CITRUS RESEARCH BOARD

# HLB EXTERNAL SCIENTIFIC REVIEW

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This document was presented to the CRB Board on 11/02/16.

# Executive Summary:
# Strengths, Gaps, and Recommendations

The summary that immediately follows presents a brief accounting of the panelists' views on strengths, gaps/weaknesses and recommendations according to each CRB-funded research area. For a more detailed explanation of points made in the executive summary, please refer to **Accomplishments from Individual Research Presentations**, for a full accounting of individual research project accomplishments. **Appendix 1** details the in-depth discussions and recommendations/suggestions from scientific experts who served as panelists and recorders, CRB-funded researchers, regulatory officials from CDFA and APHIS, and members of the CRB. **Appendix 2** lists short-term recommendations, and **Appendix 3** lists final, overall recommendations resulting from 2.5 days of discussions.

For the review itself, research projects were assigned to each of the five panelists based on their area of expertise. Due to the number of researchers giving presentations, particularly in the area of host-pathogen-vector relationships, projects were assigned to panelists in an effort to evenly divide their responsibilities and time commitments. For the purposes of the report, the CRB-funded projects are organized into four research areas, including: **(1) Early Detection/Survey and Sampling, (2) Vector Management, (3) Host Genetics/Resistance Development, (4) Host/Pathogen/Vector Interactions.**

## Early Detection/Survey and Sampling

The risk-based analysis for sampling and survey is an essential component of the research portfolio, optimizing how and where the CDFA and other groups should target their resources. However, one survey per year is not enough, and should increase to 2-3 surveys each year, if resources can be provided. Opportunities for self-survey and scouting for growers are needed that can be coordinated with CDFA.

Both areas of improved, direct detection of *C*Las and indirect/early detection technologies (EDTs) are important aspects of the CRB research portfolio. As direct and EDT methods progress, coordination with APHIS and CDFA for validation will be needed. The most promising EDTs will ultimately need to be correlated with the APHIS-approved protocols. The CRB is encouraged to coordinate this research, focus on development of deliverables, and help determine how EDTs can be deployed in early scouting. This coordinated research will also be a means of developing more confidence in the EDTs, and in separating HLB-positive reactions from other biotic and abiotic stresses. Affordable methods of quickly distinguishing trees with

HLB from trees with stubborn or other confounding biotic and abiotic stressors need to be made available to growers.

The infrastructure provided through the Contained Research Facility in Davis is required to manage several of the research programs funded by the CRB:  for comparisons between the direct and EDT methods, to determine the value for use of EDT's for pre-screening and survey, and for screening transgenic and conventionally-bred citrus lines for HLB responses (resistance/tolerance/susceptibility).  If resources were available to duplicate lines for evaluation in field or greenhouse trials in Florida, it could help speed the evaluation process.

## Vector Management

The research projects supported by the CRB include conventional and organic insecticide programs, recommendations to growers by outreach/extension, impressive biocontrol programs, and promising new developments toward lures used in an attract-and-kill approach for ACP.  All of these projects have made important contributions to vector control that can be deployed now and into the future.  A gap that received extensive discussion was how to correlate the flushing of trees with timing of pesticide applications, especially through an area-wide approach, something that seems to be lacking in California.  Understanding the correlation between appropriate vector management strategies with tree growth and flushing is an important area to consider, and provides a needed link between research areas.  Management of flushing could also be integrated into development of additional cultural practices, based on work done in Florida showing ACP infestations often initiate at the edges of contiguous blocks. Edge management could include border barriers, trap crops or more intensive pesticide applications.  This will require additional expertise from plant physiologists and botanists, coordinated with other disciplines.

Another recommended area for research is in the discovery of new compounds and delivery methods for ACP control, in particular for organic citrus production, which is significant in California.  These compounds could include GRAS (generally regarded as safe) materials and biologicals.  Since current pesticides for organic citrus are not as persistent as conventional ones, organic groves are likely to become a source of infectious psyllids, unless materials are found with longer activity, or if organic growers are persuaded to spray more frequently to maintain control.

Regarding maintenance of biocontrol agents long-term, research directed toward creating natural insectaries is suggested, since the current approach is costly.  Citrus relatives that are attractive to the ACP but are not hosts for *C*Las may exist (e.g., curry leaf) that could serve to maintain *Tamarixia radiata* populations, but this needs to be confirmed. Research efforts to maintain biocontrol agents are essential until true host resistance is established.

## Host Genetics/Resistance Development

Classical and biotech approaches for resistant citrus variety development funded by the CRB is impressive, and supports several diverse, cutting-edge technologies. However, in the biotech area progress would be enhanced if research was coordinated through a team of researchers who could assess and prioritize the numerous methods, genes, promoters, terminators, and other genetic components, across a common set of conditions so results can be compared. Involving Ralph Scorza in these discussions is also important, as he has taken the 'HoneySweet' *Plum pox virus*-resistant plum from inception to deregulation by EPS. Since space for evaluation in the CRF is limited, duplicate tests should be established in Florida, where HLB is endemic. A team from the CRB and funded researchers should initiate meetings with the key regulatory agencies (APHIS, EPA, and FDA) as was done several years ago, to provide (and receive) input on the new technologies (e.g., RMCE). Classical breeding is revealing resistance to HLB in citrus relatives. The speed of breeding can be accelerated by combining classical with biotech approaches such as rapid cycle breeding. Biotechnologies can be used to identify these resistance genes which could then be transferred to commercial citrus, perhaps reducing the regulatory burden associated with resistance genes transferred from other organisms. These classical and biotech approaches are long-term, but are essential for the California citrus industry of the future. The CRB should work closely with the Florida and Texas programs in all genetic-based approaches to HLB resistance and issues surrounding these technologies. Genetic resistance holds promise for the long term, but research and development in this area must be consistently pushed to produce cultivars approved for cultivation and made available to growers as quickly as possible at a price they can afford.

A research summit, organized by the CRB was suggested that focuses on transgenic development of perennial trees, which could include: *Prunus* species, walnut, Arctic Apple®, American chestnut, and poplar.

## Host/Pathogen/Vector Interactions

Research in this area included using *Xylella fastidiosa*, which is being used as a surrogate to identify virulence genes in *C*Las, nanoparticles as a means of delivering antimicrobials or key nutrients directed to the phloem, viral vectors to introduce therapeutic molecules to the citrus host, and proteomics to evaluate and ultimately block the ability of the ACP to acquire and/or transmit *C*Las. The surrogate system with the culturable *X. fastidiosa* provides a unique means of developing anti-virulence strategies for *C*Las. Research with viral vectors and small RNAs should continue, but should build on what has been done in Florida with the T36 CTV isolate. The question of why a separate CTV vector needs to be made from the California isolate was raised. Can this be confirmed? If the Florida CTV vector could be used in California, time and resources would be saved. Could the antimicrobials identified by Lin's program be used in the nanoparticle research and tested for delivery to the citrus host or ACP?

In summary, the CRB is funding excellent research, but implementation of the most effective methods is needed. The Board can play a leading role in the future coordination and advancement of research toward deliverables based on current knowledge of the best available strategies. Some of the methods presented in the review could be fast-tracked toward deliverables. As improved methods develop, they can replace existing technologies. This is especially critical for the short term in the areas of ACP control and disease detection and diagnostics, and will be essential to protect the current California citrus industry. Knowledge, new tools, and strategies for the future California industry will be enhanced through increased coordination with Florida and Texas researchers and funding entities. This is especially important in evaluation of disease resistant or tolerant citrus cultivars, where Florida can serve as a living laboratory, and resistant/tolerant materials will preserve the national citrus industry.

# Introduction:
# HLB External Scientific Review

Five scientific experts were selected for the review panel of CRB-funded research. Selection was based on the diversity of scientific disciplines that represented the major component of HLB and its psyllid vector, and preeminence in their respective fields. The panelists were: Neal Van Alfen, plant pathologist from UC Davis (retired); Jan Leach, plant pathologist from Colorado State University; Robert Shatters, entomologist from USDA-ARS; Ralph Scorza, plant geneticist from USDA-ARS (retired); and Bruce Hay, molecular biologist from Cal Tech. Gail Wisler, retired from the USDA-ARS Office of National Programs, served as panel chair. The CRB review of HLB research was introduced by statements addressing the likelihood that an HLB epidemic in California commercial citrus is imminent. Although over 200 million dollars have been spent nationally on solutions to HLB and its vector, the Asian citrus psyllid (ACP), and research progress on diverse solutions is promising, the citrus community currently is still limited by the basic approaches to HLB management that include: (1) maintaining extremely low ACP populations, (2) removing infected trees as soon as they are detected, and (3) replanting with healthy trees, while maintaining these trees free of HLB and ACP for the expected life of a productive tree.

