Citrus Research and Development Foundation, Inc.

MEMORANDUM

TO: nuPsyllid Management
FROM: Tom Turpen, Project Director
DATE: December 23, 2014
RE: Quarterly Interim Report

Rear and Release Psyllids as Biological Control Agents – An Economical and Feasible Mid-Term Solution For Huanglongbing (HLB) Disease Of Citrus

Project Mission and Organization

The purpose of this NIFA-CAPS is to create attractive 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 (CLas). Achieving this outcome will require progress in the following three areas of emphasis – An *Effector Mechanism*, A *Driver System*, and *Diffusion*. The current conditions threatening citrus production nationally require our key personnel to work concurrently on parallel technical plans and to accelerate the leading alternatives based on assessments by our team leaders, advisors and management.

These assessments have suggested a near term application of this research for the protection of new solid block plantings from HLB. This concept "Psyllid Shield" is being evaluated for field trials to demonstrate efficacy in protecting. 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 field results under various scenarios and has selected 5 RNAi sequences as field trial candidates based on the results of indoor experiments with caged insects.

A large team Annual meeting is being coordinated by our administrative team for Feb. 8th in advance of the upcoming HLB International conference in Orlando, Feb. 9-13th, 2015.

TECHNICAL PROGRESS

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 including bioinformatics, proteomics, yeast two-hybrid (Y2H), peptide-ligand and scFV-ligand libraries.

- There is a growing list of candidate effectors generated from bioinformatics (proteomic and transcriptomic), genetic (yeast two-hybrid) and physical methods (Far-Westerns--immunoprecepitations and mass spectrometry). In the case of the yeast two-hybrid method numerous candidates are being tested reciprocally to confirm results. This workflow of the Effector team has already generated more high quality targets than can be analyzed in bioassays. In many cases loss of gene expression through RNAi is highly toxic to psyllids. We have only conceived of two tools to use to disrupt the Effector Mechanism, RNAi and competitive protein ligand inhibitors (proteins, such as scFV antibodies or peptides). Secondary metabolites or RNA aptamers are potential additional options. In order to use an Effector for insect replacement, we need to disrupt these interactions while maintaining psyllid fitness.
- The extensive transcriptome data set (the Transcriptome Computational Workbench) has been created from whole adults and nymphs as well as dissected salivary glands and guts of insects infected or not infected with CLas is available to the research community at www.sohomopter.org/ACPPOP. See also: "Comparison of Potato and Asian Citrus Psyllid Adult and Nymph Transcriptomes Identified Vector Transcripts with Potential Involvement in Circulative, Propagative Liberibacter Transmission" published in *Pathogens* (3: 875-907; doi:10.3390/pathogens3040875).
- Using the yeast two-hybrid method, gut and salivary gland library matings (30/ea) have been performed and the results reinforce the likely role of several complexes and individual proteins identified in the level 2 biological processes of the Gene Ontology categories of Adhesion, Nutrition, Invasion and Immune functions. An interaction between a potential CLas virulence factor and a psyllid signaling molecule that regulates fundamental cellular processes, including cell growth, migration and survival was identified as well as an interaction between a psyllid and *Wolbachia*-associated protein that indicates the potential importance of tripartite interactions for pathogen invasion. A CLas phage protein and an ACP protein predicted to be important in CLas invasion of host tissues contains two putative domains; a coat protein and a highly conserved known virulence factor. RNAi knockdowns studies for 16 candidates are in progress while at least 5 of these targets show a significant reduction in CLas transmission in a functional transmission bioassay to date.
- 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. Optimized acquisition assays have been developed and are now being used to test for peptide inhibition of CLas acquisition. In this assay, acquisition is measured (using PCR detection of CLas) as an inhibition of movement of the bacterium into the salivary glands and/or an overall reduction in the titer of CLas in the psyllids.
- Twenty different scFVs were identified that bind separate surface antigens on CLas and the genes encoding these scFVs have been transformed into citrus. 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. These viruses include a Reovirus (dsRNA), two *Iflavirus*-like isolates (+sense, ssRNA) and a Densovirus (ssDNA). Because the *Ifla*-like viruses are most readily manipulated as a gene vector, efforts are now focused on obtaining the full genome sequence.
- The *Ifla*-like viruses discovered (DcIV) in this work may be members of a new genus because parts of the genome show highly significant similarity to know members of the genus *Iflavirus* whereas other regions are more similar in genome organization to the genus *Dicistrovirus*.
- The tentative full-length sequence of DcIV is 8389 nucleotides with the 5` UTR of 489 nt and 3` UTR of 491 nt. Efforts are now focused on cloning and confirming the 5' termini of each of the genomic RNAs to enable a recombinant vector system to be implemented for reverse genetics.
- Multi locus sequence typing (MLST) of female *D. citri* from locations throughout Florida indicates there are three genetically distinct types of *Wolbachia* (all are strain B). Screening of *Wolbachia* genes coxA, hcpA, gatB, fbpA, ftsZ and wsp was conducted using endpoint PCR. One type is prevalent in all populations, with two rare sequence types occurring in distinct regions. Individuals with rare sequence types have been isolated to create isofemale breeding lines for laboratory evaluations of Las transmission, life history, and cytoplasmic incompatibility.
- Initial analysis indicates that symbiont densities including Liberibacter are inversely related to *Wolbachia* density, which suggests that while native *D. citri Wolbachia* may not completely outcompete endosymbionts, it may still reduce endosymbiont densities through a mechanism such as competitive exclusion. All endosymbiont titers were assessed using qPCR and related to a *D. citri* housekeeping gene (Wg). Endosymbiont titers were assessed by relative standard curves. *Wolbachia* titers were assessed using a newly developed TaqMan qPCR assay, targeting the hcpA gene. This gene imparts greater specificity than the Wolbachia wsp gene used previously. Whether individual *Wolbachia* strains might limit symbiont titers in the psyllid, and whether reduced Liberibacter titers in *D. citri* correspond to reduced inoculation of Citrus hosts is currently being investigated.
- Stable *Wolbachia* infections were detected following microinjections of *Cuerna costalis* leafhoppers as a proof-of-concept, indicating that this may be a viable strategy for artificially infecting Hemipterans with non-native Wolbachia infections. The passage of *Wolbachia* to subsequent generations and localization of *Wolbachia* in insect tissues is currently being evaluated. Co-colonization of *D. citri* with a microinjected *Wolbachia* culture obtained from *Drosophila* is in progress.
- Proof of concept has been established in a model system for chromosomal-based gene drive systems for population replacement in the psyllid. However, the challenge with single or two-locus underdominance is to fully rescue the siblings that should be protected by the antidote in an engineered toxin-antidote system.

