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 June 30, 2014

PROJECT DIRECTOR SUMMARY

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 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 assess the minimum field trial plot size required to demonstrate efficacy in protecting new solid block plantings from HLB.

An Annual Meeting of Team Leaders, Scientific and Regulatory Advisors was held at the Dallas Fort Worth Airport on May 12th and 13th. The team discussed revisions to the current technical plan based on the progress outlined below, as well as budget to plan variances. MaryLou Polek and Tom Turpen provided an update on current HLB conditions. The team is planning for a larger Annual meeting that will include all researchers associated with the project and has proposed a day just in advance of the upcoming HLB International conference in Orlando, Feb. 9-13, 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 and scFV libraries.

- There is a growing list of candidate effectors generated from bioinformatics (proteomic and transcriptomic), genetic (yeast two-hybrid) and physical methods (Far-Westerns). 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 or peptide fragments). Secondary metabolites are a potential third option. In order to use an Effector for insect replacement, we need to disrupt these interactions while maintaining psyllid fitness. Two CLasY2H candidates likely involved in adhesion and quorum sensing were selected to optimize immunoprecipitation (IP) and Co-IP assays because of the high number of ACP interactors that were identified from them in Y2H assays. It is anticipated that the approach will yield several native interacting proteins for mass spectrometry identification following confirmation by western analysis.

- A system for screening peptide libraries for protein ligand inhibitors was established based on the elements listed below. These results led to the decision to use 4-amino acid peptide libraries for the screening program. These libraries are now made and the screening protocol is being initiated.
    - Citrus flush takes up peptides in xylem and phloem when leaf petioles are placed in solutions containing the peptides. About 20% of the psyllids (nymphs or adults) that have fed on these leaves take up these peptides and these peptides can be detected throughout the alimentary canal. Previous work on Asian citrus psyllid feeding has shown that about 20% of the time they feed on the xylem.
    - The assay is based on feeding young nymphs on leaves placed in the peptide solution and then removing these nymphs in the fourth or fifth instar stage and placing them on citrus leaves that test positive for CLas. Once these insects have emerged as adults they are transferred to uninfected leaves in a single leaf transmission assay using a published PCR-based method.
    - Current results show that nymphal psyllids can ingest CLas and transmit as adults indicating that adults have acquired CLas.
    - Peptide combinatorial biotinylated libraries varying in length from between 4 and 10 amino acids (plus the double gly tag spacer) were tested to determine the optimal length of the library to screen.
    - Psyllids fed on artificial diet containing either 4, 5 or 8 random amino acid chain length libraries were >60% positive for peptide binding to the alimentary canal membrane.
    - By reducing the biotin concentration and increasing the wash stringency, 0% binding in the negative controls was obtained and the tetrapeptide libraries were chose for further experiments.
    - To date 80 biotinylated tetrapeptide libraries have been screened where in each library one of the four positions was locked to any one of the twenty possible amino acids and preferential binding to homogenized gut membranes was quantified by fluorescence emission.

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 from 18 locations around the world. These viruses include a Reovirus (dsRNA), two Iflavirus isolates (+sense, ssRNA) and a Densovirus (ssDNA). Because the Iflaviruses are most readily manipulated as a gene vector, efforts are now focused on obtaining the full genome sequence.
- Sequence data generated to date yields most of the expected genomes for the Brazil (~8,000 nt) and China (~7,000 nt) *D.citri Iflavirus* isolates. (Known iflavirus genomes range in size from 8,800 to 10,000nt). These two virus isolates differ slightly in nucleotide sequence but are the same virus species. However, they are distinct from all other known iflavirus sequences (only ~35% identify at the amino acid level) that are available for comparison. Based on NGS sequence data, RT-PCR followed by Sanger sequencing, 5' RACE and 3' RACE, sequences are available for ~85% and 75 % of the genomes for the Brazil and China iflaviruses, respectively.
- To date, 7,956 nt of genome sequence of the *Diaphorina citri iflavirus* strain Brazil (DcIV-BZ) and 7,350 nt of the *Diaphorina citri iflavirus* strain China (DcIV-CH) have been generated. Efforts are now focused on identifying and confirming the 5' termini of each of the genomic RNAs.
- There are significant regional differences in *Wolbachia* infection levels among Florida psyllid populations assessed using multi locus sequence typing (MLST). All investigated D. citri populations harbor wDi from supergroup A. Several unique *Wolbachia* wDi genotypes were identified using five gene loci (coxA, ftsZ, fbpA, hcpA and wsp) in different D. citri populations from Florida. The genetic variation of the gene cytochrome c oxidase subunit 1 (coxA) was notably distinct among wDi populations.
- There are a total of 12 different genotypes of *Wolbachia* in Florida with a decreasing diversity from north to south. Initial phylogenetic analyses of all gene loci indicate there are at least three unique sequence types (ST) of *Wolbachia*, based on genetic differences in coxA, fbpA and wsp genes.
- An artificial feeding system has been developed to introduce *Wolbachia* into ACP for the purpose of screening psyllids for vector and host competence. Inoculation of ACP with new *Wolbachia* infections must occur during the nymphal stage in order for the infection to establish and be maintained in the insect.
- Cell cultures of several *Wolbachia* strains, including several that normally infect various Hemipteran species, *Drosophila melanogaster* and the Asian tiger mosquito, *Aedes albopictus* are established. Novel *Wolbachia* strains have been introduced into ACP at the nymphal stage. Infected adults will be mated so that progeny can be evaluated for subsequent transmission, CI, and fitness assays. Using this system, foreign *Wolbachia* infections in ACP have been confirmed in adult ACP via PCR.
- In a model system, the toxin-antidote systems for haplolethals and reciprocal chromosomal translocation underdominance continue to be improved by tuning expression of the respective genes. The challenge with the haplolethal system is in maintaining fitness of the homozygotes.
- Proof of concept has been established with the translocation drive mechanism through precise engineering of the translocation sites. This system shows great potential for ACP-HLB control because it is a high introduction threshold mechanism

robust to mutations anticipated to inactivate drive while genes of interested can be easily linked to the translocation breakpoint.

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.

- Dr. Tim Gottwald and Dr. Jed Kessling and co-workers have developed modeling tools useful for the prediction of the spread of nuPsyllid within an existing wild type population and for the performance of the Psyllid Shield trials.
- There is a substantial effort to rear and release any type of nuPsyllid under development. This group has organized a set of key questions for an upcoming conference call discussion:
    - Will nuPsyllid rearing efforts will be piggybacked onto existing rearing programs for the ACP parasitic wasp, *Tamarixia* or be conducted at separate locations?
    - Will initial field tests be conducted in Florida, Texas, and California? Where will the nuPsyllids for these tests be reared; i.e., at a single facility in each state (Florida, Texas, and California) or at a single facility for distribution nationwide? How will it be decided where the nuPsyllids be reared?
    - What is a realistic estimate of the numbers of nuPsyllids that will need to be produced for initial field tests? Is it realistic to assume that there will be no fitness costs to nuPsyllids in terms of rearing efforts?
    - Will nuPsyllid be required to be reared in secured/quarantine level facilities? Are there other regulatory concerns foreseen at this point?
- The Outreach Team is planning a questionnaire on GMO and nuPsyllid concepts for use with a PowerPoint presentation and electronic quizzing. This will be used initially to assess knowledge and acceptance of the technology with growers.

SUMMARY

Because of the progress with the effector characterization and viral drivers, it is an important time for the team to continue to:

- select and prioritize effectors;
- obtain antibody reagents for top effector candidates;
- determine a 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;
- 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;
- 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).