appear. According to the model, the infection takes over a grove long before the first symptoms appear. The model takes into account factors such as psyllid movement patterns, psyllid aging & mortality, & citrus flushing patterns to make its predictions. This model is highly adaptable & is capable of incorporating factors such as pesticide spraying, invasion of psyllids from other groves, & the various effects that RNAi constructs may have on the psyllid population or CLas transmission. For this project, the model will be refined to be capable of accurately predicting the impact of different RNAi constructs & delivery means (CTV and/or D. citri virus) on the spread of HLB in conjunction with other strategies to insure that new citrus plantings can be protected. Objective 5-A market modeling approach will be used to estimate the changes in welfare for consumers & producers of oranges, lemon and tangerines in Florida and California. The market model contains linear equations for demand by consumers, total market supply, production by growers in each region, & trade. It is used to estimate the percentage changes in market supply, price, production and demand from an exogenous shift in the supply curve due to a sudden change in crop management, such as what occurs when an exotic pest becomes established, or a technological change. The percentage changes in prices, quantity demanded & production are used to calculate the changes in welfare to consumers & producers. For Florida, the model will be simulated based on current estimates of rates of spread & disease progression. For California, the model will be simulated based on expected values extrapolated from the Florida data. Objective 6-We will develop an extension outreach plan that enhances existing extension educator programming in citrus. There are four primary outreach approaches for this proposal.

ACCESSION NO: 1005515 **SUBFILE:** CRIS
PROJ NO: FLAW-2014-10141 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23030 **PROPOSAL NO:** 2014-10141
START: 15 JAN 2015 **TERM:** 14 JAN 2019
GRANT AMT: $3,495,832 **GRANT YR:** 2015
AWARD TOTAL: $3,495,832
INITIAL AWARD YEAR: 2015

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STEAM-GENERATED SUPPLEMENTARY HEAT THERMOTHERAPY AS AN IMMEDIATE TREATMENT FOR PROLONGING PRODUCTIVITY OF HLB-INFECTED CITRUS TREES

NON-TECHNICAL SUMMARY: This four-year multi-state multi-disciplinary research and extension project aims to address the urgent need of citrus growers in Florida to an immediate treatment for HLB-affected citrus trees by utilizing steam for rapid thermotherapy. The overall goal of this project is to provide an immediate scalable technique using steam as well as synthesize scientific explanations on how the technique works in mitigating HLB and prolonging the production life of infected citrus trees. The central hypothesis of the project is that the HLB-affected trees can be treated for inoculum reduction using a rapid steam-based thermotherapy technique in a manner that is biologically and economically feasible with minimal environmental impact. This project covers both the practical and theoretical aspects of the thermotheraphy through an interdiscplinary approach involving researchers with expertise in plant pathology, plant physiology, engineering, nematology, economics, and plant biology. The project objectives include enhancement of the steaming system and operations used for the treatment, investigation of the effects of steam-based treatments on the survivability of CLas and the expression of HLB symptoms, determination of treatment efficacy and effectiveness, development of a comprehensive steaming system which includes treatment of the roots, studying the effects of heat treatment on yield and fruit quality, and conducting comprehensive economic analyses of the system. The enhanced steaming system will be used to treat citrus trees on a quarterly basis from different sites in collaboration with local citrus growers. An strong extension program is planned to disseminate the outcome of this research.

OBJECTIVES: Objective 1: Enhance the existing steam-generated supplementary heat thermotherapy system so that it will generate consistent heat and provide a uniform temperature to the canopy and roots. Objective 2: Determine the effect of steam treatment on CLas recolonization, overall tree health, and fruit yield and quality. Objective 3: Determine the efficacy of steam-based thermotherapy related to CLas viability. Objective 4: Evaluate the effectiveness of steam-based thermotherapy considering the pretreatment condition of roots as affected by pests and diseases and characterize the effects of the treatment on pests and diseases. Objective 5: To determine time-temperature combinations for the inactivation of CLas that do not result in tree defoliation. Objective 6: As a canopy post-treatment, horticultural mineral oil (HMO) applications to tree surfaces will improve the thermotherapy process and the

development of new application technology. Objective -7: Conduct comprehensive economic analyses of the steam-generated supplementary heat thermotherapy system.