The charge to the review panel by the CRB was to "objectively evaluate, at the highest scientific level, the HLB research currently funded by the CRB to have a positive impact on future California citrus production", with the outcome being "a thorough analysis of the work done by CRB-funded researchers on HLB and ACP that will help guide decision-making regarding future HLB research funding".

The review panel agreed that excellent progress has been made on medium to long-term solutions, including direct and indirect (pre-symptomatic) detection of HLB infections and risk-management to guide survey and sampling; ACP management using conventional and organic pesticides and biocontrol; biotech and classical breeding approaches toward tolerant/resistant citrus cultivars; therapies for treating infected trees including antimicrobial and nutritional treatments; virus-based vectors to deliver antimicrobials; and biotech approaches to ACP management by blocking the psyllid's ability to transmit *C*Las.

A key suggestion made by the panel for the overall planning of CRB research is to develop a "systems working group", where all relevant technologies are considered together to determine how best to deploy interrelated approaches and technologies. Rather than focusing on "research silos" that constitute the independent researcher paradigm, the CRB can lead groups with members representing the major research areas presented below to consider the entire biological system (host, pathogen, vector, environment, and associated organisms) toward maintaining a healthy citrus industry in the growing presence of the ACP and the HLB disease that ultimately follows. This team (or teams) of scientists could assist in directing research efforts to support a productive citrus industry of the future.

# Accomplishments from Individual Research Presentations

## Research Area 1:

### Early Detection/Survey and Sampling

**Risk-based survey for decision making in the management of HLB (Gottwald):** The risk-based surveys provide predictions of highest risk areas in residential and commercial citrus production for finding HLB-positive trees and psyllids. APHIS and CDFA base their surveys and sampling efforts on these models, which are re-evaluated every year. These models align well with the two residential neighborhoods where HLB was found; Hacienda Heights and San Gabriel. CDFA resources are targeted to high risk areas for survey and sampling, but require significant "manpower". There is 98% correlation between citrus transportation corridors and ACP incidence, thus ACP movement by these pathways must be stopped. Farmers' markets, budwood "swap meets", and certain ethnic celebrations that require particular citrus species are all opportunities for HLB-positive plants and psyllids to be distributed.

- **One survey and sampling effort by CDFA each year is not sufficient. Two to three surveys/year are needed that sample the same areas for re-testing.**
- **Opportunities for self-survey by growers are needed. Current methods deployed by CDFA are "manpower-intensive", and more resources are needed. Coordination between growers/industry and CDFA is especially important. How can that be affected, and where can growers send samples?**
- **Mixed landscape with residential and commercial properties are especially problematic.**
- **Precautions must be taken to avoid spread of ACP by trucks transporting citrus. Other options include packing of fruit where it is picked.**

**Please note**: the next three projects address direct detection of *C*Las. The five projects that follow address indirect detection of *C*Las-infection (termed "Early Detection Techniques").

**Improved detection of *C*Las in citrus and ACP (McCollum)**: How and where to sample within a tree is critical due to the uneven distribution of the *C*Las in the host plant. Latency of symptom expression also makes early detection problematic. Current, APHIS-approved HLB diagnosis is based on direct detection of *C*Las, which is coincident with the presence of HLB symptoms. But in California where citrus stubborn is endemic, those symptoms can be confused with HLB disease symptoms.

Current PCR and qPCR validated methods do not reflect false positives, but are more likely to provide false negatives. That is how the validated protocols are intended to work, but false negatives create the danger of infected trees going unnoticed while providing inoculum for the ACP. Thus, "absence of confirmation is not confirmation of absence". Absolute confirmation requires standard PCR plus the *C*Las 16S rDNA sequence.

- **Digital PCR (dPCR) results were strongly correlated with qPCR, but not as high-throughput as qPCR, and can be used as confirmatory test following qPCR, if approved by APHIS.**
- **The 16S rDNA primers for qPCR are transferrable to dPCR, with the sensitivity of detecting one copy number of *C*Las.**
- **Four labs performed a ring test using APHIS-approved protocol, and all were very consistent. It was apparent there was no need to run more than 38 cycles in qPCR, and any Ct value ≤ 38 should be considered suspect.**
- **Extensive greenhouse studies using infectious ACP to inoculate trees showed *C*Las transmission can occur in less than 24 hours. Thus sampling young flushes where ACP feed exclusively, especially if eggs/nymphs are present, improves the probability of detecting nascent *C*Las infections.**
- **A nested PCR was also developed as a confirmatory test, followed by restriction enzyme digestion and sequencing. APHIS is interested in the protocol. It improves the conventional PCR sensitivity for detection of *C*Las with 32 and 36 Ct values.**

**A phage/prophage-based PCR for sensitive and specific detection of *C*Las and *S. citri* (Chen)**: The goal is to improve the current PCR assay (based on the 16S rDNA), not replace it. The 16S rDNA has 3 copies in the *C*Las genome. The proof of concept for this study was completed in *S. citri*, where the prophage gene assay (over 10 copies) was more sensitive than using the spiralin gene (1 copy).

As more *C*Las positive trees are detected, those sequences will be needed to further understand the diversity of *C*Las in California. It is important to note that while these additional PCR techniques may increase sensitivity of detection, they do not get around the problem of where to sample in the tree for detection.

- **With samples tested so far, primers for the RNR (ribonuclease reductase) gene from *C*Las prophage is 3X more sensitive than the primers for the 16S rDNA, and are conserved across all 10 *C*Las genomes sequenced to date.**
- **APHIS is aware of this work and is engaged.**

- **The phage assay, although more sensitive, is still dependent on the presence of *C*Las in samples tested.**
- **The RNR primers detected two distinct phage types between the Hacienda Heights (type 2 prophage) and San Gabriel (type 1 prophage) samples.**

**Co-detection of *C*Las and *Spiroplasma citri* (Pagliaccia, Osman):** Mixed infections with *C*Las and *S. citri* create a unique problem for California, as citrus stubborn symptoms are similar to those of HLB. A multiplex assay was designed for these pathogens that works as well as a singleplex assay for each.

- **Multiplex assay detected all three Liberibacter species from around the world and with the Hacienda Heights sample.**
- **The cost for this assay is a few cents more than the singleplex.**
- **The *S. citri* Ct values were elevated, so may need better primer development, aside from the spiralin gene currently used. Perhaps this would benefit from the phage primers for *S. citri* as described in the Chen project.**

**Contained Research Facility; UC Davis: Effect of mixed infections on detection of HLB using two early detection methods (Godfrey):** The contained research facility (CRF) at UC Davis plays a vital role in providing the infrastructure to compare methods of direct *C*Las detection and indirect methods for detecting HLB-positive trees. The CRF was established to work with HLB under Biosafety Level 3 (BSL3) containment, under the direction of Kris Godfrey. Collaborators are using indirect methods of HLB infection compared to the APHIS-approved protocol of direct *C*Las detection. These methods include metabolomics, proteomics, volatile organic compounds, phytobiome communities, and small RNA (sRNA). Research will also test the sensitivity and specificity of *C*Las in mixed infections (with *Citrus tristeza virus* and citrus stubborn), and the ability of ACP to transmit *C*Las in mixed infections.

