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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 and to extend the breadth of communications about new technologies for disease intervention such as the current study in progress by the National Academies, Board on Agriculture and Natural Resources, “A Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting A Ravaging Disease.”

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. These budget amendments are being finalized with the respective institutions.

## 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 and made available to researchers ([www.sohomoptera.org/ACPPoP](http://www.sohomoptera.org/ACPPoP)). 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. Data revealed that extreme temperature impacts bacterial titer within the psyllid vector equally, suggesting the activity of specific gene(s) at non-optimal temperatures, not the amount of bacteria, plays a key role in altering transmission parameters. This hypothesis was confirmed from differential expression analysis, in that data did show that high temperature had a greater impact on psyllids, regardless of infection status, based on the larger number of genes that were significantly changed. Functional analysis of the genes mis-expressed at this higher temperature extreme are underway. Preliminary data suggests that functions known to be important in pathogen-host interactions are affected.
- Proteome expression profiling: During this report, TCW was updated to allow for protein sequence and corresponding spectral count data to be analyzed. In total (average of 3 technical replicates) 608 unique proteins were identified by mass spectrometer in adult whole body, gut and salivary gland tissues. Results showed that 55% (304 proteins) had modified changes in abundance due to *Liberibacter* presence

or absence in adult whole body, gut, and salivary gland tissues. The predicted functions of these proteins were investigated using Gene Ontology and KEGG programs and results confirm that were previously reported regarding our invasion model. Additionally, comparative *in silico* analyses identified 22 genes that were modified by *Liberibacter* presence in both the transcriptome (expression) and proteome (abundance). To date 4 of these genes (previously reported 2) have been tested using RNAi with one showing a reduction in transmission and therefore a promising target for control.

- Yeast two-hybrid: Of the 109 matings and 83 co-transformations completed to date, 47 ACP, 35 CLAs and 10 phage genes have been selected for additional analysis. Of those a total 31 ACP, 16 CLAs, 6 phage, and 2 endosymbiont-associated genes were selected for further study. Those that showed interactions had a variety of functions initially reported in virus-host pathogen systems, and more recently, for bacterial pathogen-interactions with their hosts.
- Co-immunoprecipitation: In total, roughly 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 that 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. In all 30 interacting partners are recognized as important. Both psyllid genes were subjected to RNAi analysis and the bacterial and phage genes were subjected to qPCR experiments. One manuscript using Y2H and CoIP findings is in preparation from this project, with an expected submission date of August 2017.
- TEM/SEM studies: 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 last year.
- Validation of effectors: RNAi testing 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 3 different expression profiles e.g. proteome and transcriptome, and/or protein-protein interaction (standard yeast-two hybrid, specific bait prey co-transformation and co-immunoprecipitation 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. To better understand the impact of "gene stacking" in RNAi assays, four genes that have shown very promising results were selected for more extensive analysis. All possible dsRNA stacking combinations (15 total) were studied. Both persistence and gene knockdown were examined. Treated insects were tested immediately after a 3 day-dsRNA feeding period (oral delivery), followed by a 7d holding period on plants. Results after the 3- day feeding from all dsRNA combinations showed gene knockdown (i.e., significant decrease in relative gene expression). Testing of insects after the 3-day dsRNA feed and 7-day holding period show the same level of knockdown but interestingly in more than a few combinations a gradual decrease in the level of knockdown, indicating a possible effect on persistence that could negatively affect efficacy of dsRNA treatments. Furthermore, preliminary results for one of the four genes in specific combinations shows an actual upregulation of the

relative gene expression. Indicating not only a possible problem with persistence, but possibly some form of trans-activating transcription regulation. These results are preliminary, but warrant further study to better characterize this putative yet important phenomenon and shed light on how best to provide more effective combinations of RNAi candidates. Putative functions of the 12 genes strongly suggest an invasion model similar to other known bacterial pathogens, including endo-exocytosis involving at least a clathrin-mediated step. As a next step, additional genes are being selected for RNAi analysis based on these results.