APPROACH: Objective 1:An existing heat treatment machine will be enhanced to provide rapid, precisely controlled, and uniform supplementary heat treatment into the canopy, roots, and surrounding soil of HLB-affected trees. Associated tasks include: (a) construction of an enhanced steaming system, (b) development of a mathematical model to predict both the heat transfer through the tree and the soil as the steam is applied and the temperature along the depth and direction of canopy and soil, (c) design and construction of a feedback control system to efficiently control the heat thermotherapy process, (d) development of a new mechanism for heat treating the root system, and (e) development of a post-treatment mineral oil application to improve the effect of the steam thermotherapy (objective 6).Objective 2:The steaming system from objective 1 will be used to conduct a comprehensive field trial in 'Valencia' sweet orange. The trial will be designed as a split-plot design with timing of treatment application being the main-plot. Main-plots will be steam applications done at quarterly intervals to determine if there is an optimal time of year for CLas reduction in the canopy. The main-plots will be laid out as either a completely randomized design (CRD) or a randomized complete block design (RCBD) depending on the aspects of the site selected for the experiment. The main plot will consist of a minimum of 50 trees, subdivided into 10 tree sub-plots. There will be five replications of the main plots. For the first year of study, the sub-plot treatments will include five levels of heat treatment: 1) no heat treatment (control); 2) 58°C for 15 s; 3) 58°C for 60 s; 4) 58°C for 90 s; 5) 58°C for 120 s; and 6) 60°C for 30 seconds. These heat and time combinations have been selected to create two extreme set-ups including one combination in which steam is applied but no defoliation occurs while the other combination will cause total defoliation based on prior experiments. Before the steam treatments in each main-plot, an HLB symptom assessment (scale 1= Vigorous, asymptomatic, 2= Slight decline, symptomatic, 3= Moderate decline, symptomatic, 4= Severe decline, symptomatic, 5= Nonviable, will not recover) and sampling for CLas will be done. Objective 3:The viability of CLas after heat treatments will be used to assess the effectiveness of the treatment to reduce or eliminate the bacteria in the tree. Two different approaches will be used to assess bacterial viability. One will be based on the propidium monoazide (PMA) real-time PCR assay adapted for CLas detection. The second approach to assessing CLas viability will be based on a comparison of specific mRNA populations in the bacteria either with total genomic DNA (CLas) or CLas 23 rRNA levels. The rationale is that mRNAs in bacteria (and other organisms) show much higher turnover rates compared to genomic DNA or 23S rRNA. Hence, fluctuations in mRNA population size can provide a sensitive means to assess the overall viability of bacteria soon after the heat treatment. The expectation is that the exposure to high temperatures will result in drastic changes in the pattern of RNA abundance within minutes to hours after treatment. Objective 4:Sub-objectives related Objective 4 are to i) characterize the effects of the treatment on soilborne pests and diseases and ii) determine whether additional IPM practices improve the thermotherapy outcome. In addition to the field experiment described in Objective 2, we will select groves based on the infestation status for three key pests of the citrus root system: the root weevil Diaprepes abbreviatus the phytoparasitic, nematode Tylenchulus semipenetrans, and the oomycete Phytophthora nicotianae. Tree responses to thermotherapy in groves infested and not infested by each pest will be compared. Additionally, in some infested groves, thermotherapy will be applied to trees that are treated or not treated with chemical and biological pesticides capable of reducing pest population densities. Objective 5:Sustained productivity of citrus trees after heat treatment requires that the metabolic functions of the tree be maintained and that CLas populations be reduced to minimize symptom development and tree decline; our work indicates both are possible. Two key underlying factors for the long-term success of thermal treatment will be established. First, mathematical functions that describe the amount of time that a leaf or twig can

be exposed to steam at a given temperature without dying will be derived and verified for CLas inactivation in planta, and a rapid enzymatic method developed for assessing leaf viability. Understanding the rate of heat transfer between steam and trees is critical to establish a dependable system. In the proposed system, as steam is injected into the canopy, convective streams of steam and air mixtures are produced; optimization of the system requires characterization of heat transfer to individual tree components. Secondly, to understand the thermal death kinetics of CLas in citrus branches, D-values and the z-value will be established. When microorganisms are heated at a constant temperature, the decrease of viable organisms follows a first order reaction, commonly defined by D-values (the time to inactivate 90% (1-log) of microorganisms). The temperature dependence of D-values are represented by z-values (the temperature change needed to change D-values by a factor of 10). D- and z- values allow for the determination of equivalent rates of microbial kill under varying time and temperature treatment combinations. Objective 6:In this objective, the effect of post-treatment with three HMO aerosols to improve effectiveness of thermotherapy on HLB-affected canopies will be evaluated. The HMO selection, application volume, and concentration (< 2%) will be based on preliminary laboratory studies (year 1). In regards to appropriate application technologies, an ultra-low volume (ULV) applicator will be evaluated to post-treat the enclosed citrus canopies. The ULV-based aerosols with < 50 um droplets are expected to have a thin, even coating of the material on canopies. Preliminary studies on effectiveness of an ULV applicator on coverage and deposition rates in varied parts of canopies will be conducted (year 1). The ULV application treatments, with an oil soluble fluorescent tracer dye, will be conducted to evaluate the deposition with fluorometry and coverage with oil sensitive paper based image analysis. Objective 7:Economic analysis into steam-generated thermotherapy will proceed in four steps: 1) estimate of capital costs; 2) estimate of annual operational costs; 3) assess the long-term impact of thermotherapy on yield and fruit quality; and 4) comparing the net present value (NPV) of thermotherapy with alternative HLB management strategies.

