NIFA Award No. 2012-51181-20086
Citrus Research and Development Foundation, Inc.

NuPsyllid: Rear and Release Psyllids as Biological Control Agents – An Economical and Feasible Mid-Term Solution for Huanglongbing (HLB) Disease of Citrus

Quarterly Report for the Period ending May 31, 2016

PROJECT DIRECTOR SUMMARY

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. This research has established a broad foundational knowledge base of molecular interactions between host, pathogen and vector that is now contributing to additional NIFA-funded programs. Part of our outreach in the final phase of this program will be to integrate our progress with others focused on the HLB challenge.

These assessments have suggested a near term application of this research for the protection of new solid block plantings from HLB. We continue to evaluate the "Psyllid Shield" control strategy. 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 has identified a key stakeholder partner to plan for larger scale field trials of this disease management concept.

The consensus of the team leaders and stakeholder advisors developed at an Annual meeting this quarter was to continue with the concurrent work plan originally proposed with respect to the Driver and Effector research. The team has updated project objectives and budgets for the remaining term of the funded work. Management has determined are all within the scope of our original proposal and are in the process of submitting revised work plans to NIFA for approval in order to synchronize our remaining cash flow with those priorities.

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). 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.
- Extensive transcriptome data sets (the Transcriptome Computational Workbench) have been created from whole adults and nymphs infected or uninfected with CLas or CLso, and made available to the research community, at www.sohomoptera.org/ACPPoP. Research using this data-rich resource has resulted in the publication of several manuscripts: "*Asian citrus psyllid expression profiles suggest Candidatus Liberibacter asiaticus-mediated alteration of adult nutrition and metabolism, and of nymphal development and immunity*", *"Comparison of potato and Asian citrus psyllid adult and nymph transcriptomes identified vector transcripts with potential involvement in circulative, propagative Liberibacter transmission*", *"'Candidatus Liberibacter solanacearum' and evidence for surface appendages in the potato psyllid vector'"* published in PLOS ONE, Pathogens, and Phytopathogly, respectively.
- During this quarter, preliminary RNASeq data was obtained from the CLas-infected and -uninfected ACP instar study (1^{st}-3^{rd} nymphs, 4^{th}/5^{th} nymphs, and adults). In total, 28,330 contigs were identified of which 63% (17,791) were annotatable using NCBI and UniProt databases. Data suggests that the younger instars (1^{st}-3^{rd}) are more responsive to CLas infection, since 6% of contigs were significantly (p<0.05) differentially expressed, compared to the older stages (4^{th}/5^{th} instar and adult) that 1 and 2% of contigs were significantly differentially expressed, respectively. Also, expression profiles suggest that although very few differences are apparent between younger and older nymphal stage groups (1-3 vs 4,5) (1% of contigs differentially expressed between uninfected), in the presence of CLas major differences between the nymphal stages are evident (6% of contigs differentially expressed between CLas-infected). Interestingly, Gene Ontology analyses shows that only the differentially expressed contigs of the 4^{th}/5^{th} instar stage are involved in immune system processes (GO: 0002376). Data from this study will allow for strategic stage-specific RNAi candiates to be selected and/or validated.
- During this quarter, 6 phage genes that our combined data mining approaches (transcriptome and proteome) have highlighted as key effectors, were selected for expression analysis in psyllid nymphs and adults by qPCR. Using this approach, 3 genes were further validated to be important effectors based on their differential expressional profiles between the different nymphal stages (1^{st}/2^{nd} and 4^{th}/5^{th}) and adults. One phage gene (autotransporter-like) was 3x higher expressed in early nymph compared to older nymph and adult. Another phage gene (capsid associated) was 3x higher expressed in adult compared to young and older nymphs. The final phage gene (peroxidase-like) was significantly higher expressed in the young nymph and adult compared to the older nymph. One of these phages gene have been moved into RNAi to determine if the technique can

be used to further validate these types of effectors. The second manuscript reports differential transcipt and protein profiles in gut and salivary glands libraries (essential for selection of some of our currently most promising genes). In total (from the average of two biological experiments with replicates), 353, 403, and 252 unique proteins were indentified in adult whole body, gut and salivary gland tissues, respectively. Of these, 19, 16, and 24% showed significantly different levels between Liberibacter-infected and -uninfected treatments, respectively.

