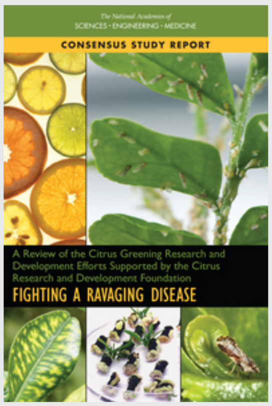

A Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting a Ravaging Disease (2018)

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FIGHTING A RAVAGING DISEASE

Committee on a Review of the Citrus Greening
Research and Development Efforts
Supported by the Citrus Research and Development Foundation:
Fighting a Ravaging Disease

Board on Agriculture and Natural Resources

Division on Earth and Life Studies

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The National Academies of

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Preface

Citrus huanglongbing (HLB) is a complex, challenging, and devastating disease that threatens a major U.S. crop despite intense management efforts. Research on HLB over the past decade has involved scientists, growers, industry representatives, regulators, and administrators who have explored multiple facets of the disease syndrome and the many environmental, horticultural, sociological, and economic factors that impact it. This National Academies of Sciences, Engineering, and Medicine study committee, following its statement of task to review and assess these research efforts, has relied upon many sources of information, including refereed scientific journal articles, extension publications, websites, presentations by researchers and others at the 2017 International Research Conference on Huanglongbing as well as in a series of the National Academies–hosted scientific open forums and webinars targeted to specific research topics, project progress reports, and personal communications. The committee is grateful to each of the individuals who contributed to the committee's work by sharing information about their research, observations, perspectives, and experience. The information gathered from these sources and during these interactions was essential to the committee's understanding of the issues and to the writing of this report. Many of these people are acknowledged by name in Appendix B; we realize that we may have missed some contributors and regret any inadvertent omissions.

The committee owes much to Dr. Camilla Y. Ables, Study Director, whose knowledge of citrus production and pathology, as well as her professional and capable guidance, contributions, and support at each stage of the committee's work, were invaluable to this process. We also thank

Robin A. Schoen, Director of the National Academies' Board on Agriculture and Natural Resources, for her wise and thoughtful input and advice throughout the study, and Jenna Briscoe, Research Assistant, whose IT skills, meeting organization support, and good cheer made these aspects of our work move seamlessly. We thank Dr. Harold Browning, Chief Operations Officer of our study sponsor, the Citrus Research and Development Foundation (CRDF), and the CRDF staff, who worked closely and tirelessly with us to assure that we had access to needed information. Thanks are due also to representatives of other HLB funding agencies and federal agencies (the California Citrus Research Board, USDA NIFA, USDA MAC, USDA APHIS, and USDA ARS), involved in HLB research and management, for providing information on their responsibilities, activities, and projects.

Sincere thanks are due to the members of the study committee, a group of outstanding and experienced scientists who dug deeply into the literature, entered thoughtfully and enthusiastically into discussions with one another and with HLB information providers, and used their remarkable skills in analysis and composition to create a report that is not only comprehensive but perceptive and forward thinking. Over the past year, as the committee participated in four project meetings/research forums and three webinars in addition to numerous conference calls, members maintained a high level of energy, sustained by trust that their science-based and experience-informed findings, conclusions, and recommendations will provide support to HLB stakeholder groups for future decision making and research prioritization on this challenging disease.

Jacqueline Fletcher
Chair, Committee on a Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting a Ravaging Disease

Acknowledgments

This Consensus Study Report was reviewed in draft form by individuals chosen for their diverse perspectives and technical expertise. The purpose of this independent review is to provide candid and critical comments that will assist the National Academies of Sciences, Engineering, and Medicine in making each published report as sound as possible and to ensure that it meets the institutional standards for quality, objectivity, evidence, and responsiveness to the study charge. The review comments and draft manuscript remain confidential to protect the integrity of the deliberative process.

We thank the following individuals for their review of this report:

Blake Bextine, The University of Texas at Tyler
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[1] National Academy of Sciences.
[2] National Academy of Sciences.

Although the reviewers listed above provided many constructive comments and suggestions, they were not asked to endorse the conclusions or recommendations of this report nor did they see the final draft before its release. The review of this report was overseen by Jeffery Dangl, NAS,[3] University of North Carolina, and Michael Ladisch, NAE,[4] Purdue University. They were responsible for making certain that an independent examination of this report was carried out in accordance with the standards of the National Academies and that all review comments were carefully considered. Responsibility for the final content rests entirely with the authoring committee and the National Academies.