PROGRESS: 2015/01 TO 2016/01
Target Audience:The target audience is citrus growers affected by HLB, as well as grove equipment managers, extension agents and third parties interested in or actively engaged in promotion, production or operation of in-field thermotherapy technology. Additional stakeholders include other specialty crop growers which could directly adapt thermotherapy technology. Changes/Problems: Nothing Reported What opportunities for training and professional development has the project provided? Materials describing the type of experiments and how thermotherapy is performed have been presented as part of the UF-CREC extension program at major grower events such as the Citrus Expo. We have also distributed general materials about HLB to the grower community. How have the results been disseminated to communities of interest? Nothing Reported What do you plan to do during the next reporting period to accomplish the goals? Objective 1: Minor uneven thermal distribution still exists within the existing tree enclosure, with the two sides where steam is introduced maintaining a slightly higher temperature (2-3 ºC) than the other two sides. Further design improvements will be made in the first part of 2016 to eliminate this issue. Additionally, a second tree enclosure cover has been purchased to replace the original cover, which did not provide an adequate seal with the ground. This allowed steam to escape out of the bottom of the tree enclosure during windy conditions and should be corrected with the installation of the new cover. The student design team from UCF will make final adjustments to their designs of the GUI and small-scale thermotherapy system from testing and possible implementation in the second quarter of 2016. Successful design concepts from the small-scale system and GUI will be implemented in the existing thermotherapy system. Two presentations are also planned for the 2016 ASABE International Meeting to be held in Orlando in June 2016 detailing the progress made thus far by Reza Ehsani's and Yunjun Xu's research teams. Objective 2: The remaining thermotherapy

treatments in the first major field trial will be completed and the effect of the treatments on CLas and yield will continue to be evaluated. We will investigate the selection of a second site for a repetition of the field trial with treatments and seasonal timing selected based on results of the first trial and any results that develop from Objective 5. The time of initiation of the second trial will be dependent on ongoing results collected from the first trial. Objective 3: We plan to conduct a detailed study of the RNA profile from the related C. Liberibacter crescens as a surrogate for CLas in response to exposure to elevated temperatures using liquid cultures in a 65 oC water bath in a study modeled on a similar study in B. subtilis. The B. subtilis study showed a definite correlation in the ratios of the 16S and 23S rRNA amounts at a series of elevated temperatures. The goal is to construct an RNA-based index of CLas viability as related to temperature exposure based on analogies with the CLas crescens model. If the temperature of exposure is known, then an accurate prediction of viability can be inferred. Once a strong link has been established between the temperature experienced by the leaf and the viability of CLas, assessments of the efficacy of the heat treatment in the field can be reduced to a determination of the thermal profile of the canopy during steam treatment. The idea is to reduce the biological aspects of the project to studies under the controlled conditions of the laboratory and reduce the field testing to a problem of achieving a uniform temperature profile in the canopy. Using this approach, we should be able to predict the efficacy of the heat treatment in the field based on the characteristics of a three-dimensional temperature profile of the individual canopies. Realization of this goal provides a practical means to estimate the efficacy of field treatments quickly. Objective 4: In the second year, we shall continue pest management treatments and initiate and evaluate the effect of the thermotherapy treatment of infested/non-infested trees and pest-managed/non-managed trees. Root densities, root dieback, and growth will continue to be monitored in the treated trees and the winter and spring treatments of the field trial in objective 2.We will use the phytophthora collected in the large field trial to prepare and plan for a small trial focused in a heavily infested citrus orchard. Objective 5:The first round of temperature-time treatments will be performed and initial results collected. Objective 6: Taking the hydrogel idea forward, we hope to do initial lab test to establish feasibility, followed by a small field test in March 2016.The three areas of attention will be a) pretreatment to enhance thermal conductivity of steam, b) the application of warm water in the absence of steam, c) the use of as part of a solar heating system. Results will be analyzed to confirm the applicability of thermotherapy treatment for the control of pear psyllids. Based on the outcomes, we will fine-tune the citrus HLB management protocols to be followed in 2016 field trails. If successful, it will greatly impact present control practices and provide sustainable method of organic control for pear psyllids in the Pacific Northwest. Consequently, the impact of this experiment can be envisaged in the form of environment-friendly sustainable organic pest management practice. Experimental treatments and procedures have been finalized in this reporting period. the WSU team plans to conduct those experiments and transfer the learnings from pear psyllid management to citrus HLB management. Our Q1-Q2 efforts will be focused on development of experimental set-up; simultaneously calibrate the sensors; conduct laboratory experiments for pear psyllid management at two growth stages; pertinent data analysis; 2016 ASABE meeting manuscript/ presentation, preparation and discuss the application of findings towards effective citrus HLB management. Objective 7: Following harvest of the first crop during the first quarter of 2016, data will be collected which will be used in the economic analysis. Yield data from subsequent years will provide long-term economic data which will be used for completion of this objective.

IMPACT: 2015/01 TO 2016/01

What was accomplished under these goals? Objective 1: Extensive field trials were conducted in the later part of 2015 to better understand the operation of the system as well as experiment with placement of fans, steam nozzles, temperature sensors, and new electronic controls. Construction of the existing system was completed in June 2015, with the first trials conducted later that

month. Gradual improvements were made from the original design throughout the remainder of the year. These additions reduced the typical treatment time from 3 minutes to 2 minutes and resulted in a nearly 40% reduction in per-tree treatment costs. Thermal distribution was also improved with the addition of steam nozzles around the perimeter of the base and the addition of a second fan to the ceiling of the tree enclosure. A LabVIEW Graphical User Interface has been developed as a command module for the operation of the thermotherapy system. The GUI panels include controls and instrumentation to monitor and adjust systems critical to the thermotherapy process. Particular focus has been placed on improving the efficiency of both equipment operation and the thermotherapy process. In addition to the work conducted on the GUI, the student design team also conducted thermal analysis of their proposed small-scale thermotherapy system to determine the optimal placement of fans within the tree enclosure. Objective 2: The field trial location was identified and mapped. The summer and fall thermotherapy treatments have been applied at multiple time-temperature combinations and compared to untreated controls. Before and after leaf and root samples have been collected with continuing sampling occurring over time to determine the proportion of bacteria killed and the movement of bacteria from roots to the treated branches and leaves. Damage caused to the canopy by thermotherapy is being quantified. Both fresh weight of wood pruned so that the machine will fit over the trees and the dry weight of branches killed by the steam treatment have been measured. Fruit drop resulting from thermal damage to fruit on the tree was also assessed. Yield and fruit quality assessment will not be done until the Valencia fruit are ripe in the spring. An absence of CLas from the treated leaf material was not observed from the summer treatment. A migration of bacteria from roots to leaves was detected in the untreated control, so it was impossible to determine if this was due to a failure to reach a killing temperature or if it was rapid recolonization from the roots. The bacterial titer was lower in the treated leaves than the untreated control. Objective 3: Initial tests of the PMA viability assay in the field suggested that there was a considerable degree of variation in the viability of CLas within the canopy regardless of whether the tree had been heat-treated or not. Large variations in the copy number of CLas amplicons within the canopy of a single tree was not surprising; however, extensive variation in the % viable obtained using the PMA assay was not expected. Data was collected for 16 trees in the initial test block. Although the results are still preliminary, they appears to be consistent with the early finding that the viability of CLas in severely heat-treated samples was high (ranging from 61.3% to 71.5%). Perhaps this is more evidence that re-infection from the roots is a common occurrence in steam-treated canopies. An RNA viability is still a primary objective; however, due to the unexpected variability in viability and the relatively large sample size needed to determine the mean viability of a treatment plot before and after treatment, the same problem regarding resources and time to conduct the assay still exists whether the assay is PMA- or RNA-based. Objective 4: Soil beneath ten individual 10-year-old Valencia trees on Swingle citrumelo rootstock was treated with a hot water generating apparatus on August 17, 2015. The water was injected at a depth of 15 cm via 11 probes spaced 15 cm distant from one another in a semicircular pattern (60 cm radius) in the undercanopy soil on the north and south side of each tree. Two weeks later, soil in the treated zones was sampled by combining 4 cores of soil from each side of the treated trees and from the same relative positions of 10 additional untreated trees. The experimental design was a paired t-test. Objective 5: Preliminary experiments have been initiated to determine time-temperature combinations for the inactivation of CLas. Plant material is being prepared and optimal temperature and time ranges are being investigated. Objective 6: The goal is to identify an advanced material that will enhance the plant thermotherapy process. In this regard, there are three areas: (1) a pretreatment that would weaken but not kill HLB prior to thermotherapy, (2)a treatment prior to thermotherapy that would enhance heat transfer and (3) a post treatment to protect the tree against reinfection by the vector. Current efforts focus on (1) and (2).Based on an extensive literature review of additional literature, the direction is toward using a modified Hydrogel. We are examining pectin as a

starting point for the agricultural hydrogels. Objective 7: This objective will move forward after the data from the first year of study becomes available.

PUBLICATIONS (not previously reported): 2015/01 TO 2016/01
No publications reported this period.

ACCESSION NO: 1005557 **SUBFILE:** CRIS
PROJ NO: FLAW-2014-10120 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23010 **PROPOSAL NO:** 2014-10120
START: 01 MAR 2015 **TERM:** 29 FEB 2020
GRANT AMT: $4,613,838 **GRANT YR:** 2015
AWARD TOTAL: $4,613,838
INITIAL AWARD YEAR: 2015

INVESTIGATOR: Johnson, EV, G.

PERFORMING INSTITUTION:
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ZINKICIDE A NANOTHERAPEUTIC FOR HLB

NON-TECHNICAL SUMMARY: Huanglongbing (HLB) is an invasive disease of citrus that is devastating the largest citrus industry in the US and threatening the other major citrus producing regions. This disease causes significant crop loss for citrus growers because of increased premature fruit drop and reduced fruit quality (i.e. small, unpallatable flavor). The bacteria that causes the disease (Candidatus Liberibacter asiaticus) is transmitted by insects (Asian citrus psyllid). Control of bacterial plant pathogens is difficult because of the limited bactericides available and because they only act as a protective film on the outside of the plant. Insect transmission bypasses this protective barrier and insect control alone cannot prevent disease spread.This project aims to develop a specially formulated bactericidal particle that is small enough to enter the plant vascular tissue where the bacteria lives. It is made from plant nutrient and plant derived compounds to develop novel bactericidal activity not found in the raw ingredients. These particles are designed to breakdown into these nutrients after it has performed its bactericidal function. In conjunction with testing how effective this new bactericide is in controling HLB in citrus orchards, the safety and fate of the bactericide particles will be investigated to ensure safe use of the product. The effect of this new bactericide on citrus tree health, fruit production and quality will be determined. Crop improvement data from field trials will be used in economic analyses to determine if it will allow citrus growers impacted by HLB to return to profitable production. Once shown to be safe and effective for control of HLB, it will likely be useful for control of many other bacterial pathogens that significantly limit food production and threaten farmers livelihoods.

OBJECTIVES: The goal of this project is to develop an economical bactericide for control of HLB-affected trees in established citrus orchards, allowing growers to maintain production and profitability in the presence of endemic HLB. We have developed a prototype plant nutrient-based nanoparticle (novel vacancy-engineered (VE) Zinc oxide (ZnO) particle formulation) with unique bactericidal activity that can translocate into plant tissue with the goal of providing an economical HLB management option for infected trees that will allow efficient and profitable citrus production in the presence of HLB. We will pursue this goal with the following research and outreach objectives:Research objectivesObjective 1. Development, improvement, and characterization of Zinkicide nanoparticles.Objective 2. Test the efficacy of new Zinkicide formulations and optimize field application for HLB control while minimizing non-target effects on beneficial organisms.Objective 3. Determine residue lifespan of Zinkicide in planta and

toxicology on non-target organisms to ensure safety and expedite product registration for grower use to combat HLB.Objective 4. Evaluate the economic feasibility of using Zinkicide to manage HLB compared to existing methods of citriculture in the presence of HLB.Outreach objectivesObjective 1. Develop interactive media tools including a website and related tools to educate citrus growers about the efficacy, viability, and best use practices of Zinkicide as an HLB management optionObjective 2. Provide training for safe handling and field use of nanoparticlesObjective 3. Provide in-service training on Zinkicide for extension agents from major citrus producing regions across the U.S.

APPROACH: Objective 1. Production of the Zinkicide nanoparticles is done in a unique single pot reaction under conditions that don't require a second purification stage. This process will be optimized for large scale synthesis. To be able to identify nanoparticle half-life and location in plants methods will be developed to detect and quantify the Zinkicide particles instead of just their chemical components using multiple microscopy techniques based on the particles unique emission spectra.Objective 2. Greenhouse and field trials on grapefruit and sweet orange will be performed to determine efficacy and the most effective method and timing of application. Currently used field application methods (i.e. foliar spray and soil drench) will be the methods tested to maintain economic sustainability of the treatment. Efficacy will be determined based on fruit production and quality. Samples will also be taken from these trees to determine the systemic movement and residue of the particles using the detection techniques under development in objective 1. Using special microfluidic chamber techniques, developed to study vascular bacterial pathogens, the mode of action of Zinkicide against Liberibacter and/or related bacteria will be determined.Objective 3. Residue analysis of trees treated in objective 2 will be done to determine the duration of effective concentrations in the tree. These residue concentrations will also be incorporated into standard toxicity assays for non-target organisms to provide information on the safety of the treatment.Objective 4. To determine the economic sustainability for the target audience (citrus growers) a benefit-cost analysis will be done to inform the growers on the most cost-effective way of using the treatment to get the best yield productivity return with the minimum input cost.The progress of each method will be evaluated at yearly stakeholder advisory committee meetings where progress on each objective will be presented to the entire research group and stakeholder advisors. Based on these results and the advice of the stakeholders, the plan for each objective will be assessed and modified as needed to ensure the most efficient use of resources towards the final goal of developing an effective management strategy for HLB.Feedback will also be acquired from grower outreach and extension events including field days and workshops to assess the value of the knowledge provided to the grower stakeholders.

ACCESSION NO: 1005575 **SUBFILE:** CRIS
PROJ NO: FLAW-2014-10146 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23029 **PROPOSAL NO:** 2014-10146
START: 01 APR 2015 **TERM:** 31 MAR 2018
GRANT AMT: $2,096,540 **GRANT YR:** 2015
AWARD TOTAL: $2,096,540
INITIAL AWARD YEAR: 2015

INVESTIGATOR: Lorca, G. L.

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A NOVEL ANTIMICROBIAL APPROACH TO COMBAT HUANGLONGBING DISEASE

NON-TECHNICAL SUMMARY: Florida growers have assumed a total infection (symptomatic or asymptomatic) of all producing groves across the state. The strategies used to reduce the effects of HLB have not been effective. Novel strategies directed to eliminate the bacteria from infected trees, and to protect new groves are needed.The goal of this proposal is to assess the efficacy of this antimicrobial therapy in active HLB-infected groves. We have designed an efficient treatment plan that is cost effective and scalable, two aspects that will be essential for any treatment discovery intended for large-scale application. Because recovery of HLB-infected plants in the greenhouse was rapid, we anticipate that the proposed therapy will deliver timely results in the mature, HLB-infected plants.. We will achieve this goal by (1) Optimizing the antimicrobial treatment in HLB-infected citrus seedlings in small scale field trials; (2) Evaluating the antimicrobial treatment in large-scale field trials. We will assess the economic, ecological, and environmental impact of each treatment; (3) Testing the antimicrobial activity of alternative organic compounds that have chemical scaffolds similar to those of the active compounds. We will have optimized the application of chemicals that are effective CLas antimicrobials.Our research team is working directly with citrus growers and groves in active production. The Stakeholder committee is comprised of large and small-scale growers, who will meet to discuss the progress and results of the project every 6 months. The results will be transferred to a broad audience through different publication media (such as the UF journal EDIS and industry publications) and presentations to grower associations.An effective antimicrobial treatment will have an immediate positive impact on the economic and social aspects of the citrus industry. The citrus industry, directly or indirectly, involves a large network of workers and companies currently operating in the state of Florida. Because the citrus industry represents a large portion of the Florida economy, the recovery of infected groves will have a substantial, positive impact on the economy in Florida as a whole.

OBJECTIVES: The goal of this project is to develop an antimicrobial treatment to cure Huanglongbing disease (HLB) in the field. We have designed a plan to efficiently carry out treatments, thoroughly evaluate results on a laboratory scale, and quickly translate treatment discoveries to groves in production. Upon completion of this project, we will have optimized treatments for field-scale applications of HLB-specific antimicrobials, including the analysis of environmental impacts (if any) associated with each of the proposed treatments. We will achieve the goal of this proposal by completing the following objectives. Objective 1. Optimization of

antimicrobial treatment in infected citrus seedlings. Infected citrus seedlings will be used to determine the optimal dosage, dosing intervals, and the elimination rate for each compound, in a controlled environment. Seedlings will also be monitored for symptoms of plant toxicity throughout the treatment period.Objective 2. Field trials: application of the antimicrobial treatment and evaluation of environmental impact. Mature, HLB-infected trees will be treated in actively producing groves. Each compound will be analyzed for effectiveness in bacterial clearance and potential environmental impacts.Objective 3. Identify and evaluate the antimicrobial activity of natural compounds that have chemical scaffolds similar to the two effective compounds we have already identified. The significance is twofold; it may allow a faster translation of chemicals into field trials, and offer a plausible treatment option for organic growers.

APPROACH: Objective 1. Determination and optimization of the antimicrobial treatment in infected citrus seedlings. For the proposed studies, we will use sweet orange (Citrus sinensis) "Valencia" seedlings. To accomplish this objective we will:1.1. Evaluate the toxicity of the selected chemicals on sweet orange seedlings. Different concentrations of each chemical will be tested.1.2. Evaluate the antimicrobial efficacy of the selected chemicals. The evaluation of the antimicrobial efficacy in plants will be performed by following the remission of HLB symptoms as well as the viability and titer of 'Ca. L. asiaticus' in tissue samples collected from new growth.1.3. Determine the stability of each compound within the citrus plant and identify residues in plant and fruit tissue as well as soil samples following treatment. The distribution, stability, and turnover of each compound will be determined in both plant tissues and soil samples. The data collected will be used to address environmental concerns and ensure compliance with EPA regulations.Objective 2. Field trials: application of the antimicrobial treatment and evaluation of environmental impact. This objective will be divided into two phases:2.1 Small scale field trial. Based on the results of the greenhouse experiments, we will test the most effective treatments on infected trees maintained by the Horticulture Science Department at the University of Florida main campus.2.2. Large-scale field trial. The chemicals found to be the most effective for treatment of HLB in the small scale field trial, will be tested in large orchards. Multiple orchards will be tested during the large-scale field trials with different varieties of HLB-infected citrus trees; sweet orange trees (Citrus sinensis, "Valencia") and grapefruit trees (Citrus paradisi, "Ruby red" or "Flame"). On each of those groves we will perform:2.2.1 Evaluation of antimicrobial efficacy. The antimicrobial efficacy of each compound will be evaluated by following the presence and viability of 'Ca. L. asiaticus', by real time PCR.2.2.2 Evaluation of the impact of each antimicrobial treatment on fruit production and quality. Oranges from each field will be collected before and after treatment, and sent to the Citrus Research and Education Center (CREC) for quality analysis.2.2.3 Determination of the stability of each compound within the citrus plant and identification of any residues in plant or fruit tissue following treatment.2.2.4 Evaluation of the impact of each antimicrobial on the host and its associated microbiota. To this end we will define the effect of each antimicrobial on the citrus microbiome and characterize plant responses to each compound. We will also elucidate the detoxification pathways for each compound.Objective 3. Identification and evaluation of antimicrobial activity of natural compounds that share a similar chemical scaffold to the initial lead compounds. We will perform an in silico identification of natural products that interact with the microbial target proteins and in vitro characterizations of protein/ligand interactions. The identified chemicals will then be evaluated (in vivo) in citrus seedlings as described in objectives 1 and 2.The data obtained on each of the objectives will be exhaustively analyzed using different statistical methods.We will also perform an economic impact analyses. The economic

component will include two primary activities. First, a traditional cost-benefit analysis of the compounds will be conducted to determine which compound has the highest net benefit per tree. The second activity will involve the construction of a dynamic bioeconomic model to determine the optimal (profit-maximizing) use of each treatment in the field.

ACCESSION NO: 1005657 **SUBFILE:** CRIS
PROJ NO: FLAW-2014-10119 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23027 **PROPOSAL NO:** 2014-10119
START: 15 MAR 2015 **TERM:** 14 MAR 2020
GRANT AMT: $3,338,248 **GRANT YR:** 2015
AWARD TOTAL: $3,338,248
INITIAL AWARD YEAR: 2015

INVESTIGATOR: Gmitter, FR, G.

PERFORMING INSTITUTION:
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DETERMINING THE ROLES OF CANDIDATE GENES IN CITRUS-HLB INTERACTIONS AND CREATING HLB-RESISTANT CITRUS CULTIVARS

NON-TECHNICAL SUMMARY: This project addresses the Citrus Production Systems priority of the SCRI/CDRE, specifically the development of HLB tolerant/resistant cultivars with acceptable horticultural and product characteristics. The current crisis in Florida citrus arising from the rapid spread of HLB disease and the subsequent decline in production threatens to spread to the other citrus producing states, as well. The US citrus industry is a multi-billion dollar contributor to the US economy, and its demise will have dire impacts not only on the communities built around this industry, but US citizens in general by removing an affordable and valuable nutrient source (citrus fruit and juice products) from our diets.The project will identify critical genetic factors controlling citrus responses to HLB among sensitive and tolerant/resistant citrus accessions, and define their roles. Through proposed over-expression experiments, and also using genome editing and transformation technologies, altered plants of commercial cultivars will be produced and tested for responses to HLB, ultimately yielding HLB-resistant lines free of transgenes. A multi-state Industry Advisory Panel, representing large processing interests, family farms producing fresh fruit, and industry organizations that interface with research, will provide guidance throughout the project on goals, objectives, and commercialization tactics. Interaction with industry at-large, as well as through an interactive website, will enable two-way communication on progress and new opportunities.The project will provide industry with new citrus cultivars exhibiting good horticultural performance, unaltered fruit or juice quality traits, and HLB-resistance, but free of GMO signatures. Consequently, commercialization should be unfettered by regulatory concerns or consumer resistance. The citrus industry can be secure, and social, environmental, or economic consequences of HLB-associated collapse of the US citrus industries can be avoided. Finally, US citizens will be well served by the continued availability of the affordable and valuable nutritional contributions that citrus fruit and juice products make to their diets and overall well-being and health.

OBJECTIVES: The long-term goal of this project is the development of HLB-tolerant or resistant citrus cultivars, using genes from citrus and its close relative and free of GMO signatures, to support the survival and revival of the US citrus industry, and thus to avoid the economic losses, environmental degradation, and sociological consequences of its potential HLB-induced demise. Such new citrus scion and rootstock cultivars must not only tolerate or resist HLB. They must meet the horticultural performance expectations of growers, and yield

fruit and juice products that meet the expectations of packers, processors, and most importantly consumers. Finally, they should be developed using contemporary genetic technologies and approaches in such a way that the cultivars will be free of GMO signatures, thus removing the impediments to their utilization and commercialization associated with regulatory requirements or consumer concerns and reluctance to purchase GMO citrus fruit or juice products. Achieving this goal will support the continued existence and expansion of the US industry, thus avoiding the calamities described above and ensuring an abundant and inexpensive supply of nutritious citrus fruits and juice for the public. Objectives:1. Validate candidate gene expression in inoculated citrus through RNAseq.2. Identify sequence polymorphisms in candidate genes from citrus accessions with different responses to HLB and dissect the gene structure and genomic organizations of candidate genes.3. Understand the roles of candidate genes by over-expressing them in HLB-susceptible citrus cultivars.4. Develop CRISPR-mediated technologies for development of non-transgenic HLB-resistant citrus.5. Precision editing of candidate genes for producing HLB-resistant citrus.6. Outreach and disseminate project results to stakeholders and the public.

APPROACH: Methods:1. Various HLB-tolerant, -resistant, and -sensitive citrus cultivars and near relatives will be graft inoculated with CLas-containing budwood sources. Leaf samples will be harvested at inoculation time and bi-weekly thereafter. RNA will be extracted using commercially available extraction kits, rRNA removed using RiboZero or equivalent, and libraries prepared and samples barcoded to enable pooling prior to Illumina HiSeq runs, to validate candidate gene expression via RNA seq approaches.2. For each candidate gene, the transcribed region and 2-kb upstream and downstream of the transcript will be extracted from the rough lemon and Poncirus genome sequence and used as the input template for capture probe design. Illumina sequencing libraries will be prepared and enriched for candidate genes using the above-described SureSelect system. The citrus genotypes used will include most of the citrus species, cultivars, or citrus relatives that had been evaluated previously for HLB responses and several dozens of additional citrus genotypes that are being evaluated. Enriched libraries will be sequenced on the HiSeq 2000. Sequence reads will be aligned to the citrus genome sequences. Sequence contigs for candidate genes will be aligned with mRNA sequences from Objective 1 to understand gene structures and aligned with the available citrus genome sequences to understand the candidate genes' genome-wide organization and evolution.3. Candidate genes will be cloned into transformation vectors, which will be used to transform and regenerate sweet orange and grapefruit plants. These plants will be identified, and confirmed, then characterized for gene integration and expression. Once confirmed, plant will be inoculated with CLas and their relative sensitivity or tolerance of HLB disease will be determined by comparing growth, symptom severity, and CLas titer between transformed and control plants. Finally, those plants with apparently better resistance or tolerance will be entered inti field trials.4. CRISPR cassettes will be designed and employed for citrus transformation. Resulting modified plants will be characterized, as above, for their resistance or tolerance to HLB disease.5. Target genes and sites for editing will be determined, based on the criteria of being highly expressed in HLB-susceptible citrus genotypes but little expressed or down-regulated in HLB-resistant/tolerant Poncirus and rough lemon. gRNAs will be designed based on the genomic sequences from Objective 2. Agrobacterium transformation will be used to deliver CRISPR cassettes into orange and grapefruit, and plants regenerated. Induced genome sequence changes will be validated, gene expression levels will be determined, and plant responses to HLB disease will be characterized as above, in greenhouse and field environments.Efforts:Throughout the course of the project, information generated will be submitted for scientific publication. Further, progress in achieving

the goals established will be reported through our website, as well as at conferences, scientific meetings, and citrus industry forums.Evaluation:Progress will be assessed by comparing the timelines established for each individual objective and outcomes as they are realized. We will publicize the CRISPR technologies we develop through scientific publications, oral and poster presentations at scientific conferences/meetings/seminars and a website to be developed under this project. Upon request, we will also provide appropriate CRISPR gene cassettes for research use (with the authorization of a standard material transfer agreement) and for new citrus cultivar development (with a fully executed licensing agreement). The impacts of our technologies can be measured in several ways. 1) Short-term: The number of requests received for the CRISPR cassettes; 2) Medium-term: The number of applications of our CRISPR technologies as documented in scientific publications, and 3) Long-term: The number of new cultivars developed using our CRISPR technologies. The overall impact of the project will be assessed by conducting written, short-answer surveys at the beginning and at the end of this project to document industry responses to the development and use of citrus cultivars with genetically engineered HLB resistance and non-GMO HLB resistance. Changes in knowledge gain, perception, and behavior will be extracted by comparing the survey results between Year 1 and Year 5.

ACCESSION NO: 1005600 **SUBFILE:** CRIS
PROJ NO: KS603372 **AGENCY:** NIFA KAN
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23028 **PROPOSAL NO:** 2014-10154
START: 01 MAR 2015 **TERM:** 28 FEB 2017
GRANT AMT: $3,734,480 **GRANT YR:** 2015
AWARD TOTAL: $3,734,480
INITIAL AWARD YEAR: 2015

INVESTIGATOR: Brown, S. J.

PERFORMING INSTITUTION:
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DEVELOPING AN INFRASTRUCTURE AND PRODUCT TEST PIPELINE TO DELIVER NOVEL THERAPIES FOR CITRUS GREENING DISEASE

NON-TECHNICAL SUMMARY: U.S. citrus growers have a critical need for grove-deployable management practices that keep healthy citrus from becoming infected and infected trees from becoming symptomatic. We present a systems-based pipeline approach delivering commercial, grove-deployable solutions using a novel therapeutic delivery strategy and citrus transgenics. A data integration and analysis platform combining existing complex -omics/biological data with molecular/cellular research will steer hypothesis-driven testing of inhibitors of multiple molecular pathways to provide solutions that can be optimized by combinatorial delivery to citrus. Research areas will include: 1) molecular interaction inhibitor discovery (gut membrane binding peptides, RNA aptamers and non-toxic chemical library screening) to block psyllid acquisition/ transmission of HLB and/or growth in the plant; 2) dsRNA delivery to induce psyllid RNAi responses that block HLB transmission or kill the psyllid (or both). The proposed delivery system has negligible environmental impact, is economical in comparison to current control strategies and is highly tractable, allowing it to function as a delivery vehicle for different solution strategies. Co-delivery with bactericides (previously shown to be effective against HLB through laborious injection methods) will be evaluated as complementary methods of control. To translate these therapeutic treatments into long-term solutions, transgenic research will be initiated to produce interdiction molecules (peptides and dsRNA) expressed in the phloem. By engaging stakeholders in design and testing stages, and educating the public, we will deliver acceptable solutions that are applicable to citrus greening and extensible to a wide variety of related economically important pathogens of citrus and other specialty crops.

OBJECTIVES: Research:Improved understanding of HLB transmission and interactions in psyllid and plant phloem.Grove deployable solutions and suite of effective management practices identified to keep current citrus production, maintain high fruit quality, and prevent fruit drop.Extension/OutreachIncreased growers/public understanding of biotechnology methods of disease management.Researchers understand consumer attitudes towards biotechnologies.Increased awareness of novel therapeutic application technologies and products by industry segments.Industry understands economic feasibility and practicality of management recommendations.Students increase knowledge of bioinformatics, genome analysis, database construction: New data visualization tools.Understanding of industry/ consumer knowledge about HLB and its impact on U.S. citrus production.

APPROACH: Objective 1: Data integration / Analysis Combine -omics data into single platform.Manually curate target gene sets in psyllid. Model biochemical pathways. Create visual digital library to access organ or organ system data. Objective 2:HLB Science to SolutionPerform PPI and proteomic studies on gut proteins. Mine interactome and screen dsRNA, RNA aptamer and nontoxic small molecule libraries for blocker molecules. Test candidate molecules in soil application, RNAi and transgenic plant.Test grove-deployable strategies.Conduct metabolomics analysis of treatment efficacy. Evaluate delivery strategies in greenhouse experiments. Objective 3 Engagement, Extension, Education. Utilize industry-relevant Extension and Engagement Methods to introduce emerging agricultural strategies to the citrus industry. Investigate consumer attitudes toward and acceptance of HLB reduction and prevention technologies to inform consumer engagement and outreach