- These advancements to elaborate our model of gut invasion has improved our ablity to select better candidates for reverse genetics to probe bacterial effector mechanism(s), and to facilitate better selection of single and multiple psyllid dsRNA candidates. To date, 39 RNAi experiments (with at least two replicates) have been conducted for 30 different psyllid genes (previously reported 27) with 12 (previously reported 8) of these targets showing a reduced CLso transmission in functional transmission bioassays using the single-gene and/or stacked (multiple-gene) RNAi approach and either CLso-infected (born and reared) or -uninfected psyllids (introduced to a CLso source plant). Also, RNAi of 3 (previously reported 2) of these genes have caused significant psyllid mortality, compared to untreated controls.
- During the past quarter, we've modified our 'topical' dsRNA delivery system to be consistent with the ACP/CLas system so that results from both systems can be better compared. This method allows for the exact dsRNA dose (amount and time) to be known during the delivery process, and therefore applied precisely between labs. Preliminary results indicate that RNAi via this modified topical delivery results in no significant mortality, compared to untreated controls and therefore can be an effective means of delivery.
- One manuscript, "*Colonization and intrusive invasion of potato psyllid by 'Ca. Liberibacter solanacearum*", has been revised and re-submitted (in review). It reports results of TEM/SEM studies that support the yeast two-hybrid (Y2H), co-immunoprecipitation (CoIP), and *in silico* transcriptional profile data that suggest Liberibacter uses exocytosis to exit psyllid gut cells into the hemolymph, and probably endocytosis to enter salivary glands. The paper provides the first visual evidence of endocytosis-exocytosis interactions of CLso with its psyllid vector, which support our "invasion model" in which CLas/CLso transforms the endocytic/exocytic host pathways to facilitate internalization, infection, and circulation in the psyllid host/vector.
- Two additional manuscripts are *in preparation.* The first manuscript reports our results from standard yeast two-hybrid protein-protein interactions experiments for ACP gut and salivary gland library matings (36/ea), CLas library matings (37), specific bait-prey cotransformations (82; previously reported 70) and co-immunoprecipitation (CoIP), or pull-downs (10). Findings from these additional experiments provide further support that the SC1-gp060 (Colicin 1A-like) that is known in other systems to have toxin-like activity that kills other bacteria, which is predicted from our studies to serve as an effector in the CLas/ACP system, which may operate in conjuction with a chimeric form of the ACP WAPL gene (wings apart-like protein homolog) to alter the function of ACP-clathrin light chain, ACP-clathrin heavy chain, ACP-kinesin and ACP-adaptor protein 1, all essential for cytoskeletal functioning, collectively, key effector-psyllid protein interactors in our invasion model.
- Candidate single gene products that may stop the Asian citrus psyllids ability to acquire/transmit (AcTrans) the '*Candidatus*' Liberibacter asiaticus (CLas) bacterium have been identified in functional assays. A mixture of 3 peptides (hexameric peptides) has been identified that significantly reduce the movement of the bacterium into the salivary glands. This activity was observed when the peptides were introduced orally in artificial diets fed to psyllid nymphs.

- Peptides were also identified that could prevent successful acquisition of CLas by the psyllid due to their direct effect on CLas survival. A set of peptides between 10 and 20 amino acids that have bactericidal activity when introduced into citrus leaves reduce the CLas titer by as much as 90% after 7 days within regions of the petiole/midrib of the leaf. Studies are currently underway to evaluate the effect of these peptides on CLas acquisition within the psyllid. We have characterized the activity of one of these peptides and show that it is a unique antimicrobial peptide (does not share specific motifs identified in other antimicrobial peptide classes) with activity against *Escherichia coli* in liquid culture. Furthermore, the likely targeting of protein synthesis by this peptide has been demonstrated through: a) structural modeling showing favorable interaction with *E. coli* ribosomes; effect on *in vitro* protein synthesis in *E. coli* cell-free extracts; and identification of the importance of a specific *E. coli* membrane transport protein known to be involved in peptide transport for optimal activity of this peptide.
- Single chain Antibodies targeting surface antigens on CLas have been created that interact with 12 different predicted surface epitopes. These antigens include the major outer membrane protein OmpA, two flagellar antigens, and the capsular polysaccharide synthase, and two pili components. Some of these have been expressed in transgenic citrus and others have been expressed and purified using a 6X histone tag strategy. These will be used for laboratory bioassays developed to study acquisition and CLas survival. Citrus rootstocks expressing two scFv have been made at Fort Pierce. A scFv selected to bind a surface exposed epitope of TolC = NodD (secretory pore) and a scFv selected against InvA, a protein produced by CLas believed to prevent apoptosis of infected cells. Multiple scFv selections have been introduced in citrus and multiple transformation events (~400 in all) are currently under evaluation for their effect on CLas survival in the plant, and acquisition/transmission 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 while efforts continue to identify viruses from worldwide collections of *D. citri*. The Diaphorina citri picorna-like virus (DcPLV). See Nouri, S., Salem, N., Nigg, J. C., Falk, B. W. 2016. *Diverse array of new viral sequences identified in worldwide populations of the Asian citrus psyllid (Diaphorina citri) using viral metagenomics*. J. Virology 90: 2434 – 2445). DcPLV is a leading candidate vector that might be of use for a paratransgenesis delivery system but there are others possible that are being pursued with an additional NIFA SCRI grant on virus-based RNAi approaches towards *D. citri* control. DcPLV was the first *D. citri* virus identified in this work. DcPLV has been identified in samples of *D. citri* from Taiwan, China, and Brazil but not yet from any U. S. collected *D. citri*. DcPLV is a novel insect virus with an unusual genome organization. DcPLV has a positive-sense ssRNA genome of 10,222 nucleotides and contains a single ORF coding sequence of 8,757 nucleotides. The intent remains to develop a recombinant DcPLV that can be introduced back into naïve *D. citri* however, it appears that some region(s) of the DcPLV cDNA may have toxic effects on *E.*

coli, thus additional low copy plasmids and recombinant minus *E. coli* strains are being used to continue efforts to clone the complete genome as cDNA.

- With separate SCRI funding, other *D. citri* viruses as being assessed as potential vehicles for transgenesis. These are *Diaphorina citri* densovirus (DcDNV) (Nigg, J.C., Nouri, S., and Falk, B.W. 2016. *Complete genome sequence of a putative densovirus of the Asian citrus psyllid, Diaphorina citri.* Genome Announcements, In Press). *Diaphorina citri*- associated C virus (DcACV) (Nouri, S., Salem, N., Falk, B.W. 2016. *Complete genome sequence of Diaphorina citri Associated C virus (DcACV), a novel putative RNA virus of the Asian citrus psyllid.* Genome Announcements, In Press) and *Diaphorina citri* flavi-like virus (DcFLV) (Matsumura et al., 2016, In Preparation). Unlike for DcPLV, there is evidence that these three viruses are in some, but not all, U.S. *D. citri* populations.
- Experiments are also in progress to determine if Flock House Virus (FHV, a well characterized ssRNA nodavirus with a genome organization similar to that of DcACV), can infect *D. citri*. If so, this would at least be useful for experimental studies assessing efficacy of given effectors. Additionally, infection of cells, as opposed to whole insects, may offer a rapid approach for evaluating the efficacy of paratransgenic constructs. Various established insect cell lines are being tested but a *D. citri* cell line would be very valuable for these efforts. In feeding tests there is evidence that suggests DcDNV can be orally transmitted to naïve *D. citri* and that viral sequences are retained in some proportion of subsequent *D. citri* generations. Analyses so far suggest that these sequences do not represent episomal virus, but may be integrated into the *D. citri* genome. Since densoviruses have been used in other insect systems, even for paratransgenesis, DcDNV may prove to be a good virus for our objectives.
- As previously reported, the genetic diversity of *Wolbachia* in *D. citri*, was compared across sequences of five Multilocus Sequence Typing System (MLST) alleles as well as the *wsp* gene of *Wolbachia* across samples collected worldwide. For each gene, one representative sample from each wDi profile group was selected and used for library construction. Alleles that were (1) present in two libraries derived from independent PCR reactions and (2) conformed to their original chromatogram were considered true alleles. Ten libraries were sequenced for each gene, each with ~40-48 clones. Two wDi strains (ST-1 and ST-2) were detected among the samples tested. Some populations were co-infected with multiple wDi strains.The mtCOI sequences correlated with the wDi profiles; however, mtCOI sequences of co-infected populations were different from those of singly-infected population. Our findings suggest that Wolbachia-induced cytoplasmic incompatibility may exist in *D. citri*, and that both single infection by different wDi strains and co-infection by additional strains could lead to such responses.
- As previously reported, *D. citri* was transfected with a supergroup A *Wolbachia* of *Drosophila melanogaster* (wMel). The wMel cells used in these experiments were obtained directly from *D. melanogaster* adults or extracted from *D. melanogaster* S2 cell cultures harboring wMel.s Microinjections were carried out with a FemtoJet Microinjector (Eppendorf, Inc., Fremont. CA). Adult psyllids of a *Candidatus* Liberibacter asiaticus (*C*Las)-free colony were held on *Murraya koenigii* for over 5 d, divided by gender, and subjected to microinjection. Approximately 40 individuals of both genders were used in both Experiments A and B. The injected individuals were placed on *M. koenigii* and 8-10 individuals and sampled 24 h, 5 d and 10 d post-injection. To monitor the presence and growth of wMel and wDi in *D. citri*, primers were designed that only amplify either the wMel *ftsZ* gene or the wDi *ftsZ* gene. qPCR assays were used to determine whether wMel can colonize *D. citri*, and whether its presence could influence the growth of wDi. Native *Wolbachia* infections in adults and nymphs have been reduced for use in co-infection experiments. Due to the low

number of individuals (5 per ca. 100 microinjections) produced, establishing co-infected isolines for use in experiments is ongoing.

