Citrus Research and Development Foundation, Inc.

MEMORANDUM

TO: nuPsyllid Management
FROM: Tom Turpen, Project Director
DATE: December 31, 2015

RE: Quarterly Interim Report as of November 30, 2015

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. 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. CRDF is seeking additional stakeholders to plan for larger scale field trials of this disease management concept. A prospective lead partner for this development effort has been identified.

The consensus of the team leaders and stakeholder advisors developed at the last Annual meeting was to continuing with the concurrent work plan originally proposed with respect to the Driver and Effector. The team has updated project objectives that management has determined are all within the scope of our original proposal. We are working now to synchronize our remaining cash flow with those priorities.

700 Experiment Station Road • Lake Alfred, FL 33850 • Phone: (863) 956-5894

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 sets (the Transcriptome Computational Workbench) created from whole adults and nymphs infected or not infected with CLas or CLso are available to the research community at www.sohomoptera.org/ACPPoP. The manuscripts entitled: "*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.
- The manuscript entitled "*Localization of a rod-shaped morphotype of 'Candidatus Liberibacter solanacearum' and evidence for surface appendages in the potato psyllid vector*" (PMID: 26551449) was published, which confirms that flagella and pilus-like appendages are assembled into visible structures, providing for a means of attachment for establishment in the psyllid, and motility for circulation in hemolymph, respectively, collectively, supporting their involvement in *Ca.* Liberibacter pathogenesis in the psyllid vector, and perhaps also in the plant host (Cicero et al., 2015; http://dx.doi.org/10.1094/PHYTO-04-15-0088-R). A manuscript entitled: '*Propagation, circulation, and biofilms associated with Ca. Liberibacter solanacearum infection of the potato psyllid'* (Cicero et al., December 2015) was also submitted.
- Transcriptome annotations are being used to update the proteome identifications for psyllid salivary glands and gut proteins, determined in a previous project funded by the Florida citrus producers. A comparison of the results of both data sets is underway to identify and illuminate the role of additional protein-transcript combinations in psyllid organs with which CLso/CLas interact.

- The re-annoted transcriptomes for the dissected salivary glands (1000/ea.) and guts (1000/ea.) of psyllids infected or not infected with CLas. or CLso were finalized, resulting in 35,288 and 36,995 contigs, respectively. More than 70% of contigs were able to be annotated using the NCBI and UniProt sequence databases, which includes the *Diaphorina citri* genome (source: NCBI). Preliminary differential expressional, Gene Ontology, and KEGG analyses support findings observed in the adult and nymph transcriptomes and more importantly identified additional candidates for targets that may, if silenced using dsRNA, interfere with Liberibacter transmission and psyllid survivability. Manuscript preparation based on our results from multiple studies, including; the standard yeast two-hybrid method using ACP gut and salivary gland library matings (35/ea.), CLas library matings (37), non-standard Y2H specific "Bait" to "Prey" matings (42), and co-immunoprecipitation (CoIP) experiments. The objective of this manuscript is to highlight our findings leading to the construction of a model in which both prophage and CLas effectors transform the endocytic host pathway into pathogen-mediated phagocytosis. This process in part involves membrane ruffling to facilitate invasion and possibly dissemination (spreading) within the psyllid host once CLas cells exit gut biofilms. Our proposed "invasion model" suggests that CLas first adheres at least with CLas OmpA to both integrins and ABC transporters. Subsequently, a prophage protein (SC1-gp060, Colicin 1A-like), usually a toxin to kill other bacteria, is used as an effector by utiliizing its own TolA region and a Liberibacter host ABC transporter, which alters the normal function of clathrin light chain (CLC) and adaptor protein 1 (AP1) proteins to facilitate endocytosis *via* membrane ruffling.
- This working model of invasion has enhanced our ablity to select new cadidates to elaborate on the associated effector mechanism(s), and facilitated the selection of single, and in combination (stacking) dsRNA candidates. To date, RNAi studies have been conducted for 25 candidates with 8 of these targets showing a reduction in CLso transmission in functional transmission bioassays using the traditional single-gene RNAi approach.
- The two most recent genes tested that show an effect on CLso transmission, both resulting in a reduction of 12.5% compared to experimental controls, were also tested using a "stacked-gene" RNAi approach. This additional RNAi method of "stacking" interacting or networking genes is being employed to perhaps obtain a greater impact on transmission interference. Preliminary results from the stacking of these two genes along with another candidate gene showed a reduction in transmission by 30% compared to controls. Replicates of this three-gene dsRNA combination are ongoing.
- The identification of peptides that bind the psyllid digestive tract (DT) epithelium and the different and reproducible binding dynamics for these peptides have been described previously using an acquisition asay. The acquisition assay is used to monitor movement of the *Candidatus* Liberibacter asiaticus bacterium (CLas) from the gut lumen and into the salivary glands. Acquisition is measured (using PCR detection of CLas) as the detected movement of the bacterium into the salivary glands and/or an overall increase in the titer of CLas in the psyllids. Using this assay replicated studies were conducted on the effect of gut binding peptides on CLas acquisition. One combination of three gut binding peptides was able to reduce (greater than 3-fold) the percentage of adults that acquired CLas in their salivary glands when these peptides were added to artificial diet fed to forth instar nymphs that developed into the tested adults.
- During the peptide screening process an in-leaf assay was developed for measuring mobility of peptides within a citrus leaf and to test the effect of the peptides on the

CLas bacterium present within this leaf. Using this assay, a decameric peptide was identified that reduces the CLas titer within leaves when the leaf is removed from the citrus tree and the petiole is placed in a solution containing the peptide. These results are being replicated further for validation.

