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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 March 31, 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.

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). Draft manuscripts on the transcriptome analysis are ready for publication and public release of the data. 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.

- 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. Several RNAi candidates are being evaluated for the Psyllid Shield concept for interim use until full insect replacement can be achieved.
- 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 PCR-based method that was previously published.
    - 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. Whereas, less than 5% of the psyllids that fed on any of the negative controls (diet containing water, biotin, or 2 or 4 specific amino acid peptides) had alimentary canals that tested positive for the presence of peptides.
    - By reducing the biotin concentration and increasing the wash stringency, 0% binding in the negative controls was obtained.

- Regulatory Advisor Jim White attended a FIFRA Scientific Advisory Panel meeting at the EPA entitled “RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment.”

Related Applications

The efficacy of RNAi suggests 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. Transient viral vectors based on CTV or other suitable constructs would be used to inoculate solid blocks and deliver 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, Taiwan and Brazil but not yet confirmed in populations from Florida, Texas or California. Efforts are increasing to find additional isolates in the US.
- 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.
- Psyllid *Wolbachia* is now available in two insect cell culture lines as well as additional *Wolbachia* strains isolated from other insects (including *Drosophila*). Cell cultures are being used as a model for determining whether the psyllid *Wolbachia* can co-exist or be replaced by other *Wolbachia* strains prior to CI and transmission assays in psyllids.
- An artificial feeding system has been developed to artificially infect *Wolbachia* +/- psyllids with *Liberibacter* for the purpose of a) determining when and where the infection process occurs and b) developing an efficient method for screening psyllids for vector and host competence.
- There are significant regional differences in *Wolbachia* infection levels among Florida psyllid populations assessed using multi locus sequence typing (MLST).
- 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. Important control experiments demonstrated that fitness costs observed previously are a consequence of the selected chromosomal docking site or marker gene inserted at this site.

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.
- 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 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