- Our goal is also to develop a chromosomal gene drive system for population replacement in the psyllid. Several chromosome translocation-based drive elements have been generated in Drosophila. To generate a chromosomal driver, efforts continue to streamline the process for generating insects that carry reciprocal translocations. As discussed in previous reports, there has been some success in generating translocations using a strategy in which transgene insertions are first created on two different chromosomes, and recombination between them follows induction of double-stranded DNA breaks on each of these sites. While this procedure works, it requires a number of steps and crosses. It may be possible to simplify the process by creating a translocation directly in the germline of an injected embryo. This would allow generation these rearrangements much more directly, at many different sites.
- In addition to the above, work continues on achieving transgenesis at other life stages, such as the nymph and the adult using some newly published gene delivery agents along with more traditional ones such as jetPrime and other related lipofection reagents.
- Publications describing the high threshold gene drive using translocations, in Drosophila are in preparation. Discussions continue JPL collaborators on the custom gene gun ideas for insect transformation. This is likely to be timed for an official DARPA funding call (probably sometime this summer) in order to approach them for funding.

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.
- The development of 'super-stimuli' which are strong behavioral elicitors, may provide a means of boosting the efficacy of synthetic attractants by enabling them to outcompete

background stimuli. Plant pathogens elicit the production of super stimuli in their host plants to make infected plants more attractive to insect vectors; examination of pathosystems may reveal the identity of potentially useful super-stimuli. Of significance to the Las-ACP-citrus pathosystem, Dr. Lukasz Stelinski (UF) and his associates have shown that Las-infected foliage emits the volatile signaling compound methyl salicylate, and that it acts as a super-stimulus in attracting uninfected ACP to Las-infected trees. The emission of methyl salicylate (MeSA) is governed by the production of salicylic acid (SA), an internal signaler that is induced by pathogen infection. The Stelinski and Sétamou (TAMU) labs are developing scent attractants containing methyl salicylate.

- Another important attack/stressor signaler system in plants is the jasmonic acid/methyl jasmonate (MeJA) system. Exogenous application of MeJA to potted Las- and Las+ Valencia orange trees significantly altered volatile emission both quantitatively and qualitatively. In behavioral assays, ACP significantly aggregated at higher levels of MeJA-treated foliage.
- Exogenous applications of salicylic acid on volatile emission in Las- and Las+ Valencia trees are being tested. The data collected so far indicate that SA-treated trees: 1) Emit a quantitatively greater amount of volatiles; 2) Produce high levels of MeSA, with this compound comprising 50% of the total amount of volatiles emitted; and 3) Do not emit indole, E-jasmone and other compounds induced by the application of MeJA.
- Studies will soon be underway to: 1) Determine the effect of SA- and MeJA-treatment on ACP colonization; 2) Identify the volatiles emitted by SA- and MeJA-treated foliage that influence ACP behavior; 3) Develop ACP scent attractants based on SA- and MeJA-treated foliage.
- A paper outlining the conditions required for successful adoption of gene-drive technology for control of agricultural pest species (such as ACP) was submitted to the Journal of Responsible Adoption by Paul Mitchell (UW Madison, team member), McRoberts and Zach Brown from NC State.
- Senior analyst from the McRoberts lab, Carla Thomas, interviewed USDA-APHIS field response personnel in Southern California about factors affecting the success of regulatory programs. A follow up set of interviews has been arranged for later in the summer of 2016 to finalize the transfer of information from the USDA-APHIS field team to the nuPsyllid modeling team.
- The outreach team conducts monthly teleconference calls to create a list of ACP and HLB funded projects to select projects that represent insect, plant and bacterial solutions to the HLB problem. The team will interview the researchers, and develop grower-level explanations of the research for the 'Science for Citrus Health' website.

SUMMARY

There are a number of excellent candidate effector targets including several identified in a functional screen. 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 and be prepared to use others that might 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 (either transient CTV viral vectors expressing psyllid-targeted RNAi or small molecule sprays);
- engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB.