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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 November 30, 2016**

**PROJECT DIRECTOR SUMMARY**

**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. This research has established a broad foundational knowledge base of molecular interactions between host, pathogen and vector that is now contributing to additional NIFA-funded programs. Part of our outreach in the final phase of this program will be to integrate our progress with others focused on the HLB challenge.

To successfully use an Effector for insect replacement, we need to disrupt interactions required for the spread of HLB while adequately maintaining psyllid fitness. New actives discovered in this program that are specifically toxic to psyllids may be used for novel insect suppression technologies. While this is not the proposed population replacement, if genetic or other modes of conditional delivery can be developed then new forms of biological control will be feasible. For example, these assessments have suggested a near term application of this research for the protection of new solid block plantings from HLB. We continue to evaluate the “Psyllid Shield” control strategy. 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. A key stakeholder partner identified by CRDF is investing in regulatory approvals necessary for field trials of this disease management concept.

Our team has updated project objectives and budgets for the remaining term of the funded work to synchronize our remaining cash flow with priorities set in the last Annual meeting.

## 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--immunoprecipitations and mass spectrometry). 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.
- Transcriptome expression profiling: Extensive transcriptome data sets (the Transcriptome Computational Workbench; TCW) have been created from whole adults, adult salivary glands, adult guts, and nymphs infected or uninfected with CLas or CLso and are continually updated with datasets associated with published manuscripts made available to the research community ([www.sohomoptera.org/ACPPoP](http://www.sohomoptera.org/ACPPoP)). Because accurate database annotations are critical for interpreting transcriptome analyses, the bioinformatics team is assessing the quality of these datasets. Published manuscripts include; “Asian citrus psyllid expression profiles suggest *Candidatus Liberibacter asiaticus*-mediated alteration of adult nutrition and metabolism, and of nymphal development and immunity” in 2014, “Comparison of potato and Asian citrus psyllid adult and nymph transcriptomes identified vector transcripts with potential involvement in circulative, propagative *Liberibacter* transmission” in 2015, “*Candidatus Liberibacter solanacearum*’ and evidence for surface appendages in the potato psyllid vector” published in PLOS ONE in 2016, and recently “Colonization and intrusive invasion of potato psyllid by *Ca. Liberibacter solanacearum*” in Phytopathology. A project studying the effect of temperature on infected and uninfected psyllids will utilize the TCW BcAN dataset. The goal is to learn the potential impacts of extreme temperature on the vector with the aim to better select effectors.
- RNASeq data from an experiment using CLas-infected and -uninfected ACP instars (1<sup>st</sup>-3<sup>rd</sup> nymphs, 4<sup>th</sup>/5<sup>th</sup> nymphs, and adults) with the aim to find additional lucrative RNAi candidates for validation was completed. Currently additional replicates are being processed to add to this dataset. Currently, 28,330 contigs have been obtained and identified of which 63% (17,791) were annotatable using NCBI and UniProt databases. Preliminary data suggests that the younger instars (1<sup>st</sup>-3<sup>rd</sup>) are more responsive to CLas infection compared to the older stages (4<sup>th</sup>/5<sup>th</sup> instar and adult). Also, expressional profiles suggest that although very little differences are apparent between younger and older nymphal stages, in the presence of CLas major differences between the nymphal stages are evident.

- During this report, TCW was updated to allow for protein sequence and corresponding spectral count data to be analyzed for proteome expression profiling. In total (average of 3 technical replicates) 608 unique proteins were identified by mass spectrometry in adult whole body, gut and salivary gland tissues. Results showed that 7% of these showed differential abundance due to *Liberibacter* presence or absence in adult whole body, gut, and salivary gland tissues. The predicted functions of these proteins are being investigated and based on the outcome will allow for the consideration of these to be effectors and submitted to further testing. To date 2 of these genes have been tested using RNAi.
- Using the yeast two-hybrid system to identify protein-protein interactors has identified 47 ACP, 35 CLAs and 10 phage genes selected for analysis. In total 25 ACP, 16 CLAs, 6 phage, and 2 endosymbiont-associated genes were selected for further study. Those that showed interactions had variety of functions initially reported in virus-host pathogen systems, and more recently, for bacterial pathogen-interactions with their hosts.
- Physically co-immunoprecipitating proteins confirms their interaction. 25 gene candidates have been selected and/or attempted for CoIP analysis. To date 5 ACP, 2 CLAs, and 2 phage genes have been tested (3 replicates) using crude protein samples extracted from both infected and uninfected adult whole body tissues. Results showed a bacterial and phage protein interacting with psyllid genes involved in endocytic processes. Another phage gene was shown to interact with a psyllid transporter gene. Both psyllid genes were subjected to RNAi analysis and the bacterial and phage genes were subjected to qPCR experiments.
- Electron micrographs revealed putative endocytic/exocytic processes, biofilms that require complex cellular communication, and an intravesicular bacterial lifestyle evidenced for the first time allowing for additional effector candidates to be explored. Two manuscripts describing these efforts were published this year. Cicero, J. M., Fisher, T.W., and Brown, J.K. 2016. Localization of '*Ca. Liberibacter solanacearum*' and evidence for surface appendages in the potato psyllid vector. *Phytopathology* 106:142-154 and Cicero, J.M., Fisher, T.W., Qureshi, J.A., Stansly, P.A., and Brown, J.K. 2016. Colonization and intrusive invasion of potato psyllid by '*Ca. Liberibacter solanacearum*'. *Phytopathology* <http://dx.doi.org/10.1094/PHYTO-03-16-0149-R>.
- To validate effector candidates, RNAi has been conducted for 31 different psyllid genes using the single-gene and/or stacked (multiple-gene) RNAi approach and either CLso-infected (born and reared) or -uninfected (introduced to a CLso source plant) nymph and/or adult psyllids to date. Genes are selected as 'transmission interference candidates' by literature review of other pathosystems, from expression profiling of our proteome and transcriptome, and/or yeast-two hybrid analyses. Thus far, positive results (reduced transmission) have been obtained for 12 genes in functional transmission bioassays. In addition, 3 other genes have caused some psyllid mortality. Putative functions of the 12 genes strongly suggest an invasion model similar to other known bacterial pathogens. As a next step, additional genes are being selected for RNAi analysis based on these results.
- Using quantitative PCR (qPCR), expressional profiles were obtained from early instars (1<sup>st</sup>-2<sup>nd</sup>), late instars (4<sup>th</sup>-5<sup>th</sup>), teneral, and non-teneral adult psyllids. To date, 14 genes including psyllid, phage and *Liberibacter* genes have been analyzed. One *Liberibacter* gene showed significantly higher relative expression (3-6x) in later instars to adults life stages. This gene is putatively involved in cell motility and showed significant interaction in Y2H studies. Another *Liberibacter* gene, putatively involved in cell

communication, showed a similar expression profile (high expression in late instars and adults). Interestingly a phage gene with a putative function implicated in virulence in other well-known pathosystems, was significantly higher expressed (2.5x) in the early instar. Low expression profiles for two phage genes with putative functions known to aid in virulence in other pathosystems, suggest that these are not key effectors in the CLAs/ACP system. In addition, preliminary data for a unique psyllid gene predicted to be a key factor in invasion, shows differential expression between infected nymphs (1.3 fold increase) and adults (0.7-0.03 fold decrease).

- Candidate single gene products that may stop the Asian citrus psyllids ability to acquire/transmit (AcTrans) CLAs have been identified in functional assays. One set of three hexameric peptides significantly reduces a psyllids subsequent ability to acquire/transmit the bacterium when fed to nymphs (AcTrans blockers). These peptides can be taken up into the leaf vascular tissue where they are mobile in the leaf vascular tissue and reduce leaf bacterial titer by greater than 80% in 7 days.
- Peptides were also identified that could prevent successful acquisition of CLAs by the psyllid due to their direct effect on CLAs survival. Two separate bactericidal peptides between 10 and 20 amino acids that have bactericidal activity when introduced into citrus leaves reduce the CLAs titer by as much as 90% after 7 days within regions of the petiole/midrib of the leaf. Studies are currently underway to evaluate the effect of these peptides on CLAs acquisition within the psyllid. One of these is a unique antimicrobial peptide (does not share specific motifs identified in other antimicrobial peptide classes) with activity against *Escherichia coli* in liquid culture. Furthermore, the likely targeting of protein synthesis by this peptide has been demonstrated through: a) structural modeling showing favorable interaction with *E. coli* ribosomes; effect on *in vitro* protein synthesis in *E. coli* cell-free extracts; and identification of the importance of a specific *E. coli* membrane transport protein known to be involved in peptide transport for optimal activity of this peptide.
- By combining both AcTrans blockers and bactericidal peptides, induction of greater than 95% mortality was observed in developing psyllid nymphs and none of the surviving nymphs have successfully acquired the CLAs bacterium.
- Such high nymphal toxicity was not expected because the AcTrans blockers induce minor mortality alone (<20%) and the antimicrobial peptides alone induce ~ 50% mortality. Dose response studies are in progress because for use in developing a replacement nuPsyllid population, it would be desirable to find a dose that maintains nymphal fitness while maintaining complete inhibition of successful acquisition.
- Single chain antibodies targeting surface antigens on CLAs have been created that interact with 12 different predicted surface epitopes. These antigens include the major outer membrane protein OmpA, two flagellar antigens, and the capsular polysaccharide synthase, and two pili components. Some of these have been expressed in transgenic citrus and others have been expressed and purified using a 6X histone tag strategy. These will be used for laboratory bioassays developed to study acquisition and CLAs survival. Citrus rootstocks expressing two scFv have been made at Fort Pierce. A scFv selected to bind a surface exposed epitope of TolC = NodD (secretory pore) and a scFv selected against InvA, a protein produced by CLAs believed to prevent apoptosis of infected cells. Multiple scFv selections have been introduced in citrus and multiple transformation events (~400 in all) are currently under evaluation for their effect on CLAs survival in the plant, and acquisition/transmission 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.

- From sequencing worldwide collections of *D. citri* and bioinformatics analysis, several potential candidate viruses have been discovered that might be useful for paratransgenesis delivery systems for inducing desirable traits in *D. citri*. Efforts continue to develop some of these for use as tools in this project.
- The Diaphorina citri picorna-like virus (DcPLV) was identified in *D. citri* from Taiwan, China, and Brazil but not yet from any U. S. collected *D. citri*. DcPLV is a novel insect virus with an unusual genome organization. DcPLV has a positive-sense ssRNA genome of 10,222 nucleotides and contains a single ORF coding sequence of 8,757 nucleotides. It has not been possible yet to clone the entire genome obtained through the extension overlap PCR strategy as cDNA using different strains of *E. coli* cells including JM109, DHB10 and MDS. Creating a reverse genetics system for DcPLV will require first, the ability to clone and maintain the complete genome sequence in *E. coli*, and secondly, a functional and efficient bioassay. Efforts are currently focused on expanded cell culture systems to address both challenges.
- One of the other viruses originally discovered in the nuPsyllid work is *Diaphorina citri* Reo-like virus (DcRV). Live *D. citri* (Hawaiian collection) infected with DcRV does not appear to induce obvious phenotypic effects in *D. citri* – HI and *D. citri* – CA lacks DcRV. Attempts to transmit DcRV to naïve California *D. citri* are in progress. While DcRV is unlikely to be a good candidate virus for complete reverse genetics approaches (because it has 11 dsRNA genome segments making it not a simple candidate for cDNA cloning), its potential for inducing negative phenotypes and affecting *D. citri* will be evaluated.
- The virus discovery program has stored lyophilized, frozen, and ethanol preserved *D. citri* containing DcPLV, *Diaphorina citri*-associated C virus (DcACV), and *Diaphorina citri* densovirus (DcDV). These insect stocks serve as sources of viral nucleic acid for cloning efforts as well as viral reservoirs for virus transmission studies. An expanded NIFA-funded effort is focused on DcACV and DcDV.
- DcACV has a small bipartite positive- sense ssRNA genome, and so far this virus is taxonomically unclassified, but has some similarities to nodaviruses (e.g. FHV). The complete genome of DcACV is cloned under the control of an inducible promoter and insect cell transfection experiments are in progress.
- The complete genome of DcDV is not yet cloned but in artificial diet feeding tests there is evidence suggesting oral transmission DcDV to naïve *D. citri* and that viral sequences are retained in some proportion of subsequent *D. citri* generations. Analyses so far suggest that these sequences do not represent episomal virus, but may be integrated into the *D. citri* genome. Since densoviruses have been used in other insect systems, even for paratransgenesis, DcDENV may prove to be a good virus for our objectives.
- These results have been published in the following papers; Nouri, S., Salem, N., Nigg, J. C., Falk, B. W. 2016. Diverse array of new viral sequences identified in worldwide populations of the Asian citrus psyllid (*Diaphorina citri*) using viral metagenomics. *J. Virology* 90: 2434 – 2445; Matsumura EE, Nerva L, Nigg JC, Falk BW, Nouri S. 2016.

Complete Genome Sequence of the Largest Known Flavi-Like Virus, *Diaphorina citri* flavi-like virus, a Novel Virus of the Asian Citrus Psyllid, *Diaphorina citri*. *Genome Announc.* 2016 Sep 8;4(5). pii: e00946-16. doi: 10.1128/genomeA.00946-16; Nigg JC, Nouri S, Falk BW. 2016. Complete Genome Sequence of a Putative Densovirus of the Asian Citrus Psyllid, *Diaphorina citri*. *Genome Announc.* 2016 Jul 28;4(4). pii: e00589-16. doi: 10.1128/genomeA.00589-16; Nouri S, Salem N, Falk BW. 2016. Complete Genome Sequence of *Diaphorina citri*-associated C virus, a Novel Putative RNA Virus of the Asian Citrus Psyllid, *Diaphorina citri*. *Genome Announc.* 2016 Jul 21;4(4). pii: e00639-16. doi: 10.1128/genomeA.00639-16 and meetings: Nouri, S. Diverse array of new viral sequences identified in worldwide populations of the Asian citrus psyllid (*Diaphorina citri*) using viral metagenomics. Society for Invertebrate Pathology, July 27, 2016, Tours, France; Falk, B. W. 2016. RNAi-based strategies against insect vectors of plant pathogens. Plenary lecture. Sept 20, 2016, International Citrus Congress. Foz do Iguacu, Brazil; Falk, B. W. 2016. RNAi-based strategies against the Asian citrus psyllid, *Diaphorina citri*. Fundecitrus, Araraquara, Brazil. Dec. 16, 2016.

- The analysis of *Wolbachia* (wDi) genetic variation was completed among 24 *D. citri* populations located across multiple countries. Six *Wolbachia* genes, including the surface protein gene (*wsp*) and genes used in the multilocus sequence typing (MLST) procedure, *coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*, were sequenced for every sample to allow sensitive and robust analyses of wDi diversity. The MLST procedure recognizes different sequences of the same gene as separate alleles and unambiguously characterizes each bacterial strain based on the allele profiles of the genes analyzed. Sequencing of wDi genes and subsequent clone library screening allowed identification of prevalent wDi profiles and the possible occurrence of *Wolbachia* co-infection among the *D. citri* populations tested.
- *Wolbachia* was detected in all DNA samples tested. Compilation of the sequencing data revealed that most of the populations surveyed exhibited one of two wDi profiles. One wDi profile includes all five MLST alleles matching ST-173 in the MLST database; this profile was detected in all individuals sampled from China, Singapore, and Argentina. A different profile was detected in all samples originated from Florida, Hawaii, Texas, American Samoa, Belize, Mexico, Pakistan, and Colombia; the alleles of this profile did not completely match to any known profiles in the MLST database, but were identical to sequences of a published *Wolbachia* genomic assembly obtained from Florida-reared *D. citri*; this profile is hereafter referred to as ST-FL. Overall, only the Thailand, Trinidad, Barbados, and Puerto Rico populations had samples exhibiting wDi profiles different from ST-173 and ST-FL.
- The distributions of the two prevalent wDi strain identified in this study, ST-173 and ST-FL, were not entirely restricted to specific geographic regions, suggesting that factors such as human transportation may have played a major role in distributing *D. citri* (along with their wDi strains) to various parts of the world. Overall, the distribution patterns of profiles ST-173 and ST-FL also corroborated findings from previous works.
- Co-infection of more than one dominant wDi strains was detected in populations located in Thailand (Co-1) and the West Indies (Co-2). Interestingly, the *Wolbachia* strains that the Co-1 and Co-2 populations carry included ST-173 and ST-FL, respectively. Since Thailand is located near China and Singapore (ST-173 areas), and the Co-2 populations are distributed near ST-FL populations, it is plausible that *D. citri* in Co-1 and Co-2 populations may have originated from their surrounding areas. Psyllids in the Co-2 populations may have acquired additional wDi strains as they moved into the West Indies.

- Similar to a previous study focusing on wDi *wsp* and mtCOI, the current analyses including multiple wDi genes showed that populations carrying different wDi strains have different mtCOI, and that samples with different haplotypes do not co-occur within the same population. These findings support the hypothesis that infection with different wDi strains could induce bi-directional CI, a phenomenon that may facilitate genetic divergence. Interestingly, data shows that the Co-2 populations are co-infected with strains that were undetected in all of the ST-FL populations, and that these *D. citri* had the most different mtCOI sequence compared to the haplotypes of ST-FL populations. In some insect species, co-infection with additional *Wolbachia* strains could have an additional effect, such that crosses between co-infected and singly infected insects may result in unidirectional CI. That the geographically distant North American, Hawaiian and Belizean populations all have the same ST-FL profile, yet Co-2 (West Indies) populations and their surrounding populations have relatively different mtCOI sequences further supports the possibility that wDi may induce CI and reduce inter-population gene flow. In strategies used to control insect pests or insect-borne pathogens, *Wolbachia*-induced CI could play an important role in reducing insect population size or acting as a drive system for disseminating desirable genes/alleles. The data presented here support our investigation into the possibility of using *Wolbachia*-based strategies to control *D. citri* and citrus greening. The results presented here have been submitted for publication.
- It remains possible that the ST-173 and ST-FL strains confer different phenotypic effects on their respective vectors. One goal for the project going forward is to procure ST-173 *Wolbachia* for evaluation in CLas transmission experiments. This would entail artificially infecting *Wolbachia*-free *D. citri* for comparison against ST-FL *D. citri*. The challenge will be obtaining the required permitting for acquiring live specimen from a foreign source. Given recent results from an ongoing investigation of the *D. citri* immune system, it appears unlikely that these *Wolbachia* strains will affect the vector immune system to disrupt transmission, although investigations of non-native (e.g. Dipteran) *Wolbachia* effects on *D. citri* physiology are ongoing.
- Our goal is also to develop a chromosomal gene drive system for population replacement in the psyllid. Several chromosome translocation-based drive elements have been generated in *Drosophila*. To generate a chromosomal driver, efforts continue to streamline the process for generating insects that carry reciprocal translocations. As discussed in previous reports, there has been some success in generating translocations using a strategy in which transgene insertions are first created on two different chromosomes, and recombination between them follows induction of double-stranded DNA breaks on each of these sites. While this procedure works, it requires a number of steps and crosses. It may be possible to simplify the process by creating a translocation directly in the germline of an injected embryo. This would allow generation these rearrangements much more directly, at many different sites.
- A paper describing how to generate chromosome translocations, their predicted drive properties, and their actual properties, has been submitted to PLoS Biology. It can also be found online at bioRxiv.org, <http://biorxiv.org/content/early/2016/11/17/088393>. As discussed above efforts continue to streamline the process for generating insects that carry reciprocal translocations. Our original work used a three transgene approach that required a number of generations and crosses to generate translocations. We have now generated a number of translocations in *Drosophila* using a two transgene approach that involves two strains, one expressing Cas9 and a second line expressing