- A third drive mechanism, engineered translocations has been constructed and implemented in a model system and future work will focus on this 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.

Diffusion

Once a nuPsyllid population is developed, its successful use will depend on series of factors based on the overall phenotype and fitness of the population in the environment and most importantly, will depend on human adoption, including the behavior of regulatory agencies, growers and consumers. All of these attributes must be modeled accurately for a nuPsyllid release to be used effectively. As for any other innovation, diffusion is the rate of change. Several aspects of the technical and communication plan 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.

- 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 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 nuPsyllid colonies be housed in a controlled/quarantine facility. Potential sites in each state were identified.
    - An estimated population size for a nuPsyllid 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 design of release and monitoring studies will also need to be postponed until the driver and effector mechanisms are selected. Modeling studies that will help predict the spread of nuPsyllid have been developed and are being refined and validated with historical datasets on HLB spread.

- 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.

- Together with molecular biologist and extension expert Peggy Lemaux, Powerpoint presentation materials are being developed to be used by extension specialists in the various states to teach growers about genetic engineering and its potential uses for the ACP/HLB situation.

SUMMARY

Review of the data obtained to date supports an "invasion" theme whereby CLas hijacks host endocytic pathways for entry into host tissues, which has been seen in other pathosystems. Because of the progress with the effector characterization and driver options, it is an important time for the team to continue to:

- select and prioritize effectors;
- obtain antibody reagents for top effector candidates;
- use the bioassay platform for comparative testing of the phenotypes in ACP, maximizing transmission blockage and minimizing fitness loss;
- accelerate development of a viral vector 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;
- determine if *Wolbachia* transformation is a feasible goal;
- develop ACP transformation capacity at any level of efficiency;
- continue to ready the engineered translocation contructs;
- begin to model the logistics of rearing and releasing nuPsyllid around hypothetical specifications and explicit assumptions;
- model the trial design to demonstrate the impact of Psyllid Shield in protecting new plantings (transient CTV viral vectors expressing psyllid-targeted RNAi);
- continue to engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB.