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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 February 28, 2015

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 solid block plantings from HLB. This concept "Psyllid Shield" is being evaluated for field trials to demonstrate efficacy in protecting. 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. This work laid the foundation for a more comprehensive and systematic approach recently funded through the NIFA CDREE from Project Director, Bryce Falk a Team Leader and PI of this project.

A large team Annual meeting was held on Feb. 8th in advance of the HLB International conference in Orlando, FL. This was a great opportunity for the Driver and Effector teams, who are primarily involved in attempting isolate or build a nuPsyllid colony to present the status and results to those involved in the ultimate downstream release and adoption of this technology. The entire group of participants and stakeholders used a risk matrix methodology to rank whether they perceived any of the three driver systems were most likely to be implemented. Because there is no compelling winner (or loser) from this analysis, we are continuing with the concurrent work plan originally proposed with respect to the Driver and Effector teams. A draft update to the project objectives and budget is in progress.

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 set (the Transcriptome Computational Workbench) has been created from whole adults and nymphs as well as dissected salivary glands and guts of insects infected or not infected with CLas is available to the research community at www.sohomopter.org/ACPPOP.
- Using the yeast two-hybrid method, gut and salivary gland library matings (30/ea) have been performed and the results reinforce the likely role of several complexes and individual proteins identified in the level 2 biological processes of the Gene Ontology categories of Adhesion, Nutrition, Invasion and Immune functions. An interaction between a potential CLas virulence factor and a psyllid signaling molecule that regulates fundamental cellular processes, including cell growth, migration and survival was identified as well as an interaction between a psyllid and *Wolbachia*-associated protein that indicates the potential importance of tripartite interactions for pathogen invasion. A CLas phage protein and an ACP protein predicted to be important in CLas invasion of host tissues contains two putative domains; a coat protein and a highly conserved known virulence factor. RNAi knockdowns studies for 16 candidates are in progress while at least 5 of these targets show a significant reduction in CLas transmission in a functional transmission bioassay to date. Expression is currently being confirmed for these and other effector candidates by mass spectrometry analysis.
- A system for screening 4-amino acid peptide libraries for binding to psyllid digestive tract epithelium has identified at least 8 candidate ligands that bind specifically at submicromolar concentrations with different binding kinetics. Optimized acquisition assays have been developed and validated and are now being used to test for peptide inhibition of CLas acquisition. In this assay, acquisition is measured (using PCR detection of CLas) as an inhibition of movement of the bacterium into the salivary glands and/or an overall reduction in the titer of CLas in the psyllids.
- Sixteen different scFVs were identified that bind to three separate surface antigens on CLas and the genes encoding these scFVs have been transformed into citrus. 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.

- Viruses infecting psyllids were found by deep sequencing and bioinformatic analysis of small RNA libraries from over 30 diverse ACP collections from 18 locations around the world. These viruses include a Reovirus (dsRNA), two *Iflavirus or Picorna*-like isolates (+sense, ssRNA) and a Densovirus (ssDNA) and possibly others.
- Now that a virus that looks amendable to use as a driver and gene vector has been identified, the primary objective will be to develop the *Diaphorina citri picorna*-like virus (DCPLV) into a recombinant vector system to be implemented for reverse genetics and to understand some of the biology of DCPLV.
- Isofemale lines carrying different *Wolbachia* types are being expanded and evaluated. In addition to Florida populations, ACPs from outside of Florida, including as Texas, California and Hawaii are being sampled. Preliminary results indicate that high prevalence of ACPs from one of such populations (located in Hawaii) carry a *Wolbachia* strain that is not found in Florida populations. The process of obtaining a permit from USDA AHPIS to import live psyllids to Florida has been initiated. Isofemale breeding lines will enable laboratory evaluations of Las transmission, life history, and cytoplasmic incompatibility.
- 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.

- 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. An abstract of the work was submitted to the Agriculture and Applied Economics Association meeting in San Francisco, July 26-28, 2015.
- The Outreach Team has determined that 1) because the effector and driver systems are all progressing equally it will not be possible to eliminate one or more from the outreach efforts and 2) an educational program should focus on the context of genetic technologies in general so that the nuPsyllid option for disease management is contrasted for example with a genetically modified citrus host and other technology options.
- Together with molecular biologist and extension expert Peggy Lemaux, PowerPoint presentation materials have been drafted and are being reviewed with the extension group.

SUMMARY

Review of the data obtained to date supports an “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 candidate 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.