- **Preliminary studies in the CRF indicated that *C*Las infection is predicted by indirect methods prior to the *C*Las being detected directly with qPCR.**
- **To date, graft transmission of *C*Las works better than by using infectious ACP, but they are working on improving their ability to successfully infect trees using ACP, since this more closely represents what occurs in the field.**
- **The BSL3 facility in Riverside is anticipated to be open for business in 2017, and will help in capacity.**
- **The CRF will also plan for space to accommodate GE plants that will be evaluated for reactions to *C*Las.**
- **Space is limited, so those needing access should contact Kris Godfrey.**

**Comparative study of early detection techniques; Texas 2 (LeVesque, McRoberts):** The goal of this project was to compare the different approaches currently being considered as "early detection technologies", and to compare their effectiveness to qPCR. Eleven diagnostic procedures were evaluated in a blind study in Texas by testing trees that were known to be positive for HLB, known healthy trees, and trees with unknown status. Methods included: digital droplet PCR and qPCR (both direct assays for *C*Las), two methods based on volatile profiles, and one each using a metabolite assay, immunoassay of *C*Las effector proteins, phytobiome population differences, and small RNAs. The metabolomics and phytobiome assays discriminated between infected and healthy trees with high levels of accuracy, higher than qPCR, which was accurate for 80-90% of known samples, which was consistent across PCR labs. The qPCR tests were able to detect infected trees with a high level of accuracy – generally in the range from 80% to 90% if the initially tested branch was included (from previous samples), but this figure declined to around 25-30% when the initially-tested branch was excluded from the evaluation. There was little variation among the different PCR labs in terms of overall accuracy. One problem in the experimental design was that the healthy trees were grown in a screen house a significant distance from the field-grown trees, and that the numbers of trees per variety did not match the field trial.

- **Methods that target the whole tree (systemic) response show less inter-sample variation compared to the PCR approaches compared.**
- **A problem with the TX2 is that the "unknown" trees were not tented after sampling to prevent further feeding by ACP that potentially carry *C*Las, so the research team cannot go back to the same tree and be assured that its status of infection has not changed.**
- **Any method that can detect a systemic response to *C*Las should be put into the hands of the growers. Reaction by one of the EDTs is no cause for regulatory action, but it could trigger a high intensity tree sampling to account for within-tree variation for *C*Las by confirmatory PCR. How can this be coordinated with CDFA?**

**Biomarkers based on metabolomics for HLB detection (Slupsky):** Metabolomics is a measure of plant metabolism, not a generalized response, and differs with different pathogens. The goal is to use these biomarkers for early detection and test as markers to identify plant immunity/resistance. The system could detect HLB infection 6 weeks post inoculation vs 10 weeks for qPCR in the CRF at UC Davis. The same patterns appear in greenhouse trees that were ACP-infected in FL. Metabolic profiling for HLB specific detection improves with increased data. Leaves proved to be better samples than fruit and roots. This needs to be tested on different citrus relatives from citrus germplasm collections. Variety and regional differences alter metabolite concentrations, but infection with *C*Las can be discriminated from healthy or other disease states. According to Slupsky, the more samples we run the stronger the model becomes on identifying consistent patterns and develop high throughput.

Texas experiments indicated that the method performed very well (91-100%) and the sampling was not an issue for the method like qPCR. They are working on automating sample preparation and data analysis so the method can become high throughput. Method can be used to identify possible HLB "hot spots" to be followed up with qPCR or other confirmatory tests.

- **This method could be used to test new varieties for their reaction to HLB, as a faster method to screen for resistance or susceptibility.**
- **Unlike direct detection of *C*Las by PCR, this method is not completely dependent on where samples are taken.**
- **Different metabolic profiles are seen for HLB, stubborn, and CTV.**
- **From a survey of 700 trees in several counties, several trees are suspect and should be followed up with thorough sampling and direct *C*Las detection. This should be coordinated with CDFA.**

***C*Las-induced small RNAs for early diagnosis of HLB; understanding natural defense mechanisms (Jin):** Three small RNAs (sRNA) have been shown to be upregulated in citrus seedlings grafted with HLB-positive buds 2 months post grafting. The microRNAs 399, 1005, and 109 are induced in samples that have been *C*Las infected by ACP or by graft inoculation. These small RNAs are detectable throughout the tree, meaning that this approach for detection would overcome the problem of uneven distribution of the pathogen in the tree.

*C*Las-positive plants have reduced levels of phosphorus compared to healthy plants indicating possible phosphorus starvation upon infection. An experiment with University of Florida, of adding phosphorus to trees showed increase in vigor and yield, but impact in *C*Las titers was not tested. **Note:** Be careful about drawing conclusions about adding a specific nutrient to a tree and correlating it with therapeutic effects.

- **The HLB-tolerant rootstock hybrids US 897 and 942 had numerous sRNAs that were downregulated after *C*Las infection, but were not downregulated in the HLB-sensitive Cleopatra mandarin, so sRNAs may be used as biomarkers for resistance selection.**

- **US-942 takes up phosphorus more efficiently, and sRNAs indicative of phosphorus-starvation are highly elevated in US-942. Efficient phosphorus uptake may be why it is more tolerant/resistant to HLB.**

***C*Las effector proteins (Coaker)**: The goal of this research is to identify core effectors that are conserved across multiple *C*Las strains and apply this knowledge to diagnostics and opportunities to interfere with the HLB disease response. Effector proteins are required for infection, are produced by pathogen, and secreted into plant cells to cause disease. *C*Las infection significantly alters the phloem proteome and induces multiple host defense responses, including cysteine protease, which is induced post *C*Las infection.

- **Tissue blots immunoassay effectively detected effector proteins from plants that were inoculated by grafting the Hacienda Heights *C*Las isolate in the CRF at UC Davis.**
- **Cysteine proteases; secreted host immune proteases are selectively inhibited in citrus phloem. This creates potential targets for CRISPR/Cas9 or for transgenic plant development. If cysteine protease is overexpressed in the host, it can enhance immunity.**
- **Need to obtain as much *C*Las effector sequences as possible; about 13 core effectors from *C*Las have been sequenced.**
- **Coaker indicated a need for more *C*Las genomes to be sequenced**

# Research Area 2:

## Vector Management

This section addresses research on conventional and organic chemical management of the ACP and alternative ACP management strategies, including biocontrol.

**Optimizing chemical control of ACP in California (Morse):** California is buying time using chemicals to control ACP. IPM in Florida has been virtually eliminated due to pesticide use to minimize ACP populations throughout the citrus-growing areas of the State. California is trying not to upset the balance of IPM programs. California citrus flushing cycles are very different from what is seen in Florida and Texas; California experiences less flushing than other areas, based on low rainfall and timing of irrigation. There is an excellent biocontrol ongoing in California, and they want pesticides to be as least destructive as possible to the environment and balance of IPM.
This research is coordinated with numerous researchers and extension specialists who provide outreach. Information on the numerous insecticides for ACP control has been disseminated to the public in a variety of formats. The goal is to optimize biocontrol, manage resistance development to insecticides, optimize use of systemics (Imidacloprid most widely used neonicotinoid). The soil type and dry climate in California influences better soil-drench uptake of systemics compared to Florida. Frank Byrne is looking at soil amendments that may influence update of systemics.

- **ACP must be controlled in the presence of HLB; a few non-compliant growers can make HLB management ineffective.**
- **Timing of flushes is critical to coordinate with sprays. Can timing of flushes be managed on an areawide basis? There was extensive discussion at the meeting regarding managing flushes to correspond to insecticide application**
- **Need to incorporate new products as they become available and new products with different modes of action are needed to manage concerns over development of insect resistance to currently available pesticides.**
- **Need better organic pesticides with longer persistence**
- **Grower's participation in areawide treatments is essential**
- **Need to minimize costs while achieving adequate control.**
- **The current nursery pre-treatment protocol needs re-evaluation; shorten treatment interval prior to shipment is warranted, and determine if 150 days of control is achieved (CDFA is scoping proposal). From production nursery to retail outlet; need 30 days treatment prior to shipment. Should shorten that interval to 3-5 days, so would not have to re-treat in retail environment.**
- **A gap in the evaluation of alternative cultural practices was noted here. This includes the use of grove border strategies that would take advantage of findings in Florida showing that psyllid infestation often initiates at the edges of contiguous blocks. Edge management strategies could include border barriers, trap crops, or more frequent border pesticide applications. Effectiveness of these strategies in California needs to be evaluated.**

**ACP Management for Organic Citrus; Florida Studies (Qureshi):** ACP has completely changed the dynamics of IPM in Florida. Commercial, residential and abandoned groves are key to area-wide management. Dormant winter treatments are important to target adult ACP; eggs and nymphs not present at that time, BUT, warmer winters are problematic (winter of 2016) resulting in poorer control.

- **Organics + oil or soap; better than organics alone.**
- **Other parasite/predator populations have decreased significantly in Florida so they are hard to find in commercial citrus now.**
- **New organic materials needed; including GRAS and natural compounds.**
- ***Tamarixia radiata* has been released in all programs but are recovered more from organic groves than conventional.**
- **Biocontrol agents, parasites and predators, are still are not fully compatible with pesticides.**
- **Hard to find new organic materials that are effective AND persistent.**

- **Incentive or financial support is needed for organic growers to make additional applications. Because of the potential of organic groves becoming a source of infected psyllids, effective control of psyllids in organic groves is a high priority issue for all the citrus growers.**
- **Organic citrus production is a bigger industry in California than Florida, so important to extend research to California.**

**New trapping and control methods for ACP (Stelinski, Davis):** Prior research to find mating pheromones have not been successful for ACP. An effective lure was developed based on upregulated volatile signatures of HLB-positive citrus that are effective regardless of seasonal variability and citrus type. ACP are not sessile, and can move over two miles on their own capacity, and beyond 400 miles with the wind. ACP uses non-host plants to refuel when moving. Behavioral management is key to the success of a lure. Because ACP and HLB are at low levels in California, aggressive sampling is necessary to quickly identify the appearance of ACP and the ACP/HLB complex.

- **The lure is based on seven signature volatiles from the initial 13 identified, that were found to be the most effective in mimicking HLB-positive trees and altering ACP behavior; over two times the number of ACP trapped compared to un-baited traps used by researchers.**
- **3-D shapes with lure are more attractive than 2-D, with spheres more attractive than a cube.**
- **Application of lures: attract-and-kill, and monitoring of urban areas and grove borders, more accurate forecasting.**
- **This lure is currently being developed for application to a SPLAT product; two private companies, ISCA and Alpha-Scents, are interested. May be available within a year.**
- **No regulatory challenges with this approach**
- **Potential for better ACP counts and identification of hot spots**
- **Utility patent pending, chemicals in lures commonly available**
- **Does *C*Las alter olfactory responses of ACP? Not known.**
- **It is important that the ACP from traps can still be tested for *C*Las**
- ***C*Las detection in ACP invading a new area where HLB does not occur is considered critical in finding the pathogen in a new area as fast as possible –a find may trigger action by both growers and plant health regulators. Validation of early detection methods and linking to appropriate vector management practices is an important area to continue to move forward.**

**Mass-rearing methods for *Tamarixia radiata*, to support biocontrol (Stouthamer):** Extensive research to establish genetically stable and diverse populations of *T. radiata* has been successful. *Diaphorencyrtis aligarhensis* is also an important parasitoid, but work has not advanced as much as *T. radiata.* The parasitoids are given optimal chances of successful establishment by maintaining genetic diversity of populations released. Collectively, researchers have taken a well thought-out and methodical approach that has demonstrated promising results based on establishment of the parasitoids and observed levels of parasitism. Repetitive use of insecticides against ACP generally negates effective biological control, therefore it seems unlikely that biological control will be effective in managed groves when aggressive pesticide treatments are applied. However, presented research suggested they may function in attempts to reduce urban/door yard psyllid populations, and should be deployed in "high risk" areas of citrus production that interface with door yard citrus regions and transportation routes.

- **Over 3 million *T. radiata* released in Southern California, and found about 8 miles from release sites. Over 250,000 *D. aligarhensis* released. Predators reduce ACP density.**
- **How to sustain costly biocontrol programs? Natural rearing may work in the field using curry leaf, which serves as a host for ACP, but apparently *C*Las does not replicate in curry leaf.**

  **Analysis of diverse ACP predators showed that lady beetles, lacewings and spiders are prevalent and should be considered for non-chemical control of ACP.**

**Release and monitoring of *T. radiata* in Southern California, and supplemental biocontrol by *D. aligarhensis* (Hoddle)*:*** This is a continuation and extension of the initial research to develop stable populations of *T. radiata* for release in California. Natural enemies of ACP are in Pakistan, and the Punjab region where they were collected has a 70% climate match with California (except for the monsoon season!). Many years ago, nine species of parasitoids were reported attacking ACP; most were found to be parasitoids of the parasitoids (hyperparasitoids), so not useful for ACP biocontrol.

- **Problem with establishing parasitoids: ants tend ACP nymphs and guard them from parasitoids, so parasitoids need to be protected from ants. Have demonstrated that putting biodegradable food that has a low level of insecticide in it provides effective ant control. The ants carry the bait back to queen and kill her and the colony. Organic growers need an alternative to the bait that the chemical company provides. Boric acid works well, but organic use of product is considered too small to warrant product development/regulatory costs. Need support to bring organic alternatives to market.**
- **Parasitoids have established up to 8 miles from release point, and they carry the Pakistani genetic fingerprint.**

- **A gap in evaluation of alternative cultural practices was noted here. This include the use of grove border strategies that would take advantage of findings in Florida showing that psyllid infestation often initiates at the edges of contiguous blocks. Edge management strategies could include border barriers, trap crops, or more frequent border pesticide applications. Effectiveness of these strategies in California needs to be evaluated.**
- **One area that seems to be missing is the screening of natural or Generally Regarded as Safe (GRAS) chemicals for their efficacy in managing the vector and/or the bacterium. Chemicals discovered using this strategy would have a less costly and quicker regulatory approval pathway. A collaborative effort between Florida and California would accelerate discovery. This area of work should cover, not only chemical screening, but also development of delivery methods that optimize control performance.**
- ***T. radiata* attacks 5th instar of ACP and *D. aligharensis* attacks the 3-4th instar; complimentary agents.**
- **How to sustain costly biocontrol programs? Natural rearing may work in the field using curry leaf, which serves as a host for ACP, but *C*Las does not replicate in curry leaf. Need more research/evaluation in optimizing release strategies.**

# Research Area 3:

## Host Genetics/Resistance Development

**New approach to transformation: Use of Founder Lines for improved citrus biotechnology via RMCE (Thomson):** Recombinase Mediated Cassette Exchange (RMCE) has been applied to integrate AND excise DNA into and out of host organisms using homologous recombination. This site-specific recombination technology has been demonstrated to effectively resolve complex transgene insertions to single copy, remove unwanted DNA (selectable markers), and precisely insert DNA into known genomic target sites. Thus, this approach helps speed the transition from laboratory manipulation to field production, and should help in the regulatory approval process. “Founder lines” refer to individual citrus plants that have been genetically engineered to contain a gene sequence (“landing pad”) that allows a precise integration of desired traits and ensure predictable gene expression with high and consistent transgene activity without disrupting the activity of nearby native genes “drop-in technology”. To date, 123 transgenic lines from Carrizo and all 15 candidate sweet orange founder lines were confirmed to each contain a single copy of the transgene required for further genetic manipulations. Several different disease defense genes have been incorporated into various citrus backgrounds.

- **Recombinase technology is not CRISPR, Zn fingers or TALEN technology, thus is relatively new to regulatory agencies required for approval; APHIS, EPA, and FDA. This approach should streamline the regulatory process.**
- **Trying to stack several candidate genes and work on marker removal technologies. Hope to get material to the CCPP in a year to put lines in the field for evaluation. EPA, APHIS and FDA will have to approve any lines for commercialization could be a problem/delay. Also public perception is important for commercial use of this technology.**
- **This technology is providing a platform for expression of genes required for HLB management that is faster than other systems. This project is also working on transformation technologies for citrus and the development of consumer friendly transgenic citrus.**
- **A lot of plants are available for evaluation NOW! What next? The goal is that within two years this system will be ready for CCPP disease testing through Georgios Vidalakis for field trial and clonal scale-up.**
- **The CRF needs to provide space for challenging these plants. Kris Godfrey will work with Jim Thomson to that end.**

**Development of consumer-friendly transgenic citrus with potential broad spectrum resistance to HLB, citrus canker and Phytophthora root rot (Louzada):** For these experiments, a citrus gene that triggers calcium signals called CSM1 gene, and an additional gene involved in salicylic acid pathway- NPR1, were used. CSM1 gene triggers Ca+ signals downstream of effector-mediated resistance. This is a consumer-friendly approach, with no antibiotic or other genetic markers left in the plant, using the RMCE method, in collaboration with J. Thomson.

