Citrus Research and Development Foundation, Inc.

MEMORANDUM

TO: nuPsyllid Management
FROM: Tom Turpen, Project Director
DATE: January 15, 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 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.

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.

- On December 12, 2013 an Effector Workshop was held at the USDA-ARS laboratories in Ft. Pierce to evaluate progress. Participating were Tom Turpen, Project Director, Team Leaders Bob Shatters and Judy Brown, as well as important research contributors who are supported through industry matching funds, Nabil Killiny and Michelle Cilia, and El-Desouky Ammar. Project Co-Director, MaryLou Polek joined telephonically at the end of the day. There is a growing list of candidate

effectors generated from bioinformatics (proteomic and transcriptomic), genetic (yeast two-hybrid) and physical methods (Far-Westerns). 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). In order to use an Effector for insect replacement, we need to disrupt these interactions while maintaining psyllid fitness.

- RNAi can induce dramatic phenotypes in psyllids when delivered, topically, orally, by injection or by ingestion of RNAi delivered to the phloem with a viral vector built from Citrus Tristeza Virus. The Effector Team discussed early application of RNAi for the Psyllid Shield concept for interim use until full insect replacement can be achieved. Regulatory Advisor Jim White will attend the upcoming EPA hearing on RNAi.

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), 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. These viruses were discovered in samples from China and Brazil. Efforts are increasing to find additional isolates in the US.
- Efforts to culture *Wolbachia* and other insect endosymbionts and to propagate psyllids on artificial media continue. Additionally, antibiotic treatments are being used to clear ACP nymphs and adults of *Wolbachia* infection. ACP population diversity has been characterized via multi locus sequence typing (MLST) and a quantitative real-time (qPCR) assay has been developed to assess *Wolbachia* infection rates. Project Director Tom Turpen and Project Co-Director Jackie Burns met with Team Leader, Kirsten Pelz-Stelinski and Paul Shirk as well as additional members of their laboratories to coordinate options for testing combinations of *Wolbachia* and ACP for phenotypic effects and to assess the ability to possibly transform *Wolbachia*.
- The research effort required to transform ACP was enhanced with further support from CRDF to the laboratory of Dr. Al Handler. Progress with chromosomal drivers will rely on the ability to transform ACP. In a model system, the toxin-antidote systems for haplolethals and two-locus underdominance continue to be improved by tuning expression of the respective genes. An additional and robust system of underdominance via chromosomal translocation has been constructed and is being tested for drive against wildtype.

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.

- nuPsyllid Rear, Release and Monitor Team Leader Joe Patt will meet with those involved with the logistics of rearing and releasing psyllid populations.
- The Outreach Team has established a communication forum using the UC-ANR system of collaborative tools.
- A degree-day ACP phenology model has been created and can be driven from real-time weather data from a network of thousands of weather stations across the USA. Work is in progress to tie this thermal time model to flush events for different citrus species so that the real-time dynamics of ACP populations in different locations can be modeled.
- A predictive analysis of probability to reject nuPsyllid technology for California has been developed in collaboration with Dr. Tim Gottwald's team within the GIS platform which they have developed in collaboration with UC Davis for predicting ACP/HLB risk in urban districts of California.

SUMMARY

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

- select effectors;
- 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;
- determine if *Wolbachia* transformation is a feasible goal;
- begin to model the logistics of rearing and releasing nuPsyllid around hypothetical specifications and explicit assumptions

In addition, we have organized a small group of volunteers including selected nuPsyllid Team Leaders to critique the prospects for conducting the following Psyllid Shield field trial to protect new citrus plantings from HLB. Based on results with RNAi, we appear to have the capacity to use CTV to inoculate young trees with RNAi that will not only kill psyllids but also reduce or eliminate the ability of any escape progeny that might fully develop into adults on those Psyllid Shield protected trees to vector disease. (There may be effects on blocking infection of the Psyllid Shield protected tree as well, though this has not been analyzed carefully yet). A partial list of the most relevant observations:

- In uninfected solid blocks, the pattern of disease spread is predominately but not completely from the borders.

- Any infected tree is most likely to have been inoculated by an adult that 1) acquired CLas as a developing nymph in infected flush, 2) acquired it from a nearby tree, or 3) acquired it before that tree was systemically infected, i.e. it was acquired locally.
- Several years of RNAi target evaluations have yielded a variety of phenotypes that warrant further consideration for field use. More constructs are in the pipeline and the following are in hand:
    - RNAi that kills adults and reduces fecundity in any survivors
    - RNAi that kills adults and substantially immobilizes any survivors
    - RNAi that kills adults and eliminates CLas from any survivors
- CTV can be used to inoculate trees and express RNAi and other sequences with remarkable stability.

As a management strategy, this intervention would be "stacked" with all others in practice, including area-wide psyllid control, systemic insecticides (neonics) for young trees and the release of biological control agents in non-commercial and organic environments. Any psyllids that made it through to the border (or interior) of a new solid block planting and "reared and released" on those trees that are protected by Psyllid Shield would be severely impaired in the ability to subsequently vector disease. From a variety of sources, in addition to the NIFA-CAPS program, we have substantial infrastructure in place for monitoring and modeling vector control solutions and communicating those technology alternatives to the industry both to regulators and to the public.

The trial should establish both whether the technology is useful and whether we have a mathematical model predictive of disease spread. We need a good estimate of the minimal size of an experimental plot sufficient to see a beneficial effect in order to plan the trial. We also need to validate the model of disease spread so we can further predict with some confidence the minimum size for effective commercial production based on the results of the trial.

The transmission model should predict spread in uninfected blocks of citrus with and without Psyllid Shield as a function of time, proximity to infected psyllids (frequency of inoculation), size of the plot, effectiveness of Psyllid Shield and every other relevant parameter of psyllid biology, host and the weather that can be combined to improve accuracy of the model. Effectiveness of the Psyllid Shield includes: reduction of transmission to Psyllid Shield protected trees (if any, perhaps we could find an RNAi-induced anti-feedant behavior as strong as imidacloprid?), fraction of psyllids killed on Psyllid Shield protected trees, fraction of psyllids survivors that are capable of transmission after having fed on Psyllid Shield protected trees. For example, in a limiting condition we should be able to see the infection patterns resulting from stochastic output of the model over 5 years, where there is simply no transmission at all from Psyllid Shield protected trees because 90% are killed and the rest have negligible ability to vector disease. The only infected trees will be relatively rare events from long distance psyllid movement to the interior of the planting and on the borders. The transmission events that occur in the interior will generate low numbers of psyllids, essentially incompetent for further transmission. The trees on the border may become infected but as long as they express RNAi they will continue to reduce psyllid populations and maintain a buffer zone of citrus from which any infected psyllids cannot effectively acquire and transmit CLas.