Citrus Research and Development Foundation, Inc.

MEMORANDUM

TO: nuPsyllid Management
FROM: Tom Turpen, Project Director
DATE: August 30, 2013
RE: Annual 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 already begun and will be focused on technical feasibility and on the probability of having an impact on disease control.

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, peptide and scFV libraries.

- Two de novo assemblies of the ACP transcriptome (DcWN-adult/nymph and DcGS-gut/salivary glands) were created from eight Illumina paired-end libraries which were constructed from pooled psyllids: 1) whole bodies, 2) guts, and 3) salivary glands of the uninfected adult ACP; 4) whole bodies, 5) guts, and 6) salivary glands from CLas-infected adult ACP, 7) uninfected ACP nymphs, and 8) CLas-infected ACP nymphs. Each ACP library was sequenced separately. After processing, the DcWN transcript set resulted in a total of 129,631,904 cleaned reads for (21,552,866 reads

from adult, 46,865,913 reads from CLas-infected adult, 32,265,958 reads from nymph and 28,947,167 reads from CLas-infected nymph) which were assembled into contiguous sequences (contigs), yielding a total of 45,976 consensus sequences (transcripts) with a total combined size of 50.7 Mb and an average length of 1,107 bp (150 to 26,540 bp). Of the 45,976 total transcripts, 17,598 (39%) could be annotated. The annotated transcripts comprised 35.6 Mb of sequence with an average transcript length of 1,980 bp. The average E-value for the annotated transcripts was 7.52E-13, varying from 0 to 1E-10. Approximately 17% the annotated sequences were similar to genes found in *Acyrthosiphon pisum*. After processing, the DcGS transcript set resulted in a total of 572,290,348 cleaned reads for the DcWN transcript set (44,758,331reads from gut, 30,395,320reads from CLas-infected ACP gut, 244,366,344 reads from ACP salivary glands and 252,770,353 reads from CLas-infected ACP salivary glands) which were assembled into contiguous sequences (contigs), yielding a total of 83,231consensus sequences (transcripts) with an average transcript length of 697 bp. Of the 83,231 total transcripts, 26,511 (32%) could be annotated. The transcripts from the ACP transcriptome (DcWN set) were combined with the transcripts from the similarly constructed potato psyllid transcriptome (BcWN) to form a multi-taxon transcriptome set which can be used for comparison analysis. Coding regions were identified in 57% ACP and 41% of potato psyllid (POP) transcript sequences using ESTScan and these were subjected to clustering analysis using the OrthoMCL algorithm with default parameters. Of the combined proteins from both species (60,085 in total), 67% were assigned to 8,721 orthologous groups with sizes ranging from 2 to 285 transcripts with 70% ACP and 64% POP proteins placed in orthologous groups. Thorough analysis of the transcripts from all database sets (DcWN, DcGS, and DcBcWN) is paramount in the identification of target effectors to combat Huanglongbing disease. The transcriptomes were annotated and compiled into an extensive database www.sohomoptera.org/psyllid at which multiple tools for data mining have been developed and implemented for detailed data mining, the Transcriptome Computational Workbench (Soderlund *et al*., in prep).

- Yeast-2 hybrid studies were initiated to study protein-protein interactions important in psyllid-Liberibacter interactions involved in the circulative, propagative pathway. Three cDNA libraries: uninfected gut, uninfected salivary gland and infected bacteria were made. Also three Mate & Plate “prey” libraries (uninfected gut, uninfected salivary gland and infected bacteria) were made. The Illumina transcripts in the ACP transcriptome were used as the source for identifying target psyllid effectors. The CLas genes in NCBI database (GI: 34231609) along with CLas “contaminates” detected in the ACP transcriptome, are sources for identifying target bacterial effectors. Previously it was reported that a total of 9 CLas candidate genes from a list of 19, had been moved into the Yeast 2 Hybrid (Y2H) mating experiments using the ACP gut library and the ACP salivary gland library. To date 12 gut library matings and 12 salivary gland library matings have been performed. Data analysis has been completed for 20 of those experiments with the remaining being in various stages (PCR, cloning, sequencing, etc.) moving towards completion. Through those 20 experiments we have thus far discovered roughly 44 ACP gene products (“prey”) that have high levels of interest for making them good candidates for RNAi knock down and will be moved into the RNAi phase of the project. Previously we reported the findings from the first 3 mating projects (1 midgut and 2 salivary glands) which has resulted in the identification of 4 candidate effectors (ACP gene products) that have been moved into the RNAi phase of the project. These were a putative transporter and fibronectin-attachment protein both of which may play roles in adhesion and/or

invasion, and the latter gene could be important to extracellular matrix formation by bacteria. The other ACP gene products discovered from “prey” inserts were a defense-related protein and a multi-domain protein with one domain predicted to be involved activation of the innate immune response. We have discovered more interesting candidate effectors (~40) that will be moved into the RNAi phase of the project many of which are predicted to be important in Liberibacter adherence to host tissues. One interesting candidate is an endopeptidase putatively involved in the dissolution of extracellular matrices. The knockdown of this ACP gene function would actually be of benefit to CLas so RNAi would be used to validate its function, but the overexpression of this gene could greatly interfere with CLas transmission. An ACP gene product similar to enzymes involved in signaling cascades important for bacterial communication and subsequent biofilm formation was also identified. A CLas “bait” with protease function, identified an ACP gene important for bacterial nutrition and is made available to the bacteria after processing. The “processing” makes this protease a virulence factor by destroying host resources so they are readily accessible to the pathogenic bacteria for growth and colonization within its host. An interesting finding came from using the CLas “bait” FlgL protein. No interactors have yet been discovered from the small number of gut matings attempted, only from salivary gland mating experiments, which suggests the motility of Liberibacter is most important in this tissue where inoculation (to plant) and acquitistion (from plant) take place. The interacting ACP gene products (“prey” inserts) identified from are currently being analyzed. Matings with the gut and salivary gland libraries continues. However the majority of current and future Y2H work is concentrated on using ACP gene candidates as “bait” in matings with the CLas library. To date, one candidate ACP gene putatively involved in bacterial adhesion has been mated against the CLas prey library. Several more gene candidates have been identified and are currently in various stages of being cloned, with subsequent mating experiments soon to follow.

- To date, good quality dsRNA has been synthesized using the MEGAscript RNAi kit for five psyllid genes predicted to be involved in cytoskeleton formation, defense response, vesicle transport, or transcytosis. Previously we reported on the knockdown of one of the cytoskeleton-related genes on Liberibacter transmission using the oral delivery method. Results showed that Liberibacter transmission was reduced by 18%. Recently, both microinjection and feeding studies have been conducted for actin, the other cytoskeleton-related gene whose knockdown is expected to result in high psyllid mortality and is used as a control to optimize our studies. Preliminary data from mortality assays suggest that the method of dsRNA delivery is crucial with results differing between delivery methods, and should be optimized accordingly (Tables 4 and 5). RNAi experiments for functional characterization will continue using both methods of delivery with the effectors identified in the Y2H screens as well as thought candidates identified using the transcriptome databases.

- From a large expression library screen, a set of 5 single chain antibody fragments (scFv) were identified and shown to bind to specific surface antigens of CLas. Bulk purification of the scFv antibodies is in progress under conditions required to maintain scFV binding.
- Construction of a peptide library was initiated to identify candidate effectors in a binding inhibition assay using ACP alimentary canal membrane preparations. Peptides in the library contain 4-10 amino acids, a double glycine linker and a biotin label to enable use of streptavidin-fluorescent tags.

- CLas positive citrus was used to develop an enrichment protocol for obtaining extracts from citrus that are enriched with the CLas bacterium. It was observed that the petiole and proximal leaf midrib region where enriched for CLas. Based on this observation, extracts were prepared from this region of infected leaves. A psyllid/CLas acquisition/transmission bioassay was developed by combining artificial diet feeding of this extract to psyllids followed by a single leaf transmission bioassay developed specifically for psyllid transmission studies. This assay is completed in less than 3 weeks and samples are now being analyzed for transmission using PCR methods. An initial experiment showed the detection of transmitted CLas in the excised leaves.
- A plant-based screening protocol was developed for monitoring psyllid transmission that allows full ACP life-stage development in excised plant flush. dsRNAs are delivered to plants through use of a transient expression vector (CTV). After infected ACP populations are exposed to dsRNAs that target various ACP genes by feeding *in planta*, the survivors were found to be free of CLas. The adults that emerged from nymphs, that developed on these plants from eggs laid by the original CLas positive adults, also were devoid of CLas. In controls, 15% to 20% of these newly emerged psyllids typically tested positive for CLas.

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.

- Three viruses of ACP were discovered by deep sequencing and bioinformatic analysis of small RNA libraries from diverse ACP collections. These viruses include a Reovirus (dsRNA), an Iflavirus (+sense, ssRNA) and a Densovirus (ssDNA). RNA libraries include samples from China, Taiwan, Brazil, Pakistan and Florida and will include Texas and California. A virus-free ACP colony was established for biological assays to evaluate these ACP pathogens as potential drivers. A virus-free *D. citri* colony has been established at UC Davis.

- *Wolbachia* was found to be present in all collections of ACP from 20 different Florida sites with infection frequencies of 86-96%. This population diversity has been charactierized via multi locus sequence typing (MLST) and a quantitative real-time (qPCR) assay have been developed to assess Wolbachia infection rates.
- Two isofemale lines were confirmed to be free of *Wolbachia.* These lines remain unstable, probably due to population bottle-necking. Additionally, antibiotic treatments are being used to clear ACP nymphs and adults of *Wolbachia* infection.
- Two virulent strains of *Wolbachia* have been selected for introduction into *Wolbachia*-cleared psyllid cultures via direct hemolymph transfer or cell culture transfection by microinjection.

- Chromosomal drivers were investigated in the model genetic system *Drosophila* where an invasive system modeled on the naturally occurring *Medea* element has been established. A feature that may be required for release of transgenic insects for some applications is the ability to test the release in the field at a threshold below that required for population replacement. Transgenes would spread to fixation under high

release threshold conditions and would be lost under low release threshold conditions. This concept was confirmed in a synthetic system targeting a known haplolethal gene.

- The focus now is on two-locus underdominance, a lower threshold system. In two-locus underdominance each of two nonhomologous chromosomes (A and B) carry toxin-antidote pairs in which the toxin present on one chromosome (Toxin 1) is linked to an antidote (Antidote 2) that represses Toxin 2. Toxin 2 is located on a nonhomologous chromosome, linked with Antidote 1, which represses Toxin 1. In such a system, organisms can only survive if they carry A and B chromosomes (in A/+;B+, A/A;B+, A/+;B/B, or A/A;B/B individuals), or only wild type (+) chromosomes (in +/+ individuals). 2-locus underdominance gene drive systems show an introduction threshold of 26% even in the absence of other element-associated fitness costs. This occurs because when underdominant A/B individuals are rare, they often mate with nontransgenics (wild type) and rarely with A/B, resulting in the production of many individuals that carry A or B, but not both. In this regime, the underdominant chromosomes are eliminated from the population. Conversely, once the frequency of A/B individuals surpasses a critical threshold, matings with other A/B individuals are common, while matings with wild type are rare. In this regime the underdominant chromosomes spread at the expense of wild types, resulting in the complete loss of wild type individuals from the population. Transgenes can be removed from the wild population, simply by diluting the replaced population with wild types such that the frequency of transgenics falls below ~26%. Second, transgenics are very unlikely to establish themselves at high frequency in areas linked to the source population by hitchhiking because the frequency of underdominant chromosomes will typically be less than the critical introduction threshold.

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 are of immediate use in HLB disease management applications outside of this proposal.

- Scented lures for trapping ACP are in field trials in Southern California. It appears that ACP has a similar market behavior to humans, developing a preference for what they were raised on. A similar study will be initiated in Puerto Rico in Sept.

SUMMARY

All 8 nuPsyllid Team Leaders and all 4 Scientific and Regulatory Advisors met in person on April 11 and 12th in Riverside, CA at a first Annual Meeting together with MaryLou Polek CO-PD and Tom Turpen, PD. The crux of the technical challenge is to create a driver system that will function as predicted. There is more optimism that effector mechanisms can be developed, although this must continue to be tested concurrently as planned. At this point, we do not underestimate social barriers to adoption of the technology but there is no story to communicate without a nuPsyllid and the story is subtly different depending on the

technology. All aspects of the project are proceeding as proposed, reviewed and funded. However, a primary purpose of our meeting was to identify what can be done to maximize the success. Based on the presentations and discussions during and subsequent the Annual Meeting, we have focused effort in the following areas:

- Develop and review strategies to achieving psyllid transformation
- Improve our ability to identify the phenotype we are looking for…maximize transmission blockage and minimize fitness loss
- Begin to model the complex system of release and monitoring around hypothetical specifications and explicit assumptions

Based on the foregoing analysis, CRDF approved a supplemental budget and contract extension at their Board meeting on Aug. 27th that will enhance the work plan for psyllid transformation developed by Al Handler, with Team Leaders Bruce Hay, Kirsten Pelz-Stelinski.

There are no invention disclosures reported in YR1 of the project.

These results were presented to the nuPsyllid Stakeholder Advisory Group and to Mary Lou Polek, Administrative Team Co-Director in their Annual Meeting on August 26th, in Lake Alfred, FL by Team Leaders, Bob Shatters, Joe Patt and Tom Turpen, Project Director.