- DsRNA quantification and tracking: Experiments to detect and quantify the fate of dsRNA in plants and insects are underway. To date 200 ng total dsRNA was applied to seedling tomato plants for 24 hours, followed by allowing five adult PoP to feed on those plants for 24 hours, after extraction and northern blot showed low level detection of dsRNA with digoxigenin label RNA probes detected by chemoluminescence. Also, fluorescently labeled dsRNA fed to both seedling tomato plant and adult PoP has been tracked through the phloem in the roots of the intact plant and in the gut of whole PoP by fluorescent low power microscopy. This will be followed up a closer tracking of dsRNA with tissue sectioning. These studies are necessary for monitoring the persistence and location of dsRNA in plants and psyllids as well as non-target insects.
- Quantitative PCR (qPCR) analyses have been conducted on 14 genes (previously reported 9) to date, including psyllid, phage and Liberibacter genes. Expressional profiles were obtained from early instars (1st -2nd), late instars (4th-5th), teneral, and non-teneral adult psyllids. One Liberibacter gene showed significantly higher relative expression (3-6x) in later instars to adult life stages. This gene is putatively involved in cell motility and also 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). Similar studies for 4 additional psyllid genes are in process both PoP and ACP to study relative gene expression in infected psyllids vs healthy psyllids. Work was completed to add 2 additional reference genes to add validity to the qPCR for the psyllid studies due the high level of variability in experimental conditions. Preliminary data indicates both up and down regulation varying with gene but also with life stage, pointing towards the relative importance of specific genes and life stages for further targeting efforts. In another experiment, expression profiles were obtained from adult psyllids having access to a Liberibacter source after a set period (0, 2d, 4d, 8d, 12d, 16d, 20d, and 24d). The AAP experiments were set-up and psyllids collected (3 replicates). Gene expression studies were conducted by qPCR with 12 PoP genes to date, all implemented in invasion. Five of these genes show a steady decrease in relative gene expression over the entire time course. Two showed a steady increase. The others showed inconclusive results and a shorter period time course was set up as above only collecting insects at 0, 3, 6, 12, 18, 24 and 36 hours. Four of these PoP genes were analyzed by qPCR to date. The conclusion is that their gene expression is effected very little if not at all by the onset of CLso infection over time.

- TCW databases, website, and interface for all bioinformatics: Database annotation updates are critical for completing the remaining transcriptome analyses needed to complete manuscripts anticipated during the last year of the project, and for annotating the comparative RNAseq data sets upon completion. Last quarter it was determined that quality of the data were not optimal because the computation of the read counts was inadequate. Therefore C. Soderlund and M. Willis (Gang lab, WSU) worked together to determine appropriate parameters for the mapping. The TCW databases were rebuilt with the new data, given a December 2016 update archive. A study to determine candidate transcripts that influence psyllid speciation is ongoing and one manuscript “Comparative transcriptomes of Asian Citrus Psyllid and Potato Psyllid” (C. Soderlund, T. Fisher, M. Willis, R. He, D. Gang, and J.K. Brown) was published, May 2017. To date, orthologous pairs were computed using the TCW bi-directional best-hit (BBH) algorithm with e-value 1E-05 and the restriction that the alignment must overlap at least 50% of the shortest transcript, which resulted in 8892 pairs. A comparison of the 5'UTR, CDS, and 3'UTR showed percent similarity of 60, 73, 59 and percent GC-content of 24, 34.5, 23.5, respectively. The codon analysis shows an overall 24.4% synonymous codon and 33% non-synonymous pairs, where the synonymous codons were further broken down into 10.4% 2-fold and 13.4% 4-fold degenerate. The transition differences of the three codon positions are 9.3%, 6.7% and 18.6%; the transversion differences of the three positions are 9.8%, 8.7% and 16.0%. Using TCW, the alignments were written to file for analysis by the KaKs calculator program using the method=YN, and results loaded into TCW. There were 8850 with a p-value, where 7295 have KaKs<1 and 1553 have KaKs>1, with KaKs quartiles Q1 0.02873, Q2 0.11531, Q3 0.6708. The third objective is to map the Dc transcripts to the Dc genome sequence. Initial results showed multiple ambiguities when using blast results. A more strenuous approach was developed to use dynamic programming to get the best matches and data analysis is in progress.
- RNASeq time-course: 60 RNAseq ACP libraries were constructed from two treatments (ACP\_CLas-infected vs uninfected), 5 stages (instar 1+2, instar 3, instar 4+5, teneral adult and adult) and 6 replicates, and these libraries were pooled with 12 libraries barcoded in a lane for 150 bp paired-end sequencing and 1,960 million reads totaling 296 G bp data were generated. Samples were provided by Dr. Kirsten Stelinski, collaborator. The data assembly, mapping and annotation are in process.
- Endosymbiont genome sequencing: The psyllid ovary samples from 4 psyllid species: ACP, PoP-central, PoP-western and Carrot psyllid, were used for making 4 Illumina genomic libraries and pooled on a lane for 150 bp PE-sequencing. In total, 314 million reads were produced, and total bases are 47.4 G bp. The new longer read data will be integrated with the existing data (100 bp PE reads) to help improve the assembly qualities of the endosymbiont genomes. The assembly of contigs for each genome has been completed and annotation are in progress.
- Transcriptome and gene expression analysis of Asian citrus psyllid in response to *Ca. Liberibacter asiaticus*: RNA-seq libraries were constructed from CLas-infected and CLas-free ACP samples of three different developmental stages (nymphal instars 1-3, nymphal instars 4-5, teneral and post-teneral adults). With 150 bp paired-end sequencing on the Illumina HiSeq2500, 152 Gb of sequence data were generated from 56 million reads per library/replicate, which was assembled into 34,122 contigs with 18,827 (55.2%) being annotated, which were then further analyzed for potential functional classification and potential roles in infection. The results suggested that gene expression in different developmental stages did not respond in the same

manner to CLas infection. With more contigs being up or down-regulated, nymphal instars 4-5 showed a more sensitive response to CLas infection than nymphal instars 1-3 and adults. A comprehensive analysis of the transcriptomes revealed vector life stage differences and differential gene expression in response to CLas infection, and identified specific genes with roles in nutrition, development, immune response and transmission pathways.

- Two classes of peptides that may stop the Asian citrus psyllid's ability to acquire/transmit (AcTrans) CLas have been identified in functional assays. One set of three hexameric peptides significantly reduced the psyllids subsequent ability to acquire/transmit (AcTrans blockers) the '*Candidatus*' *Liberibacter asiaticus* (CLas) bacterium when fed to psyllid nymphs. Two separate bactericidal peptides that kill CLas within infected leaf tissue when these peptides are taken up into the leaf vascular tissue have also been identified (they are mobile in the leaf vascular tissue and reduce leaf bacterial titer by greater than 80% in 7 days). In the previous report, preliminary results showing that by combining both AcTrans blockers and bactericidal peptides were presented showing induction of greater than 95% mortality in developing psyllid nymphs and while none of the surviving nymphs successfully acquired the CLas bacterium. Further replicated studies show similar results with high psyllid mortality and no complete acquisition (defined as movement into the salivary gland) of the bacterium by the surviving psyllids.
- Single AcTrans blocker peptide experiments have been initiated in combination with the antimicrobial peptides to determine the minimal combination that has the desired effects on psyllid mortality and acquisition. One of the three AcTrans blocker peptides was identified as having the most significant effect on psyllid mortality and CLas acquisition. Transgenics are being initiated with a combined expression of this AcTrans and the antimicrobial peptide.
- A topical application strategy has been developed that could be deployable in the field. Its effectiveness in delivering organic antimicrobial molecules of MW of at least 600 MW and also in systemic delivery of dsRNA molecules of up to 300 bps was demonstrated (a MW much larger than previous peptides). A 10 g batch of this peptide has been synthesized and demonstrated to have the same antimicrobial activity as the previously synthesized peptides. Plant application experiments are now being conducted.
- A transgenic citrus strategy has been developed that will produce a single phloem-cleavable peptide that, when processed, produces the desired smaller and biologically active peptides (both AcTrans blockers and antimicrobial). This system utilizes an already identified peptide cleavage system within the phloem of citrus. Plants have been produced and are currently being evaluated in the greenhouse for expression.
- 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.
- DcPLV was the first *D. citri* virus identified by us, 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. Attempts 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 but have not yet been successful. The hope is to use cultured cells to recover infectious viruses. This bacterial free strategy was recently applied to create infectious forms of a virus in the genus *Flavivirus*.
- Attempts to identify promoters to drive transcription of introduced sequences in *D. citri* by cloning putative promoters for *D. citri* actin and tubulin genes into a GFP reporter construct are in progress. This is important for delivering cloned viral constructs, such as for DcPLV, into *D. citri*. These promoters will be tested by detecting GFP expression following injection of the promoter-GFP constructs into *D. citri* insects and insect cells. This system is also being used to test the efficacy of previously described hemipteran and viral promoters. Good success in culturing cell from the hemipteran, *Anasa tristis* has been achieved.
- Another virus originally discovered early in this work was *Diaphorina citri* Reo-like virus (DcRV). We have colonies of *D. citri* (Hawaiian collection) infected with DcRV in the UC Davis CRF. We have transmitted DcRV to naïve California *D. citri* and found that the DcRV is transmitted through eggs to progeny.
- With respect to the bacterial driver system a goal for the project going forward is to develop new methodology to increase adult survivability post embryo injection. Problems arise after injected embryos hatch, since first instar nymphs prove difficult to handle and survival to the adult stage is minimal. It is hypothesized that to increase survivability of injected ACP to adulthood it is necessary to decrease ACP embryo and instar handling as much as possible. New methodologies for injecting and handling embryos were developed during the past quarter.
- To collect ACP eggs, small swingle seedlings in “cone-tainers” are inoculated with 10-15 pregnant ACP adults. Cone-tainer seedlings are selected preferentially for young flush suited for oviposition sites. ACP are returned to colony cages after approximately 3 hours or 1.5 hours or less after the time of oviposition.
- Flush with eggs is clipped from the plant and washed in 0.02% nonidet for one minute, and rinsed in DI water. After air-drying eggs are transferred with forceps to a 0.5mm

strip of double stick 3M tape attached to a coverslip. that is then placed within in a petri dish with moist filter paper.

- The cover slip with eggs are placed on a micro manipulator to be injected. Halocarbon oil can be used on the side to prevent clogged needles.
- After injection eggs are placed into a petri dish and placed in an incubator and monitored. Three days after injection eggs and tape are dusted in potato starch to prevent them from getting stuck in the tape after hatching.
- On day 4 newly emerged ACP nymphs are transferred to fresh flush on cone-tainer seedlings covered with a mesh cylinder to prevent emerging ACP from escaping. Plants with ACP nymphs may then be transferred to an environmental chamber or green house.
- Cone-tainer seedlings with transferred ACP nymphs are monitored for 14-21 days until adults begin to emerge.
- Injected ACP must be separated and sexed before they reach sexual maturity. Each ACP can be sexed under a dissection scope in a small glass vile.
- Virgin wild types should be paired with injected adults for mating. Each pair can be placed on cone-tainer seedlings and allowed time to mate and lay eggs. These eggs should be allowed 14-21 days for full development before screening.
- The first new method of rearing injected ACP from instar to adult focuses on allowing newly emerged ACP nymphs to crawl from cover slips directly to fresh flush to help decrease handling at the first instar nymph stage. The only major modification made to the initial standard operating procedure was to attach cover slides to fresh flush where nymphs were allowed to crawl directly onto flush without being handled. In this method cone-tainers were not a viable option due to their small size, orange jasmine (*Murraya Exotica*) was used for ACP nymph development. This method allows nymphs to crawl to a feeding site of their choice.
- In greenhouse assays, 226 nymphs and 0 adults emerged from the 381 embryos not injected, representing 59.3% egg hatch. Egg hatch was lower (29.1%) when embryos were injected, with 65 nymphs emerging from the 223 embryos injected. No adults emerged from either treatment.
- When ACP development occurred in incubators, rather than a greenhouse, egg hatch rates were 59.3% (294/525) and 3.2% (3/95) among non-injected and injected embryos, respectively. One adult emerged from the non-injected embryos.
- The second new method of rearing injected ACP from instar to adult involves injecting ACP embryos directly on the flush in the original oviposition sites, and development of this method is in progress. In this method embryos were left in original oviposition sites and injected accordingly. This method decreased handling of the ACP embryos and nymphs, and uninjected controls are promising. However, ACP embryos are clustered and thus difficult to inject individually and further testing is required. Of 131 embryos not injected, 105 nymphs and 5 adults emerged, representing 80.2% hatch and 3.8% survival to adulthood, respectively. No nymphs emerged from the 9 embryos that were injected.
- A third method for rearing ACP for injection forces oviposition without the need to transfer the eggs, or eggs+flush for injection, groups of males and females (2 females and 3 males) were transferred to small Petri plates with 1% agar with and without 20% sucrose and observed oviposition. An extract of new flush (the flush was ground using TissueLyser II) was added as a treatment to a set of dishes to see if the 'flush extract' would induce oviposition. After 8 days, some mortality was observed in the absence of sucrose, indicating that adults will feed on the agar (*D. citri* typically die after 3 d without

feeding). After 7 days, up to 20 eggs were observed in untreated dishes containing 20% sucrose. The following week, eggs started to hatch and nymphs successfully fed on the agar. This suggests that this method could be explored to circumvent the problem of transferring groups of eggs with flush to a different surface before the microinjections, and avoid the difficulties associated with egg clustering. Agar can be easily excised and placed on a coverslip, then removed to a fresh bed of agar. Following egg injections and hatch, nymphs could be recovered and transferred to plants. This possibility is currently being investigated.

- While nymphal hatch rates for uninjected embryos were high when eggs were retained on flush, successful individual egg injections have remained difficult for both the Pelz-Stelinski and Handler labs due to egg clustering and the inability to properly position the micro-needles for individual injections. In addition to the agar plate method under development by Pelz-Stelinski, the Handler lab has initiated protocols for DNA delivery to eggs maintained on flush by biolistics bombardment with micro-pellets coated with DNA. This is a method routinely used for plant tissue gene-transfer, with just a few reports of successful transformation of insect embryos. The most effective of these efforts was reported in 2008 where *Drosophila* was successfully transformed at rates of 3-4% using a Bio-Rad PDS-1000/He biolistic instrument with the Hepta-adaptor. The lab has obtained a previously used PDS-1000/He instrument and have been repairing and retro-fitting it for testing, as well as testing protocols for preparing eggs on flush for treatment. This includes coating the flush with 1% agar to support the eggs during bombardment. The agar overlay using Swingle flush gave considerably better support for ACP development than C35, and was not significantly different between agar treatment and controls when reared within an incubator (0, 18 and 20% adult development, on C35, Swingle + agar, and Swingle control, respectively). Although control survival was better in greenhouse rearing (29% adult emergence), maintenance of incubator conditions are simpler and more consistent, and therefore preferable. Tests for biolistics-mediated gene transfer in ACP embryos will now be initiated on Swingle flush with eggs overlaid with 1% agar and subsequent rearing in incubators.
- Transinfections with non-native hemipteran *Wolbachia* are ongoing. Development of co-infected ACP requires quantitative identification of each strain from the psyllid. While qPCR can be used to conduct whole body quantifications, localization of the new *Wolbachia* strains is also necessary because the amount of genetic material from foreign *Wolbachia* is low in individual tissues. Therefore, primers have been constructed for fluorescent in situ hybridization (FISH) to identify the unique *Wolbachia* cells in tissues. The effects of individual *Wolbachia* infection (wDi, ST-173) and co-infection on the survival and transmission capacity of CLAs-infected vs. uninfected ACP is currently underway.
- Efforts continue to streamline the process for generating insects that carry reciprocal translocations to facilitate a chromosomal gene drive system for population replacement in the psyllid. Several chromosome translocation-based drive elements have been generated in *Drosophila*.
- A paper describing how to generate chromosome translocations and their actual properties has been submitted for publication. The manuscript has been posted online at [bioRxiv.org](http://bioRxiv.org), <http://biorxiv.org/content/early/2016/11/17/088393>. As discussed in previous updates, efforts continue to streamline the process for generating insects that carry reciprocal translocations. The original work used a three-transgene approach that required a number of generations and crosses to generate translocations. A number of translocations in *Drosophila* have now been generated 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. Both of these have an unanticipated fitness cost and do not drive. It is hypothesized that expression of Cas9 remains present in these animals, and is expressed in the germline in each generation. Cas9 has been shown to have effects in other systems, and thus it is possible that this expression lowers fertility in either males or females. To eliminate this expression we took the translocation strain and injected guides into its germline that are designed to cleave and destroy Cas9. Progeny from this injection are now being tested for the presence of functional Cas9. Those that pass this test will be retested for drive.

- Great caution must be used in making the determination that any one of the lines scored as positive for being a translocation based on marker rescue with the Cas9 system. This is because, for still unclear reasons (being explored using PCR and sequencing), the act of cleavage sometimes results in marker activation that does not correspond to generation of an actual translocation. This was observed in several drive experiments. The only way to ensure that a putative translocation is really a translocation is to outcross heterozygotes and look for the 50% embryo lethality that must occur if the translocation is present. This is then followed by PCR of a number of lines to confirm. PCR confirmation works well.
- As a third approach, attempts to generate a translocation directly in the germline of an injected embryo are underway, as this would simplify the process even further. Homologous integration of both constructs in an injected embryo has been successful, but these appear not to be forming translocations when they integrate. It is not understood why the homology arms are not (at least most of the time) being used in the insertion process. One possibility is that the integration events occur at cell cycle stages when NHEJ or other repair pathways dominate over HR. To overcome this potential problem, Cas9 is being expressed under the control of S/G2 phase controls using cell cycle-dependent degradation elements.
- Efforts continue to generate psyllid cell lines. This 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.
- A number of constructs designed to either overexpress or knockdown or knockout the expression of specific genes involved in cell death and cell proliferation using psyllid-derived reagents have been generated. Sequences that should encode the psyllid ubiquitin promoter or the baculovirus IE3 promoter are used for overexpression. For the Crispr/Cas9 targeting of specific genes fluorescent reporters are being used under the control of a ubiquitous promoter, linked to homology arms for the genes being targeted, components of the hippo/warts pathway.
- Efforts continue to generate transgenics using adult injection, thus far without success. Early efforts focused primarily on females. In the last cycle work was initiated on males, which is continuing.
- Work is in progress to have cleavage-based gene drive elements working in the fly by the end of the final quarter. While these are predicted to have low or intermediate threshold drive characteristics, they have the advantage that all components of the drive element are present in a single construct, and many different genes can in principal be used to generate these elements.

## 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.
- Induction of foliar volatiles: The 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 Las- and Las+ 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 Las- and Las+ 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.

- An emulsified wax carrier (SPLAT, ISCATech, Inc.) can be used to convey MeJA to citrus foliage. A high response level was achieved using a low viscosity SPLAT formulation containing only 10 mM MeJA that was sprayed on the foliage. Further work is needed to determine the duration of the expression of foliar volatiles induced by exposure to MeJA and to devise optimal loading levels for achieving a maximal response from the foliage.
- Field tests are planned to determine if spray treatment with MeSA and MeJA influence recruitment of ACP and its natural enemies to treated v. control potted orange
- Field trials of ACP scent attractants using DPI 3D psyllid traps: Field tests of ACP traps made with a 3D printer by Florida DPI are in progress. A scent lure formulation based on the volatiles emitted by orange jasmine flush and are being tested at different concentrations of this formulation in 3D printer traps placed in orange jasmine hedges infested with ACP.
- Testing synthetic ligands of olfactory binding proteins: A new rapid screening assay is being developed to measure ACP attraction to novel synthetic ligands of olfactory binding proteins isolated from the antennae of ACP. The goal of this project is to discover highly effective scent lure attractants, either on their own or in combination with terpene compounds that are emitted naturally in the aroma of host plant flush.
- The sustainability/economics/modeling team continued to develop analytical infrastructure based on the generic needs to understand system dynamics and potential impacts of technology adoption.
- Keynote papers by Ryan McAllister of CSIRO on the importance of social science in invasive species responses and by Paul Mitchell on the economic impacts of HLB on Florida citrus were given at IRCHL in March.
- Short versions of these papers are available online in the Journal of Citrus Pathology: [http://escholarship.org/uc/iocv\\_journalcitruspathology](http://escholarship.org/uc/iocv_journalcitruspathology)
- The modeling team has been working with the CPDPC in California helping to disentangle the possible effects of inter-seasonal variation and ACP control treatments on the observed dynamics of ACP, particularly in the outbreak that occurred in Kern Co. in 2015-16. This work has been important in understanding the cost-effectiveness of the grower-funded urban treatment program in suppressing ACP in non-commercial situations.
- The outreach team conducts monthly teleconference calls to select and discuss projects that showcase research programs addressing the HLB problem. 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. The Science for Citrus Health web site <http://ucanr.edu/sites/scienceforcitrushealth/> was made public this quarter. It is divided into four sections: 1) Early Detection Techniques, 2) Strategies for Established Orchards, 3) Strategies that Require Replants, and 4) nuPsyllid. The team has posted the following research snapshots and additional research snapshots are planned for the next quarter. Some topics are found in two sections. Postcards announcing the web site were provided to citrus growers at meetings in Ventura, Santa Barbara, and Exeter CA during this quarter.
- Early Detection:
  - Carolyn Slupsky; Metabolite changes in the tree can help detect HLB
  - Ali Pourezza; Starch accumulation sensor for early detection of HLB
  - Cristina Davis; Using volatile changes in citrus for early detection of HLB
- Established Orchards:

Bryony Bonning; A new, Bt toxin-based strategy for suppression of the Asian citrus psyllid vector

Michelle Cilia; Managing psyllid gut cells to block transmission of CLAs

Lukasz Stelinski, Nabil Killiny; Using interference RNA to manage ACP

Bryce Falk; Using insect viruses to combat the Asian citrus psyllid

- Replants:

Bryony Bonning; A new, Bt toxin-based strategy for suppression of the Asian citrus psyllid vector

Jim Thomson; Founder lines used to improve HLB tolerance

- NuPsyllid:

Bryce Falk; Using insect viruses to combat the Asian citrus psyllid

Kirsten Pelz-Stelinski; Altering the Asian citrus psyllid's beneficial bacteria to stop HLB spread

## 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.

The combined data strongly suggest an "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 and cleavage drive constructs;
- begin to model the logistics of rearing and releasing nuPsyllid around hypothetical specifications and explicit assumptions;
- optimize trap design for monitoring and other potential control applications;
- 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.