a guide RNA that cleaves both transgene-bearing chromosomes. These are being tested for drive now.

- Success with a third approach would simplify the process even further if translocation could be generated directly in the germline of an injected embryo. Homologous integration of one of the two constructs in an injected embryo has been achieved. Studies to integrate the second construct at the same time in the proper orientation are in progress. Thus far both fragments are integrating, but one of them seems to be integrating in the wrong orientation in the transformants recovered in the experiments to date.
- Studies to generate psyllid cell lines are in progress. This novel approach involves trying to simplify the process of immortalization by taking cells from embryos and introducing oncogenes and cell death inhibitors, and/or inactivating tumor suppressor genes. These approaches can allow cells to survive and proliferate even in the absence of some growth regulators that would normally need to be present, but that are unknown. We divide the constructs up into two classes: gain of function and loss of function. With the gain of function approach an activated version of the psyllid Ras oncogene, and a potent caspase protease cell death inhibitor known as P35 are expressed. P35 is derived from baculovirus, and inhibits cell death in many organisms, including other insects. A construct that expresses both proteins under the control of a ubiquitin promoter is also being generated. The protein-coding regions are transcribed as one transcript, with the different coding regions being separated from each other by a 2A ribosome skipping sequence. With the loss of function approach Cas9 and guide RNAs will be used to bring about inactivation of a gene that normally inhibits proliferation. One pathway of particular interest is the hippo/warts pathway. Work in *Drosophila* and mammals has shown that inactivation of these proteins results in excess proliferation and in some cases inhibition of cell death. Our goal is to carry out both loss and gain of function approaches together by integrating our gain-of function construct at the hippo or warts locus.
- An alternative approach to generate loss-of function phenotypes of hippo pathway components is to use a bidirectional ubiquitin promoter derived from baculovirus. Early work on the translocation system indicated that this promoter (Hr5ie1) did have long distance enhancer activity. This suggests that it may be able to drive the expression of two cassettes. This may allow silencing of the hippo pathway components using an RNAi cassette incorporated into the gain of function cassette enabling integration anywhere in the genome.
- Efforts to generate transgenics using adult injection also continue using adult injection using males as well as females.

### *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.
- The induction of foliar volatiles or development of ‘super-stimuli’ which are strong behavioral elicitors, may provide a means of boosting the efficacy of synthetic attractants by enabling them to outcompete background stimuli. Plant pathogens elicit the production of super stimuli in their host plants to make infected plants more attractive to insect vectors; examination of pathosystems may reveal the identity of potentially useful super-stimuli. Of significance to the Las-ACP-citrus pathosystem, Dr. Lukasz Stelinski (UF) and his associates have shown that Las-infected foliage emits the volatile signaling compound methyl salicylate, and that it acts as a super-stimulus in attracting uninfected ACP to Las-infected trees. The emission of methyl salicylate (MeSA) is governed by the production of salicylic acid (SA), an internal signaler that is induced by pathogen infection. The Stelinski and Sétamou (TAMU) labs are developing scent attractants containing methyl salicylate.
- Another important attack/stressor signaler system in plants is the jasmonic acid/methyl jasmonate (MeJA) system. Exogenous application of MeJA to potted CLas- and CLas+ Valencia orange trees significantly altered volatile emission both quantitatively and qualitatively. In behavioral assays, ACP significantly aggregated at higher levels of MeJA-treated foliage.
- Exogenous applications of salicylic acid to CLas- and CLas+ Valencia trees resulted in: 1) Emission of a quantitatively greater amount of volatiles; 2) Production of high levels of MeSA, with this compound comprising 50% of the total amount of volatiles emitted; and 3) Absence of indole, E-jasmone and other compounds in the foliar odor induced by the application of MeJA.
- A study is underway to determine whether an emulsified wax carrier (SPLAT, ISCATech, Inc.) can be used to convey MeJA to citrus foliage. One of the primary aims of this study is to determine the duration of the expression of foliar volatiles induced by exposure to MeJA. Optimal loading levels for achieving a maximal response from the foliage will be determined to: 1) Determine the effect of SA- and MeJA-treatment on ACP colonization; 2) Identify the volatiles emitted by SA- and MeJA-treated foliage that influence ACP behavior; 3) Develop ACP scent attractants based on SA- and MeJA-treated foliage.
- Field trials are being conducted in southern California with the 3D traps made by the Florida Department of Plant Industry. Results show that two scent bait mixtures supplied by us, one that mimics lemon and the other orange jasmine, increase trap

captures by 20 to 30%. Since these traps are designed to preserve ACP for genetic testing, this result is significant because it shows the potential of improving scent baits for monitoring nuPsyllid.

- Work on the regulatory and social aspects of HLB prevention technologies continue. For example, a model describing the conditions required for successful adoption of gene-drive technology for control of agricultural pest species (such as ACP) was previously described by Paul Mitchell (UW Madison, team member), McRoberts and Zach Brown from NC State. Since the model essentially predicts two economic equilibria, one with wide adoption of good technology, the other with limited adoption of poor technology, it is critical to recreate those dynamics in causal models with plausible assumptions.
- Analysis of a previous grower questionnaire results continue in order to understand preferences for technology options.
- The outreach team conducts monthly teleconference calls to select and discuss projects that showcase research programs addressing the HLB problem. The projects that are discussed include nuPsyllid research as well as projects outside the nuPsyllid research program. The team has completed interviews with Pelz-Stelinski, Dawson, Killiny, Falk, Pourezza and Davis, and developed grower-oriented explanations of their research for the 'Science for Citrus Health' website <http://ucanr.edu/sites/scienceforcitrushealth/>. The current organization of these technologies is 1) Nupsyllid, 2) Strategies for Established Orchards, 3) Strategies that Require Replants, and 4) Early Detection Techniques. Some technologies cross over several categories. With funding from other agencies the subject matter has been expanded beyond the nuPsyllid program, with the goal of educating growers about new technologies (both genetic engineering and non-GE) for managing HLB.

## SUMMARY

There are a number of excellent candidate effector targets including several identified in a functional screen. It would be ideal to test these candidates in a psyllid viral vector. Data combined strongly suggest a "invasion model" in which CLAs/CLso transforms the endocytic/exocytic host pathways to facilitate internalization, infection, and circulation in the psyllid host and vector. Briefly, a model involving a putative phage gene that acts as an effector, which may operate in conjunction with a unique ACP gene to alter the function of genes associated with clathrin-mediated endocytosis, actin cytoskeletal rearrangements, and vacuolar formation, and exocytosis. 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 and be prepared to use others that might 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;
- engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB
- provide support and continuity as additional teams are funded that can build these results into existing and pending research programs seeking HLB solutions.