- **Excellent resistance responses have been observed to Phytophthora and citrus canker in transgenic citrus. HLB response is being evaluated now.**
- **Molecular markers were eliminated from transgenic Pineapple orange and Carrizo citrange using recombinase technology while maintaining the gene of interest.**
- **The establishment of the RMCE method in citrus to remove markers from transgenic plants creates the basis for the production of transgenic plants containing only plant DNA (including promoters and terminators), and thus less burdensome for the regulatory process.**

**Rapid cycling plant breeding in citrus (Moore)**: Goal of this work to promote precocious flowering in juvenile citrus using transgenic/ environmental/ or other new methodologies. These early flowering plants will produce HLB resistant citrus types that do not have to be regulated and/or overly treated with pesticides or nutrition. Early flowering gene allows for flowering, pollen and fruit production. This approach will speed the design cycle of plant generation and evaluation.

- **Flowers, fertile pollen, and fruit have been produced in the greenhouse and *in vitro* culture using these methods.**
- **This can greatly speed the process of breeding**
- **Goal is to develop methodologies for citrus improvement that do not involve genetic transformation or require regulations**
- **CTV vector (Dawson lab) has also been used to transiently induce flowering and fruiting in young greenhouse-grown trees using *ciFT3* TALEN promoter.**

**Evaluation of hybrids of citrus and citrus relatives for resistance/tolerance to HLB (Ramadugu):** Based on six years of field trails of over 100 accessions of Aurantoidea done at Fort Pierce, resistant types identified, and resistance was inherited. Sources of plant materials were from the USDA-ARS National Clonal Citrus and Date Germplasm Repository in Riverside, CA.

*Microcitrus* and *Eremocitrus* genera in particular show resistance to HLB; they are separated from citrus in evolution by 3.5 million years, and may have developed different mechanism of resistance from that seen in *Poncirus* types. Resistance reactions to HLB have been demonstrated in the field and greenhouse using hot psyllids in Ft. Pierce.

- **Conducted wide crosses using certain mandarins and trifoliates as female parents and citrus relatives as male parents.**
- **37 crosses made between these hosts and commercial citrus varieties, and hundreds of hybrids are to be evaluated in McCollum's lab. Also graft inoculation *C*Las challenge experiments have started in California.**
- **Can these genera provide resistance or some protection to susceptible trees using approach grafting/inarching or intergrafts?**
- **Transcriptome analyses are needed for these hybrids, and markers are needed to identify host response for screening potentially resistant/tolerant/immune progeny.**

**Citrus Clonal Protection Program (CCPP)-CORE Program (Vidalakis):** an excellent presentation was made regarding this UC Riverside program, led by Georgios Vidalakis. This program provides a safe mechanism for introduction of citrus varieties into California, and maintains primary sources of disease tested & true-to-type citrus trees. Not only HLB but all known vegetatively-propagated pathogens of citrus are eliminated and the goal is to flood the State with certified, pathogen-tested citrus varieties.

- **There is extensive outreach through the CCPP that is aimed at providing clean materials for homeowners and growers.**

- **Over 100 "clean" rootstocks and scions are in the process of being imported into California from Florida that have been shown to be somewhat tolerant to HLB, and are less likely to succumb to the disease.**
- **Focusing development of high-throughput assays to complement the lengthy bioassays.**

**Infrastructure Support for Research on Detection and Management of Huanglongbing and Asian Citrus Psyllid (Godfrey):** Project goal is the production of plants and insects for researchers to use in their studies that deal with various aspects of HLB and/or ACP management. Research projects include early detection methods (detection of *C*Las prior to symptom development), evaluation of rutaceous plant material for tolerance or resistance to *C*Las, detection of *C*Las in mixed infections with other phloem-limited pathogens, and efficacy of organic insecticides against ACP nymphs and/or adults.

**Progress on desired outcomes at CRF:**

- **Focusing development of high-throughput assays will complement the lengthy bioassays.**
- **Early Detection Studies – Three sets of experimental plants have been generated for early detection studies.**
- **All of the pathogens (*CTV*, *S. citri*, *C*Las) are established in host plants at the CRF for studies aimed at detection of these pathogens in mixed infections.**
- **Buds of various rutaceous plants have been grafted onto Carrizo rootstock and are growing. When the plants are large enough, they will be graft inoculated and then insect inoculated with *C*Las.**
- **Five organic insecticide products have been tested under laboratory conditions for efficacy against ACP adults and nymphs.**

# Research Area 4:

## Host/Pathogen/Vector Interactions

**Root phytobiome analysis applied to citrus tree health (Leveau, Rolshausen):** Numerous fungal, bacterial species have been identified from the citrus phytobiome (epiphytes and endophytes) in an effort to prescribe soil amendments that prevent or treat *C*Las infection for biocontrol-based management of HLB-impacted trees. This approach has also shown promise as an indirect method of HLB diagnosis in the field (TX2) that correlates well with *C*Las infection.

Distinct differences were observed between the phytobiome of healthy and HLB-positive citrus trees in TX2 experiment, and this approach was highly sensitive in identifying *C*Las-positive trees. It is important to note that the infected and healthy trees were not maintained under the same conditions: the healthy trees were grown in a screen house several miles away.

As the database grows of microbiota associated with citrus, several candidate microbial biomarkers have been identified that predict HLB/*C*Las status of trees with high accuracy using leaf swab method.

**Development of a novel target basis of anti-virulence strategy for controlling HLB (Lin):** The orthologous gene replacement method developed in this project provides a valuable tool for functional analyses of virulence gene in the unculturable *C*Las. Using *Xylella fastidiosa* (*Xf*) as a culturable surrogate (and also causes Citrus Variegated Chlorosis in citrus), specific genes in *Xf* were knocked out and replaced with orthologous gene from *C*Las, restoring gene functions. For example, (1) the twitching motility of the *Xf* cells was restored in culture when the Las *pilG* gene was inserted into *Xf* cells with the PilG knockout and (2) *in planta* experiment demonstrated that grapevines developed typical Pierce's disease when they were infected with the wild type strain of *Xf* or mutant strain complemented with *pilG* of *C*Las, suggesting that the *pilG* gene in *C*Las is required for pathogenicity.

- **Specific anti-virulence compounds have been identified in this study could provide an effective way to suppress HLB.**
- **Numerous anti-virulence compounds are being evaluated that impact the survival of *C*Las in the host. These are small molecules that are FDA approved, and are commercially available. This should speed regulatory approval.**
- **Anti-virulence molecules have no selection pressure on bacterial survival, thus no resistance will be developed, compared to antibiotics that are currently being used in Florida under a Section 18 label.**

**Photosynthate-Responsive Polymeric Nano-Carriers for Phloem-Specific Delivery in the Treatment of HLB (Summerlin):** Polymeric nanoparticles that are designed to release encapsulated cargo when exposed to elevated concentrations of sugars can be employed to facilitate delivery of active compounds specifically to the phloem to treat HLB-infected citrus trees due to changes in environmental conditions (pH, sugars). Targeted delivery and controlled release to the phloem can lead to increased efficacy and delivery of active compounds (e.g., antibiotics, nutrients). The polymer degrades into harmless amino acids.

- **Sugar-responsive nanoparticles have been synthesized and characterized that are capable of release of antibiotics and nutrients into the phloem.**

- **Proof of concept has been shown for diabetes, where a change in the surrounding environment (sugar levels) triggers release of insulin.**
- **Uptake in nanoparticles has been demonstrated in citrus cell culture (G. Moore lab) with no toxic effects.**
- **This approach should allow direct administration by growers to infected trees (injection and eventually spraying), thereby halting the effects of the HLB-causing *C*Las bacterium and limiting its proliferation via psyllids.**
- **Can this be delivered to the ACP and target it instead of the *C*Las in the host?**

**Artificial microRNA-based targeting of the ACP for HLB management (Falk, Kuo):** Engineering artificial-microRNA (amiRNA) to target mRNA in ACP genome, and analyzing naturally occurring ACP miRNAs to target and down-regulate specific ACP mRNAs.

- **Specific amiRNAs were identified from ACP and expressed in *Nicotiana benthamiana* at different levels using TMV, TAV (begomovirus component A that does not have the coat protein gene), and a binary expression vector.**
- **The abundance of two specific miRNAs were confirmed, and they are engineering amiRNAs to test against *D. citri*.**
- **Multiple amiRNAs will be cloned into binary vector for plant transformation or TAV to express in plants and evaluate for impact on ACP feeding and survival.**
- **CTV cannot be used to express amiRNAs and so therapies would need to involve transgenic trees or the use of other vectors. Possible uses of geminivirus vectors as an alternative was briefly discussed, but knowledge within the group as to its potential was limited. Further discussion with plant virologists may be useful in identifying alternative vectors.**

**Construction of a cloned infectious cDNA of *Citrus tristeza virus* (California isolate): a critical step in developing the tool for RNA interference-mediated inhibition of insect pests and pathogens of citrus in California (Ng, Yokomi):** CTV is a virus of citrus, with numerous strains identified, including a mild, virtually asymptomatic strain, that is associated with most commercial citrus trees in California. This approach has benefits over transgenic citrus development based on time to product, the possibility of providing therapy to infected trees, and faster commercialization. This approach has been well-documented by the Dawson lab for the Florida CTV isolate (T36). Drs. Ng and Yokomi are working to develop vectors using the California strains T30, T36 and RB (resistance breaking). The Dawson lab has demonstrated the efficacy of anti-microbials delivered via the CTV vector to citrus canker and HLB, and others have shown deformation of ACP wings when fed on an RNAi construct using Dawson's CTV vector.

- **Proof of concept for an intermediate consisting of some California isolates and some Florida isolates has been demonstrated in *N. benthamiana* with GFP**
- **There are many applications to this technology, but most significant for this is RNAi targeting ACP development.**
- **Currently there is bottleneck (Ng lab) in generating a fully CA strain in that some parts seem unclonable using standard approaches. A more costly, but perhaps time-saving strategy involving gene synthesis were discussed as options.**
- **It was also discussed that CTV-based expression is not permanent. The virus eventually kicks out the cargo gene. In addition, issues of naturally occurring infections and the ability of a new CA strain to infect previously infected plants (superinfection resistance) was brought up. Both of these issues are important in considering the long-term sustainability of CTV-based disease prevention/psyllid-killing strategies. They are not relevant for proof of principal experiments that will ultimately utilize transgenic trees.**

**Proteomics applied to HLB biomarkers and ACP transmission interference (Cilia):** The goal of this research is to develop novel approaches to blocking transmission using Protein Interaction Reporter (PIR) technology for characterization of protein interactions that regulate transmission of *C*Las by psyllids, and develop inhibitors of these interactions as insect vector control agents. Another component of this technology is to identify specific biomarkers that can be used as an early detection method.

- **Preliminary results from protein biomarkers demonstrate alignment with the metabolomics approach 2 weeks post-grafting, when used to test *C*Las-positive and healthy citrus in the CRF.**
- **Over 25 protein interactions identified between *C*Las and ACP gut.**
- **PIR technology was successfully applied to identify 21 protein interactions which are high-value targets to potentially disrupt *C*Las transmission by the ACP: 11 protein interactions involve *C*Las proteins, while 10 involve proteins of bacterial endosymbionts of the ACP.**
- **Two proteins in particular were identified; one of which is a phage protein with antimicrobial activity to *E.coli* in culture, and an ACP hemoglobin protein that shows multiple interactions between ACP and *C*Las.**
- **Different ACP populations vary in their ability to acquire *C*Las and acquisition and this was demonstrated to be a heritable trait.**
- **Populations of the ACP that vary in their ability to transmit *C*Las were characterized as well as the molecular and cellular responses of the ACP to *C*Las acquisition and transmission. Thus, there may be opportunities to decouple and target both acquisition and transmission of *C*Las.**
- **A significant advance in this work is getting ACP to develop from nymphs to adults, and lay eggs on artificial media. Getting about 80% survival of adults over 10 days.**

# Appendix 1:
# Detailed Discussions Organized by Research Area

## Discussion from Early Detection/Survey and Sampling

Direct methods (qPCR, dPCR, and conventional PCR or PCR followed by PCR product sequencing) of *C*Las (and associated phage DNA) detection is dependent on the presence of *C*Las in the sample. That, combined with the fact that the distribution of *C*Las in a tree is uneven, the chances of finding a leaf that gives a positive reaction leads to a high chance of false negatives, although false positives are exceedingly rare. This creates a fundamental problem in sampling asymptomatic trees directly.

Evidence was presented by multiple groups demonstrating that systemic HLB/*C*Las-specific signals could be detected earlier than PCR. This work constitutes one of the most important developments in the field. These "indirect" EDTs include metabolite profiles, plant proteases and *C*Las effector protein identification, volatile organic compound signatures of infection, plant surface microbiota identification, proteome profiles, and specific microRNAs associated with *C*Las. A significant advantage of these methods is that there is no need to sample symptomatic tissue (as with direct methods), since the signal appears to be more systemically distributed in the plant as compared to the *C*Las itself. This has not yet been quantified by any method yet, but reports from controlled greenhouse experiments show earlier detection from graft-inoculated trees, and two methods in particular (*C*Las specific metabolite profiles and leaf surface microbiota) were more sensitive than the APHIS-approved qPCR in detecting known positives in the TX2 field trials.

There is still much work to be done before any indirect method is validated. This includes understanding how these methods respond to other stresses and diseases of citrus, including citrus stubborn, Tristeza, Phytophthora, and other biotic or abiotic stresses.

It was suggested that the indirect methods of *C*Las infection could be deployed in a "tiered" approach to diagnosis. Any tree that is suspect, based on one or more indirect methods, could then be exhaustively tested for *C*Las using the APHIS-approved protocol. This must be done in partnerships among CDFA and the citrus industry, represented by CRB. This also is a means of developing more confidence in the indirect detection methods, and separating HLB-positive reactions from other stresses. CRB could incentivize movement toward commercialization through use of staged bounties that reflect real progress on sensitivity and cost. Thus, a top-down approach by CRB to evaluate and test these methods with the goal of commercialization is warranted. Since space in California is limited, Florida and Texas could be utilized for such tests.

- The CRB is supporting excellent research, but implementation is needed. California cannot wait for perfect information. We need to use the tools we currently have, and adapt/replace as better ones develop.
- Participatory research with the CDFA is needed to validate these methods, and help with sampling and to inform vector management. Metabolomics and phytobiome data are very impressive in the CRF and Texas 2 studies. Even if these methods are not perfect, they are better than waiting for APHIS-approved methods. By then it is too late.
- Is there any evidence of a positive EDT that results in full-blown HLB? This needs to be determined, and shows a flaw in the Texas 2 study, where "unknown" trees were not tented after sampling to follow up.
- There is only one place in the world where a large company (Cambuhy) in Brazil has been able to keep HLB incidence to a 2-3% level, simply by managing the ACP population, rouging trees when symptoms are expressed, and replanting with healthy trees. Southern Gardens in Florida is following the same management strategies with comparable results.

## Discussion from Vector Management

Because ACP infestations and *C*Las infections are at an incipient level in California, aggressive sampling is necessary to quickly identify appearance of the insect and insect/disease complex. In Florida, growers confronted with ACP and HLB benefit by routine scouting and sampling trees to track ACP infestations, time insecticide applications, and the evaluate efficacy of the applications. Also, Florida experiences indicate that it is beneficial in a monitoring program to relate ACP abundance to information on the presence, abundance and developmental stage of flush. Catching a flush as it begins to develop is important for timing insecticide applications. *C*Las detection in ACP invading a new area where HLB does not occur is considered critical in finding the pathogen in a new area as fast as possible –a find may trigger action by both growers and regulators. Validation of early detection methods and linking to appropriate vector management practices is an important area to continue to move forward.

**Chemical control:** Conventional insecticides have been the primary direct tactic for reducing ACP population levels, and a number are labelled for use and can be effective in conventional citrus. In Florida, insecticides in conventional citrus can be applied during the winter months when ACP populations are often at low levels, and can offset the need for additional applications for several months. But, it has been demonstrated that in California, fluctuations in psyllid population dynamics do not follow the same trends documented in Florida. Evaluations of best insecticide timings for California psyllid control remains an important area. Also, insecticides applied on an area-wide, coordinated basis is needed. Research incorporates resistance management of the ACP to insecticides, thus rotation is included in outreach and education.