- These findings create future opportunities for using these peptides in controls strategies beyond those suggested in the nuPsyllid grant. In this pursuit, the findings from this grant were the foundation of a second awarded SCRI CAPS grant "Developing an Infrastructure and Product Test Pipeline to Deliver Novel Therapies for Citrus".
- ScFv's 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 Candidatus Liberibacter asiaticus. 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.

- The virus discovery part of the nuPsyllid project is substantially completed and a manuscript on this work was recently accepted to be published in Journal of Virology. Current effort now is focused on three virus candidates: *Diaphorina citri picorna-like virus* (DcPLV), *Diaphorina citri Densovirus* (DcDNV) and *Diaphorina citri Associated C virus* (DcACV) to assess their potential as the delivery vehicle and also their biology. With funds from the nuPsyllid project DcPLV is the first choice while with new SCRI funds DcDNV and DcACV are also being pursued. Recent bioinformatics and RT-PCR confirmation analyses of additional Illumina deep sequencing datasets suggest even more viruses, some of which may be better than those identified so far.
- Because the presence of DCPLV in more than 20 US populations of *D. citri* has not been confirmed, Federal APHIS permits to import live DCPLV from Brazil, Taiwan and China populations have been obtained as well as approval to from the UC Davis IBC to conduct the work in the UC Davis BSL3 CRF.
- Efforts to clone the full genome of DcPLV as a single cDNA have not been successful. Therefore, a strategy of rebuilding the full length clone from sequence-verified subclones is being pursued.
- Live cultures of wildtype, and hopefully cloned, recombinant DCPLV at the UC Davis BSL 3P CRF will be established to determine how to transmit and spread DCPLV among *D. citri*, and perform life table studies to determine if wildtype and/or recombinant DCPLV has any negative effects on *D. citri.*
- The densities of *Wolbachia* endosymbiont in adult *D. citri* sampled from different populations using quantitative PCR have been examined. Under field conditions, the densities of all three endosymbionts positively correlated with each other, and they are associated with *D. citri* gender and locality. In addition, the infection density of *C*Las also varied across populations. Although an analysis pooling *D. citri* from different populations showed that *C*Las-infected individuals tended to have lower *Wolbachia*

densities compared to uninfected individuals, the difference was not significant when the population was included as a factor in the analysis, suggesting that other population-specific factors may have stronger effects on *Wolbachia* densities. To determine whether there is a genetic basis to the density differences, *Wolbachia* densities between aged *C*Las-negative females of two *D. citri* populations reared under standardized laboratory conditions were compared. Results suggested that inter-population variability in *Wolbachia* infection density is associated with the genotypes of the endosymbiont or the host.

- Based on these findings, three *D. citri* isolines have been developed from Florida populations for subsequent transmission studies. *Wolbachia* cultures isolated from *D. citri* populations from Hawaii and Texas and grown in stable laboratory will be used to transinfect *D. citri* to test the hypothesis that *Wolbachia* genotype drives *C*Las density and transmission. A manuscript describing these findings has recently accepted for publication in the journal *Microbial Ecology*.
- 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 work 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.

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

- Field test have been initiated to compare the efficacy of scent lures in attracting ACP to sticky card traps. The tests will compare ACP response between 'generalized' scent lures, those comprised of a mixture of volatile compounds common to a number of ACP host plants, and 'specific' scent lures, those comprised of a mixture of volatiles from a specific host plant. Synthetic compounds that act as ligands to olfactory binding proteins isolated from the ACP antenna are also being tested, alone and in combination with generalized and specialized scent lures.
- Greenhouse studies revealed that exogenous application of a plant signalling hormone, methyl jasmonate (MeJA), to the foliage of several ACP host plant species (sweet orange cultivars Valencia and Ridge Pineapple, orange jasmine, and *Citrus macrophylla*) resulted in significant changes in their foliar aroma profile. In sweet orange, this change occurred both in healthy trees and trees infected with *C*Las and in both young flush and mature flush. Laboratory tests were conducted to compare ACP response to *C. macrophylla* sprigs treated with MeJA and to untreated foliage. There was no difference in the mean numbers of psyllids settling on either MeJA-treated or untreated sprigs. However, twice as many psyllid aggregations (defined as $\geq$ 10 ACP/sprig) occurred on the MeJA-treated sprigs versus the untreated sprigs. This result indicates that the aroma emitted by MeJA-treated sprigs influences aggregation behavior in ACP. Therefore, studies of MeJA-treated foliage may identify the volatile compounds responsible for mediating ACP aggregation behavior. Conceivably, such compounds could be added to scent lure mixtures to enhance their efficacy. Tests will be conducted in the near future to measure ACP response to MeJA-treated trees in the field.
- Modeling efforts with Len Coop to include better climate suitability GIS layer for ACP are in progress. The USDA-ARS team at Ft Pierce, FL worked on incorporating Tamarixia releases into the ACP simulation model. A working version of the model was completed. Including inter-species interactions lays much of the groundwork needed for incorporating nuPsyllid-wtPsyllid interactions into the model.
- Paul Mitchell developed an initial economic model that 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 and presented the work at the Agriculture and Applied Economics Association meeting in San Francisco, July 26-28, 2015.
- The Outreach Team completed a series of 3 grower interview events in CA gathering information on grower attitudes to different potential HLB control approaches, including GM insect releases. Preliminary results were reported in Citrograph.

SUMMARY

An important publication describes the “invasion” theme whereby CLas hijacks host endocytic pathways for entry into host tissues, which has been seen in other pathosystems. There are a number of excellent candidate effector targets. It would be ideal to test these candidates in a psyllid viral vector. The translocation driver system is ready if the transformation bottleneck can be overcome. 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 the DCPLV 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 both native and 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 constructs;
- 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);
- engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB.