

Title:	Rear and Release Psyllids as Biological Control Agents - An Economical and Feasible Mid-Term Solution for Huanglongbing (HLB) Disease		
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Non-Technical Summary

This proposal presents research targeting the elimination of Huanglongbing (HLB) as an economic threat to US citrus production by blocking the ability of the psyllid insect to move the causative agent of this disease between infected and healthy trees. The primary long term goal of this project is to interfere with the spread of HLB within citrus orchards where HLB disease is established and to interfere with the invasion of disease organism into areas where the insect that transmits the causal agent is established, but in which HLB has not been detected, by strategically releasing a nuPsyllid population that is incapable of moving the disease. A further goal is to ensure the necessary adoption of the method by the social system of growers, and understanding and acceptance by consumers and the general public. Once established, this novel system of biological control would be operationally transferred to the citrus industries of U.S states (Florida, California, Texas and Arizona). Other ongoing support, if necessary, will be provided by the stakeholder organizations. We believe current management practices are not sustainable, and in any event psyllid vector eradication has never been achieved, except on small islands. Alternative HLB-management approaches must be developed as a mid-term solution to the HLB problem. Without control measures in hand, citrus growers have no incentive to replace infected trees or to replant entire orchards. The uncertainties associated with HLB will undermine the stability of the industry in currently HLB-free areas. A mid-term solution is crucial to maintain a profitable industry until citrus varieties with resistance to HLB can be developed and released. Therefore, we present a novel and more environmentally friendly alternative strategy, which we will convey to growers and the public. Grower response to this disease has resulted in a mix of increased costs, modifications to long-successful production management systems and acceptance of at least short-term yield and/or quality reductions. Total orchard loss is increasing as HLB spreads. The current situation suggests that without development of an adequate control strategy, commercial citrus production will become economically unfeasible. This disease also is impacting the millions of citrus trees grown in homeowner yards.

Accomplishments

Major goals of the project

The primary long term goal of this project is to interfere with the spread of HLB within groves where HLB is endemic and to interfere with the invasion of CLAs into areas where ACP is established but HLB has not been detected. Once released and established, the nuPsyllid population will naturally penetrate and displace the wtPsyllid population. A further goal of this proposal is to ensure the necessary adoption of the method by the social system of growers, consumers and the general public in citrus states. To achieve these goals, we propose a three-fold approach: 1). Develop a psyllid management strategy based on the development of psyllid populations incapable of transmitting CLAs (nuPsyllid) and strategically release the nuPsyllid population to displace current ACP populations that have invaded the US. 2). Provide optimized management strategies for integration of the proposed population displacement technique into current management practices: a. Southeast and Southern U.S. (FL and TX) where both the ACP and CLAs are endemic. b. Western U.S. (CA, AZ) where ACP is present and spreading while there is currently no detection of HLB. 3). Integrate the management strategies with monitoring strategies to continually assess effectiveness and provide outreach education to the grower stakeholders and citizens about the control strategy. The feasibility of the approach proposed here is supported by the experience with HLB management in Florida through the creation of Citrus Health Management Areas (CHMAs). The CHMA is based on the recognition that HLB has an important edge effect. Although insecticide applications can control ACP populations within the grove, without effective ACP management in the surrounding areas, CLAs-bearing ACP rapidly returns. A CHMA coordinates the insecticide sprays and other management activities over a large area, thus greatly reducing the edge effect of ACP re-invasion. Thus, we are proposing that most nuPsyllid releases will be focused at the periphery of CHMAs and other smaller management areas to displace the endemic population at the periphery. Vigorous ACP control measures would be continued temporarily in the interior of the management area but would be gradually tuned down to allow populations of CLAs-transmission-deficient ACP populations to become established

What was accomplished under these goals?

The purpose of this NIFA-CAPS is to create options for management of HLB by replacing the wild type insect vector (ACP) with a population that is unable to transmit the bacterial causative agent *Candidatus Liberibacter asiaticus* (CLAs). Achieving this outcome will require progress in the following three areas of emphasis - An Effector Mechanism, A Driver System, and Diffusion. At the end of the third year of NIFA support significant progress has been accomplished in each of these areas as summarized below.

Effector Mechanism - Initial assessments have not identified the required variation in CLAs transmission to occur naturally in ACP populations. However the prospects for engineering a mechanism to achieve the desired phenotype are under active investigation. The effector is the content of the phenotypic change we aim to introduce. Candidate effectors are being identified through multiple parallel methods of investigation.

- There is a promising list in various stages of validation from in silico-bioinformatics (proteomic and transcriptomic), genetic (yeast two-hybrid), physical methods (immunoprecipitation-pull down and mass spectrometry identification of proteins in the complex). In order to use an Effector for insect replacement, we need to disrupt these interactions while maintaining psyllid fitness.

- An extensive transcriptome database (the Transcriptome Computational Workbench) has been constructed and annotated for whole adults and nymphs, and from dissected salivary glands and guts of insects infected or not infected with CLAs. They have been made available to the research community at www.sohomopter.org/ACPPPOP and the manuscripts titled "Asian citrus psyllid expression profiles suggest *Candidatus Liberibacter asiaticus*-mediated alteration of adult nutrition and metabolism, and of nymphal development and immunity" and "Comparison of potato and Asian citrus psyllid adult and nymph transcriptomes identified vector transcripts with potential involvement in circulative, propagative *Liberibacter* transmission" were published in PLOS ONE and Pathogens, respectively.

- Using the yeast two-hybrid method, we have completed ACP gut and salivary gland library matings (35), CLAs library matings (37) and Y2H specific "Bait" to "Prey" matings (reciprocal confirming mating, 30), and recently, a novel batch screen was conducted between a CLAs bait-ACP prey library mating, to reinforce the potential role of several complexes and individual proteins identified using results obtained from the in silico approach.

- Co-Immunoprecipitation (CoIP or pulldowns) yield complexes of proteins from psyllid extracts reacted with the various over-expressed candidate proteins that are identified by mass spectrometry analysis. Using PCR, RT-PCR from cDNA and CoIP, the expression of three prophage gene products expressed in the psyllid has been demonstrated. All three implicate interactions in the invasion process. To date CoIP has been achieved and repeated multiple times with two ACP proteins, two CLAs proteins and one prophage protein.

- RNAi knockdowns studies for 21 candidates are completed or are in progress; at least 6 targets have shown significantly reduced *Liberibacter* transmission in a functional bioassay using the a single-gene RNAi approach.

- These results have allowed us to construct a model of invasion showing that both prophage and CLAs effectors may co-opt the clathrin coated pit endocytic pathway into a pathogen mediated phagocytosis involving 'membrane ruffling', a mode of entry/exit ascribed to several well known human pathogenic bacteria. This insight has greatly enhanced our ability to prioritize new candidates for experimentation, such that putative effectors that can be shown to act in concert can be targeted collectively, and further abate ACP-*Liberibacter* interactions essential for adhesion and multiplication in the gut, exiting the

gut, and entering the salivary glands.

- A system for screening 4-amino acid peptide libraries for binding to psyllid digestive tract epithelium has identified at least 8 candidate ligands that bind specifically at submicromolar concentrations with different binding kinetics. Competition studies with unbiotinylated versions for one have shown competitive binding indicating the biotin is not required for gut binding. Similar competition studies are underway for the other peptides. Peptide binding has been shown to be stable in adults that acquired the peptide during feeding as nymphs. Proving this stability was crucial to functional use of the peptides in the acquisition/transmission inhibitory bioassays.

- Single chain antibody fragments (ScFvs) encoding genes have been isolated and are expressed in transgenic citrus. There are 18 separate transgenic lines each producing the ScFvs recognizing separate epitopes from two different surface antigens of CLAs. These plants are at various stages of development with some ready to be used in CLAs transmission assays to determine if any scFV's, when expressed in a plant, block acquisition of CLAs by the psyllid.

Driver System - A new trait will not spread efficiently upon release within an existing population without a genetic bias of some kind. The driver is the medium of spread of the introduced phenotype--lack of CLAs transmission. The drivers under investigation are viral, endosymbiont and chromosomal.

- Viruses infecting psyllids were found by deep sequencing and bioinformatic analysis of small RNA libraries from over 30 diverse ACP collections from 18 locations around the world and the Diaphorina citri picorna-like virus (DCPLV) was selected for development into a recombinant vector system to be implemented for reverse genetics and to understand some of the biology of DCPLV.

- A collection of 6 non-native Wolbachia isolines is currently being screened for efficacy in reducing Las transmission.

- The diversity and distribution of native Wolbachia strains in existing Florida psyllid populations has been identified and phylogenetically characterized. One minor candidate strain appears to be an excellent candidate for population replacement because it is negatively correlated with the presence of other psyllid endosymbionts.

- High throughput methods for establishing and screening new Wolbachia cell cultures from psyllid samples have been developed, yielding approximately 30 new native ACP Wolbachia cell cultures.

- Proof of concept has been established for several chromosomal-based gene drive systems for population replacement in the psyllid. DNA vectors for a preferred system, engineered translocations, have been constructed and implemented in a model system predicted to yield a relatively high threshold system that will feature genetic containment and likely public acceptance advantages. In order to generate translocations by design, site-specific cleavages to promote recombination are engineered with both the cas9/guide RNA system and homing endonucleases.

- This system shows great potential for ACP-HLB control because it should be readily transferrable once ACP can be transformed and is robust to mutations anticipated to inactivate drive while genes of interest can be easily linked to the translocation breakpoint.

- Use of this technology in *D. citri* requires a transgenesis system of gene constructs and transformation of the psyllid germline. This priority is being pursued with both embryo injection and injection of adult males and females using a variety of transfection reagents and methods including the gene gun and electromagnetic rail gun.

Several aspects of the technical and communication plan for diffusion of this proposed HLB solution can be addressed most effectively only when an actual candidate nuPsyllid is available for release. The ability to rear, release and monitor psyllids has been initiated and is of immediate use in HLB disease management applications outside of this proposal.

What opportunities for training and professional development has the project provided?

Elements of this project are being conducted in University and USDA laboratories in a number of states. These sub-projects are providing considerable professional training to undergraduate and graduate students through direct involvement in the nuPsyllid project. In addition, the project employs a number of Post-Doctoral trainees in the labs, whose contributions to the research objectives serve also to provide them additional professional training. All involved in this project are being exposed to the approaches and mechanics of team research on a large scale, and team meetings involve shared experience on how component research objectives fit into the larger picture.

How have the results been disseminated to communities of interest?

A large annual Team meeting was held on Feb. 8th, 2015 in advance of the HLB International Conference in Orlando, FL including stakeholders, administrative management and advisors. This was a great opportunity for the Driver and Effector teams, who are primarily involved in attempting to isolate or build a nuPsyllid colony to present results to those responsible for the ultimate downstream release and adoption of this technology. Presentations on the project goals and objectives, as well as progress to date have occurred at meetings of the citrus growers in California, Texas and Florida numerous times during the year.

There is a substantial effort to rear and release any type of nuPsyllid under development:

- Florida, Texas, and California will each develop and maintain its own colony to provide nuPsyllids, if available, for initial greenhouse studies and pilot field releases within its borders. The decision as to where to house nuPsyllid colonies within

each state will be likely have to be made at several administrative levels.

- Regulatory agencies will likely require that nuPssyllid colonies be housed in a controlled/quarantine facility. Potential sites in each state were identified.

- An estimated population size for a nuPssyllid required for testing cannot be provided until the driver mechanism is selected. The effector mechanism may have associated fitness costs, as well, and these will have to be figured into rearing effort estimates.

- The initial plan is to piggyback nuPssyllid rearing efforts onto that of the existing parasitic wasp programs (Tamarixia) for initial testing with care to control for Tamarixia contamination.

- Modeling efforts to include better climate suitability GIS layer for ACP are in progress.

- An initial economic model was developed captures the longer-term effects of HLB on the citrus supply response and the unique complexity associated with new planting decisions for this type of perennial crop. An abstract of the work was submitted to the Agriculture and Applied Economics Association meeting in San Francisco, July 26-28, 2015.

Details of the nuPssyllid project have been made available for public consumption through inclusion in trade journal articles, through the nuPssyllid web page and through other mechanisms, including newsletters of the CRDF.

What do you plan to do during the next reporting period to accomplish the goals?

At the Annual Team meeting the entire group of participants and stakeholders used a risk matrix methodology to force rank whether they perceived any of the three driver systems to be most likely implemented. Because there was no compelling winner (or loser) from this analysis, we are continuing with the concurrent work plan originally proposed with respect to the Driver and Effector teams. The entire group discussed revisions to focus the current technical plan based on the progress achieved, as well as budget to plan variances. The administrative team has in several cases been able to propose redirecting some cash flow from within the program toward these more recently focused objectives. The priorities in the coming year are:

- accelerate development of a viral vector based on DCPLV because this is likely to be the first tool for genetic manipulations and would be immediately useful for effector prioritization;
- analyze the phenotypes of non-native Wolbachia introduced into ACP;
- develop ACP transformation capacity at any level of efficiency because of the impact of success with this bottleneck on the ability to create the desired nuPssyllid colony;
- engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB.

Participants

Actual FTE's for this Reporting Period

Role	Non-Students or faculty	Students with Staffing Roles			Computed Total by Role
		Undergraduate	Graduate	Post-Doctorate	
Scientist	4.3	0	1	5.7	11
Professional	0.5	0	0	0	0.5
Technical	5.6	0	0	0	5.6
Administrative	0.3	0	0	0	0.3
Other	0	0	0	0	0
Computed Total	10.7	0	1	5.7	17.4

Student Count by Classification of Instructional Programs (CIP) Code

Undergraduate	Graduate	Post-Doctorate	CIP Code
	1	3	26.05 Microbiological Sciences and Immunology.
	1	3	26.09 Physiology, Pathology and Related Sciences.

Target Audience

Target audiences include the primary benefactors of the research, the U.S. citrus growers. In addition, the target is the scientific community who is engaged in developmental research that has allowed this research project to be envisioned, and

on whose progress we will continue to move forward. The general public is a target of our outreach. As consumers, they are interested and concerned about how research solutions are implemented to solve practical problems, and have shown interest in the foundations of this research project. Finally, policy-makers who often are involved in funding research for Florida citrus, need to be appraised of the project, its goals, and expectations that come from progress. The Outreach Team has determined that 1) because the effector and driver systems are all progressing equally it will not be possible to eliminate one or more from the outreach efforts and 2) an educational program should focus on the context of genetic technologies in general so that the nuPsyllid option for disease management is contrasted for example with a genetically modified citrus host and other technology options. Together with molecular biologist and extension expert Peggy Lemaux, Powerpoint presentation materials have been drafted and are being reviewed with the extension group.

Products

Type	Status	Year Published	NIFA Support Acknowledged
Journal Articles	Published	2014	NO

Citation

NOTE: include J Brown Nov 2014 Publication - see page 24 of pdf file--CRDF funding acknowledged

Other Products

Product Type

Models

Description

Technology assessments have suggested a near term application of this research for the protection of new plantings. This concept "Psyllid Shield" is being evaluated for field trials. While it is not full insect replacement, it is based in part on research progress in the search for Effectors. CRDF has supplemented funding to model and assess the minimum field trial plot size and time required to demonstrate efficacy in protecting new solid block plantings from HLB with RNAi. Extensive modeling efforts have been accomplished to date and a regulatory strategy is in progress. RNAi might be delivered genetically by CTV vectors or through transgenic citrus. If exogenous delivery technology is developed and the cost of goods is inexpensive, it may be possible to delivery the actives exogenously.

Changes/Problems

Like many new projects of this magnitude communication and reporting between the 15 institutions involved in this project is not simple. The project meetings and progress reports indicate that, despite this complexity, the project is on target with its timeline of objectives. There are no major changes to the project in terms of approach and none are anticipated. However, in this continuation year 3 planning we have spent significant administrative effort in cash flow management to direct funds to the most important current objectives within the original proposed scope of work. CRDF, as the primary on this project, requires quarterly written progress reports on its funded projects, and we have included this term in all nuPsyllid project subcontracts, although it is not required under the NIFA terms and conditions. Consequently, we are receiving and posting these brief progress reports that are generated by each participant and coordinated through the team leaders up to a collective quarterly progress report submitted by the Project Director. We feel this keeps the team members focused on the goals and allows us to communicate regularly on progress.