Chemicals for ACP control in organic citrus are not as effective as conventional insecticides, and ACP spray programs for organic citrus need further attention. Pyganic in combination with insecticidal soap holds some promise, but in general, due to lower levels of toxicity and shorter residual periods of control, chemicals labeled for insect control in organic citrus should be applied more frequently than in conventional citrus. This could render the costs too high for an organic grower. Group discussions included the possibilities of providing some economic support to these growers to help keep them in business and provide better area-wide control of ACP. Discussions suggested that, because of the potential of organic groves becoming a source of infected psyllids, effective control of psyllids in organic groves is a high priority issue for all the citrus growers.

**Biocontrol**: ACP is known to be attacked by two parasitoid species (*Tamarixia radiata* and *Diaphorencyrtus aligharensis*), both of which have been imported, raised in high numbers, and released in areas of southern California. A number of indigenous predators of ACP have been identified in California citrus and some species consume many ACP. With respect to vector management, while biological control (parasitoids, predators or pathogens) can be utilized as a direct applied control tactic, for ACP to-date the primary use of biological control has been as an indirect tactic – i.e., make sure natural enemies are established, conserve them, but otherwise leave them alone to help suppress ACP populations. Collectively, researchers have taken a well thought out and methodical approach that has demonstrated promising results (up to 75% parasitism) based on establishment of the parasitoids and observed levels of parasitism. Repetitive use of insecticides against ACP generally negates effective biological control, therefore it seems it is unlikely that biological control will be effective in managed groves when aggressive pesticide treatments are applied. However, presented research suggested they may function in attempts to reduce urban/door yard psyllid populations. If disease resistance is ultimately achieved in commercial citrus varieties, the efforts to establish biocontrol agents now should be valuable in the future. The biocontrol programs are not sustainable without enormous resources of staff, infrastructure, and funding. Questions were raised regarding the possibility of a "natural insectary" in California, where a host like curry leaf (preferred host for the ACP, but apparently is not a host for *C*Las) could be planted in areas to encourage development and survival of the parasitoids. The work is important, but the concern is that this is fundamentally different from a situation in which biological control is being used to decrease losses simply by plant feeding.

It would be interesting to select for *Tamarixia* that are resistant to relevant insecticides. This should be possible, and would allow for better synergy with other control. That said, since *Tamarixia* is a specialist, it simply cannot exist without the psyllid and thus is unlikely to bring about local or area-wide extinction. But, it could help with home-based control in that it does not require human intervention. And of course, insecticide-tolerant versions would be considered natural, since they don't involve transgenesis.

**Lures, attractants:** If a potent ACP attractant could be found, it might be useful not only as a sampling/monitoring tool but also as a tool for ACP mating disruption or for attracting ACP to an eradication device charged with phagostimulants (feeding incitements) and a control chemical. There is negativity by some that no strong attractant for ACP will ever be found based on efforts over the past 10 years and the fact that no strong chemical mating pheromones are known for any psyllid species anywhere. Yet some researchers continue their efforts and presented results in this area suggest further research should continue.

No estimate of the total cost per liter or some other measure was given. Also, it was unclear how these would best be distributed so as to attract and kill, but not desensitize the olfactory system. Finally, how does one demonstrate that such a system does not have the unintended effect of attracting psyllids from a larger catchment area to the grove, with the net negative effect of trapping and killing some, but also increasing the local concentration of psyllids from distinct sources, which could increase the probability of infected individuals getting into the crop.

We see a gap in the evaluation of alternative cultural practices. This include the use of grove border strategies that would take advantage of findings in Florida showing that psyllid infestation often initiates at the edges of contiguous blocks. Edge management strategies could include border barriers, trap crops, or more frequent border pesticide applications. Effectiveness of these strategies in California needs to be evaluated. Another cultural practice being evaluated in Florida is the use of reflective mulches on the ground below trees to reduce ACP infestations. As discussed during the session, other direct management tactics that could be explored include discouraging natural flushes and those induced by topping or hedging – citrus horticulturalists, plant botanists and plant physiologists could be consulted in efforts to determine if and when flushes could be discouraged to eliminate ACP breeding sites. A breakthrough (even a moderate advance) in managing HLB via plant resistance/tolerance to *C*Las would allow growers to reduce the use of insecticides and thus better capitalize on biocontrol but this remains a medium to long-term deliverable.

A general need is seen in the development of new control options. Some new methods involving genetics/biotechnology have been presented in other sessions at this meeting; however, an area that seems to be missing is the screening of natural or Generally Regarded as Safe (GRAS) chemicals for their efficacy in managing the vector and/or the bacterium. Although not an elegant or typical research project, the advantage of this strategy is that chemicals discovered in this research would have a less costly and quicker regulatory approval pathway. There is work in this area occurring in Florida, but a collaborative effort between Florida and California would accelerate discovery. This area of work should cover not only molecule screening, but also development of delivery methods that optimize control performance. Based on open discussions at the review, the only effective HLB management program currently available is the three-pronged program: only plant trees known to be free of HLB and protect them from ACP infestation for its productive life, establish a strong ACP insecticide control program, and locate and remove diseased trees as fast as possible. Until an alternative is available (e.g., HLB-tolerant citrus), growers need to be prepared for locating and removing infected trees.

**The perceived needs are listed below:**

- **Continue funding some research on ACP chemical control. Specific product availability will change over time and close interactions with companies developing new chemistries will foster this work. This should also include involvement in research efforts to evaluate natural product/GRAS chemicals and novel delivery strategies for these. This work would support the California citrus industry's reputation as a good steward of the environment and beneficial organisms.**
- **Establish new or expanded efforts to develop ACP control tactics in organic citrus. Fund projects to evaluate ACP seasonal ecology in California citrus and relate the findings to environmental conditions, flushing patterns, and grower practices.**
- **Financially and programmatically assist extension specialists in outreach efforts to establish formal scouting programs for ACP to include assessments of ACP infestation levels and flush abundance over time.**
- **Evaluating ACP scouting and management programs in citrus before HLB becomes an issue would be extremely valuable and would get growers ready for HLB. The ACP scenario in California might end up being quite different than in Florida or Texas due to differences in environments and soil types. This should include research supporting the development of most effective action plans that link vector management practices with best monitoring practices. It seems reasonable that these action plans would evolve based on the status of psyllid and HLB incursion into production areas (i.e. some strategies will be more reasonable to employ for urban/door yard control while others are effective at the commercial production level).**
- **Entice collaborations with plant physiologists and botanists in efforts to investigate manipulating tree flushing patterns as an effort to reduce ACP population levels. Although coordination of control with flushing is important, there was not a clear sense that anything could be done, except at a very local level. Nonetheless, it was argued that it would be good to include whatever knowledge is available about flush timing so as to be able to predict best practices for intervention.**

## Discussion from Host Genetics/Resistance Development

A number of different, potential anti-pathogen genes have been incorporated into various citrus genomic backgrounds, including sweet orange types. These need to be tested in a coordinated fashion. In discussion it became clear that some of these are general stress response genes that may be expected to have some fitness or other cost to the tree. It will be important to prioritize the large number of potential transgenics coming down the pipeline, at least if they are to be characterized in California. Evaluation will need to be done in the CRF, for which space is limited. If material can be shipped to Florida for field trials, this would be preferred. Regardless of where the tests are done, there needs to be a central body that coordinates how the tests are done so that results can be compared across a common set of platforms. This is particularly important if different varieties are to be tested.

There was much discussion about the need for coordination across those developing genetic resistance, regardless of the approach. A key theme is to get away from the independent PI paradigm and into something a bit more structured, perhaps a bit more like research departments in a biotech company, who are being funded, etc., but with clear goals and top down milestones and management.

Site specific recombinases can be used to insert genes at specific positions in the citrus genome. These sites essentially constitute landing pads ("founder lines") into which different transgenes can be inserted. There is long term utility in this technology because it means that if a good site is identified that supports long term expression in phloem it can be used for other genes. The regulatory agencies have not weighed in on this RMCE technology, and it is different from other techniques currently employed for this purpose (CRISPR, TALEN Zn fingers, etc.). That said, these are still very long term experiments. Founder lines are in Florida ready to be further engineered. Researchers in Texas have transformed plants in hand that show excellent resistance to citrus canker and to Phytophthora root rot, and tests are ongoing to evaluate them for resistance or susceptibility to HLB.

The use of genes that promote rapid flowering is a promising technology, and has been successfully applied to *Prunus* species. The technique clearly still needs optimization. In addition to stimulating early flowering through overexpressing (transiently or permanently) genes such as *FT*, one need is a way of transiently silencing "anti-flowering" genes such as *TFL* at just the right time. What was described was the use of the CRISPR system to create mutant versions of the *TFL* gene, using agro-infiltration. This may also work, but could still be problematic if the gene is then permanently inactivated. It comes down to when the gene is silenced, and also knowing if silencing of only one gene is sufficient. It would be important to know if the literature teaches us any other tricks that have been used in other plant systems to make this work. In short, the work should be supported, and it may be useful to call in other experts on early flowering from other systems, where more manipulations have been successfully accomplished.

The work with citrus relatives with resistance to HLB is promising. Collaborations are valuable, and hybrids are being trialed in Florida. A system for evaluating young hybrids for resistance or susceptibility is needed. Several collaborators might help adapt their approach for diagnostics or other genomic analyses to evaluation of response to *C*Las inoculation.

To summarize specific recommendations by panel members, the breeding programs and the genetic engineering will continue to need greater communication and coordination. In the breeding, to develop a greater understanding of what germplasm is available with potential resistance to HLB, what germplasm is currently being utilized in breeding, how progeny of HLB resistance crosses are reacting to HLB, the most rapid and effective breeding approaches including rapid cycle breeding, and the most rapid and effective HLB resistance screening approaches. In the area of genetic engineering, there needs to be some type of "clearing house" to gather information on the resistance genes being used by CRB-funded projects, the vectors (promoters, terminators, and other genetic components) that they are being used with, what citrus types have been engineered utilizing these genes/vectors, and what are the results in terms of HLB resistance and unintended effects. Also, of the list of genes being tested, the potential of each to receive regulatory approval should be evaluated through interactions with the regulatory agencies. Work with those approaches that have little chance of approval would appear not to be suitable for CRB funding, but could be directed into other funding sources especially if the particular genes are of special interest. The intellectual property issues surrounding the genes and constructs being utilized require investigation so that time and money is not wasted on producing HLB resistant citrus varieties that cannot be commercialized due to IP constraints.

The best case scenario would be for the CRB-funded programs to work closely with the Florida and Texas programs in all of these genetic-based approaches to HLB resistance and the issues surrounding the technologies. Genetic resistance holds promise for long-term, stable control of HLB, but the R&D in this area must be consistently pushed to work as efficiently and as rapidly as possible to produce real products approved for cultivation and made available to growers as quickly as possible at a price that they can afford.

A suggestion was made to convene a research summit focused on transgenic development of perennial trees. That could include leaders in *Prunus* species, walnut, Arctic Apple®, American chestnut, and poplar.

## Discussion from Host/Pathogen/Vector Interactions

Research with viral vectors, small RNAs should continue, and build on what has been done in Florida with the T36 CTV isolate. The question of why a separate CTV vector needs to be made from the California isolate came up. This should be verified: that the Florida CTV vector could not be used in California.

What are the roadblocks to making proteomics cheap and high throughput; are there things that could be done to make it so? Is it feasible as a practical EDT or instead as a means of discovering the molecules and then moving to yeast or another detection system?

As discussed throughout the meeting, a tiered approach to detection was suggested, and represents a main outcome of the meeting. However, discussions and decisions on what techniques/protocols should be used in the tiered approach needs to occur, with possible redirection of funding to focus on refining and testing the selected assays.

A concern was expressed from researchers: how can we get help with Big Data capacity? ARS has an initiative in this area, and wants to contribute and be involved in any way needed.

# Appendix 2:
# Short Term Solutions

In the introduction, it was stated that there is an abundance of excellent mid-and long-term research. But the California citrus industry needs to take whatever steps necessary to stop further spread of the ACP within California and to neighboring states. But the concern was: **what do growers do in the meantime, until these longer-term solutions are ready for deployment?** The following points were taken from the discussion where all participants' viewpoints were captured.

- **Tarp all truck shipping fruit or nursery stock (spray to ensure tarping does not just distribute psyllids throughout the drive), and pack the citrus where it is harvested.**
- **Can partnerships be developed with Mexico, regarding incoming shipments to restrict ACP movement via transportation corridors from the south?**
- **Related problems arise from farmers' markets, swap meets, etc. Understanding these flows (where does the produce come from and where is it going) should be incorporated into the models if possible. It will also be critical to begin actively monitoring abandoned groves and organic growers. Since organic growers must often work harder to achieve the same control effects, they are at particular risk.**
- **The CCPP is a critical for clean budwood production and distribution, and is engaged in the outreach efforts that identify the needs of diverse ethnic populations to provide sources of clean material for their specific uses.**
- **Currently, one cycle of monitoring is done each year. This is not enough, and 2-3 cycles would be optimal, going back to the original sites for re-evaluation. This is costly, so added resources are needed for CDFA to achieve this goal. Could the EDT's, or indirect assays, be used to serve as potential monitors of disease for this purpose, as long as they are inexpensive and easy to deploy?**
- **Although the extension and outreach efforts are laudable, could more emphasis be placed on "stop the psyllid" with the current "save our citrus" message that goes out to the public, wherever citrus may be sold to the public? If traps with lures are developed in the near future, could they be provided for each tree sold from retail stores?**
- **Growers need to be incentivized to remove HLB-positive trees. This may need something like a crop insurance model in which payments are made to remove trees. Area-wide assessments may be needed to get everyone on board with control strategies, including subsidies for organic growers.**

- **A suggestion was made that citrus could be grown like some countries produce papaya where GE plants are not allowed: this is done by growing the crop in screen houses.**
- **In Florida, therapeutic treatments of two bactericides (Fireline® and Firewall®) have received emergency labels (Section 18) issued in 2016. Two seasons of data applying these chemicals with appropriate adjuvants have shown improvement in tree health and productivity, but time is needed to know if these improvements observed will be sufficient to warrant the cost. Identification of new bactericides with streamlined regulatory pathways would provide additional short-term solutions.**

# Appendix 3:
# Final Panel Recommendations

1. **CRB is to be commended for the breadth of research funded and their potential for coordinating national research groups.**
2. **The CRB, CRDF, and the Texas Citrus Producers Board and USDA, AFRI, SCRI need to be engaged and working together on the end goal in a way that avoids duplication of efforts.**
3. **Need to establish working groups as suggested above. The breeding facilitation and collaboration going should involve Ralph Scorza, as he has taken the 'HoneySweet' Plum pox-resistant plum through the process, from inception to registration by EPA.**
4. **Research needs to focus on an end result; EDTs may be the best first step in a coordinated approach to pre-screening for infected trees.**
5. **The panel agreed that a systems analysis workgroup is needed – a group to pool all relevant technologies together and how to best use them.**
6. **A second outreach to regulatory agencies, as was done 5-6 years ago, is needed to address some of the newer technologies. Active role of CRB in aiding commercial development of research outcomes would facilitate delivery or research to the commercial growers.**
7. **Vector management – Need to develop management plan for different stages of potential infestations: 1) Strategies for psyllid control prior to movement into commercial groves (much of this is in place now and several tools are available, but coordination is crucial); 2) What changes will be triggered in commercial groves when psyllids begin to move into commercial groves (what kind of area-wide or local grove treatments are recommended for areas of psyllid insipient incursions?); 3) Routine management of psyllids in commercial groves (new plantings and existing plantings- Develop IPM strategies for intense control that are ready to deploy in standard and organic production systems).**
8. **Long term research on market impacts would be beneficial– the Board has not funded this – so an economics workgroup may be needed.**
9. **Use Florida as a living laboratory, instead of dealing with greenhouse space problems in California. Utilize the CRF as much as possible but duplicate work in the field in Florida.**
10. **There is need for enhanced coordination of research. A key theme is to get away from the independent PI paradigm and into something a bit more directed research, perhaps a bit more like research departments in a biotech company, who are being funded, but with clear goals and top down milestones and management.**
11. **Find ways for researchers to share confidential information with CRB, using a confidentiality agreement, etc., to make sure the broadest survey of relevant research results is made available that look promising. Most projects are still at a stage where it should be possible for CRB to pose the question "How can we help develop or commercialize" even if IP is held independently. Researchers tend to see these discussions as often being with competitors, so it is important to find a way to structure conversations such that they lead to sharing of information in ways that make it more likely that researcher is getting close to the relevant desired outcomes and deliverables.**