[3] The National Academy of Sciences.
[4] The National Academy of Engineering.

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Acronyms and Abbreviations

ACP	Asian citrus psyllid, *Diaphorina citri*
AHL	acyl-homoserine lactone
AMP	antimicrobial peptide or antimicrobial protein
APHIS	U.S. Department of Agriculture Animal and Plant Health Inspection Service
ARS	U.S. Department of Agriculture Agricultural Research Service
Bt	*Bacillus thuringiensis*
Ca	calcium
CCTF	Core Citrus Transformation Facility (UF CREC)
cDNA	complementary DNA
CDR	constitutive disease resistance
CDRE	Citrus Disease Research and Extension (USDA NIFA SCRI)
CHMA	Citrus Health Management Area
CHRP	Citrus Health Response Program (USDA APHIS)
CLaf	*Candidatus* Liberibacter africanus
CLam	*Candidatus* Liberibacter americanum
CLas	*Candidatus* Liberibacter asiaticus
CRB	Citrus Research Board (Visalia, California)
CRDF	Citrus Research and Development Foundation (Lake Alfred, Florida)
CREC	Citrus Research and Education Center (University of Florida, Lake Alfred, Florida)

CRISPR	clustered regularly interspaced short palindromic repeats
CTV	*Citrus tristeza virus*
Cu	copper
CYPome	cytochrome p450 complement
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EDT	early detection technology
EFNP	enhanced foliar nutrition program
ENP(s)	enhanced nutritional program(s)
FAO	Food and Agriculture Organization of the United Nations
FCPRAC	Florida Citrus Production Research Advisory Council (precursor agency to CRDF)
GE	genetically engineered
GFP	green fluorescent protein
GM	genetically modified
GMO	genetically modified organism
HLB	huanglongbing (alternate name: citrus greening disease)
IFAS	University of Florida Institute of Food and Agricultural Sciences
IOCV	International Organization of Citrus Virologists
IPM	integrated pest management
IRCHLB	International Research Conference on Huanglongbing
K	potassium
LAMP	loop-mediated isothermal amplification
MAC	Multi-Agency Coordination (USDA)
MAPK	mitogen-activated protein kinase
MeSA	methyl salicylate
Mn	manganese
mRNA	messenger RNA
N	nitrogen
NAREEE	National Agricultural Research, Extension, Education, and Economics
NBS-LRR	nucleotide binding site–leucine-rich repeat
NCBI	National Center for Biotechnology Information

NI	near- to immediate-term
NIFA	National Institute of Food and Agriculture
NIH	National Institutes of Health
NRC	National Research Council
NVDMC	New Varieties Development & Management Corporation
Orco	olfactory receptor co-receptor
P	phosphorous
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PGR	plant growth regulator
PI	principal investigator
PTI	PAMP-triggered immunity
qPCR	quantitative polymerase chain reaction
R gene	resistance gene
R&D	research and development
RFA	request for applications
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SCRI	Specialty Crop Research Initiative
SDE	Sec-dependent effector
Sec	general secretory system
SNP	soft or nonclumping nanoparticles
SOD	superoxide dismutase
T1SS	Type I secretion system
TCPB	Texas Citrus Producers Board
UC	University of California
UCR	University of California, Riverside
UF	University of Florida
USDA	U.S. Department of Agriculture
UV	ultraviolet
VOC	volatile organic compound
Zn	zinc

Executive Summary

Huanglongbing[1] (HLB) or citrus greening, first observed more than a hundred years ago in Asia, is the most serious disease threat to the citrus-growing industry worldwide due to its complexity, destructiveness, and incalcitrance to management. The bacterium *Candidatus* Liberibacter asiaticus, which is associated with HLB, is transmitted by the Asian citrus psyllid (ACP). First detected in Florida in 2005, HLB is now widespread in the state and threatens the survival of the Florida citrus industry despite substantial allocation of research funds by Florida citrus growers and federal and state agencies.

In Florida, HLB research is overseen by the Citrus Research and Development Foundation (CRDF), a nonprofit corporation created in 2009 through the initiative of the state's citrus industry. In 2017, at the request of CRDF, the National Academies of Sciences, Engineering, and Medicine conducted a review of the foundation's research portfolio, with the goal of identifying ways to reconfigure HLB research to accelerate the development of tools and strategies to abate disease impacts and prevent the collapse of the Florida citrus industry (see the full statement of task in Chapter 1, Box 1-1).

In its review, the committee found that research supported by CRDF and other agencies has expanded knowledge of every aspect of HLB, yet

[1] Huanglongbing (HLB), which means yellow (huang) shoot (long) disease (bing), was unanimously adopted as the official name of the disease by the International Organization of Citrus Virologists (IOCV) at the 13th Conference of IOCV in Fuzhou, China, in 1995 (http://iocv.org/huanglongbing.htm; accessed February 22, 2018).

there have been no breakthroughs in HLB management. The reasons for the lack of breakthroughs in HLB management, despite the investments in research, are complex. Other than research on ACP in Florida, most of the available information on HLB prior to 2005 was based primarily on research performed outside the United States so researchers faced a steep learning curve. The disease itself is intractable for a variety of reasons related to the citrus host (its perennial nature, the lack of resistance in any citrus relative, and the difficulty of breeding to produce HLB-resistant cultivars); the pathogen (especially the inability to culture it in the laboratory); the insect vector (and its major role in transmission); the complexity of pathogen, vector, and host interactions; the lack of a good model system; as well as the current approach to HLB research.

The committee's analysis of HLB research outcomes allowed the committee to identify progress and pitfalls in major research areas, and to identify research efforts that the committee believes should be continued or initiated.

The committee regards the following as critical to achieve progress toward a viable HLB solution:

- Building on knowledge generated through previous research
- Supporting research on factors that influence adoption of management practices proven effective
- Greater collaboration and more-frequent venues for information sharing by scientists
- Timely and systematic communication of research outcomes and evaluation of research progress
- Increased research coordination by CRDF and other funders of HLB research

Citrus growers, particularly in Florida, still need short-term solutions to sustain the industry while researchers continue to generate longer-term approaches for managing HLB, so support of basic and applied short- and long-term research is needed. Longer-term HLB solutions are likely to involve citrus variety improvement, derived primarily from new molecular techniques such as gene editing, and those efforts should focus on targets mediating molecular interactions among plant, bacteria, and vector.

Because a single breakthrough discovery for managing HLB in Florida is unlikely, funders should support the development of sets of management approaches that can be combined in different ways, optimized, and validated for use in different locations and conditions. This approach, founded on integrated pest management, would allow optimization of management for each grower.

Economic and sociological factors that impact decision making and behaviors of growers, processors, and the public will influence the adoption and success of future HLB management efforts; hence, CRDF should consider funding these research areas and creating accessible databases to support sociological and economic modeling of HLB-related research outcomes and application projections.

During its review the committee observed inconsistency in laboratory and field experimental designs and sampling methods. Because inconsistency limits the comparison of findings across teams and institutions and the use of previous research to inform further exploration, the development of community-accepted standards to conduct, evaluate, and assess research should be supported. Improved consistency in reporting research outcomes is also needed to reduce constraints in reviewing research progress and delays in applying new information to HLB solutions. CRDF should develop a standardized format and procedure, and set a timeline for mandatory reporting of project progress and final reports, to include publications and presentations, outcomes, practical applications, and impacts.

Despite commendable efforts of multiple agencies to coordinate funding and encourage appropriate interstate, interagency, and interdisciplinary collaborations, the committee noted that decisions about research funding priorities and allocations occur largely within the domain of each agency, and it recommends that CRDF and other agencies work together to create an overarching HLB research advisory panel to develop a fresh, systems approach to HLB research prioritization and the strategic distribution of resources for research leading to effective HLB management.

Summary

Huanglongbing[1] (HLB) or citrus greening, a disease first observed more than a hundred years ago in Asia, is the most serious threat to the citrus-growing industry worldwide due to its complexity, destructiveness, and incalcitrance to management. First detected in Florida in 2005, HLB is now widespread in the state and threatens the survival of the Florida citrus industry despite substantial allocation of research funds by Florida citrus growers and federal and state agencies.

In Florida, HLB research is overseen by the Citrus Research and Development Foundation (CRDF), a nonprofit corporation created in 2009 through the initiative of the state's citrus industry. In 2010, CRDF began managing projects addressing the research and technology recommendations in the 2010 National Research Council (NRC) report *Strategic Planning for the Florida Citrus Industry: Addressing Citrus Greening Disease.* From 2010 to 2017, CRDF awarded about $124 million to 398 projects, of which nearly 90% focused on HLB. Because the research funded to date has not produced major breakthroughs in controlling HLB, CRDF contacted the Board on Agriculture and Natural Resources of the National Academies of Sciences, Engineering, and Medicine in October 2016 to request an independent review of CRDF-funded research. The overall purpose of the review was to identify ways to reconfigure HLB research to acceler-

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ate the development of tools and strategies to abate the damage caused by HLB and prevent the collapse of the Florida citrus industry (see Chapter 1, Box 1-1, for the full statement of task).

In its review, the committee found that research supported by CRDF and other agencies has expanded knowledge of every aspect of HLB, yet there have been no breakthroughs in HLB management. The reasons for the lack of breakthroughs in HLB management, despite the investments in research, are complex. Other than research on the Asian citrus psyllid (ACP) in Florida, most of the available information on HLB prior to 2005 was based primarily on research performed outside the United States so researchers faced a steep learning curve. The disease itself is intractable for a variety of reasons related to the citrus host (its perennial nature, the lack of resistance in any citrus relative, and the difficulty of breeding to produce HLB-resistant cultivars); the pathogen (especially the inability to be cultured in the laboratory); the insect vector (and its major role in transmission); the complexity of pathogen, vector, and host interactions; the lack of a good model system; as well as the current approach to HLB research.

CURRENT KNOWLEDGE OF HUANGLONGBING PATHOGEN, VECTOR, AND HOST AND THEIR INTERACTIONS

HLB is a disease of citrus associated with bacteria that are spread by a sucking insect, the Asian citrus psyllid. The following presents the current state of understanding of the disease system and the factors that influence its occurrence, severity, and incalcitrance to effective management.

Candidatus Liberibacter asiaticus

Three bacteria are known to be associated with HLB, *Candidatus* Liberibacter asiaticus (CLas), *Ca.* Liberibacter africanus (CLaf), and *Ca.* Liberibacter americanus (CLam). They are phloem-restricted *in planta*; because they are nonculturable they cannot be characterized to the extent required for official genus status, hence their "*Candidatus*" status. HLB in Florida is attributed to CLas, but because Koch's postulates cannot be tested much of what is known or hypothesized about its pathogenicity has been deduced from multiple genome sequences of the three HLB-associated bacteria.

Asian Citrus Psyllid

The HLB vector in Florida is the Asian citrus psyllid, *Diaphorina citri* Kuwayama. Development from egg to adult requires 2 to 7 weeks, adults live several months, and there are 9 or 10 generations per year.

Adults fly 25 to 50 m regularly, but can fly up to 100 m toward new leaf flushes. Movement into managed citrus groves in Florida is often from adjacent abandoned groves. Trapping data indicate no consistent ACP seasonal movement patterns, necessitating continuous monitoring.

Vector–Host Interactions

ACP infests species in at least 10 genera in the family Rutaceae (which contains about 160 genera including *Citrus*) of variable suitability for oviposition, development, and reproduction. Multimodal sensory inputs contribute to host finding; ACP orient to leaf flush via volatile signals and assess plant suitability using gustatory and visual cues. Ovipositing females prefer the plant species on which they developed, but preferences can shift after exposure to an alternative host. Adults are attracted to colors within the reflectance spectra of rutaceous plants. Infected plants are more attractive to ACP due to HLB-induced volatiles. However, infected trees are less suitable for ACP development, and psyllids leave infected plants shortly after pathogen acquisition, promoting pathogen spread.

Host–Pathogen Interactions and Host Plant Defense Mechanisms

Within citrus sieve tubes *Ca*. Liberibacters exploit cellular processes for nutrient acquisition, encoding transporters and enzymes for metabolizing host-derived nutrients. CLas effector proteins are predicted to modulate host cellular functions and disable defense reactions, benefiting CLas multiplication and phloem colonization. Citrus and its relatives reprogram their transcriptomic network in response to HLB.

Pathogen–Vector Interactions

Early-instar nymphs acquire CLas more efficiently than adults and are important in transmission. Bacterial titers in the insect change over time in ways suggestive of CLas replication in nymphs but not in adults. The pathogen survives for long periods in the insect and can be vertically transmitted at low frequency.

Diagnostics and HLB Management

Sensitive, cost-effective detection of CLas infections in citrus and ACP is critical for HLB management. Currently quantitative polymerase chain reaction (qPCR), along with conventional PCR and DNA sequencing, is used by accredited laboratories for verifying CLas infection. However, the main diagnostic challenge is sampling, which is hampered by uneven CLas spatial and temporal distribution in trees and insects and the long lag time between inoculation and bacterial detectability.

Citrus Health Management Areas (CHMAs) were established in Florida to promote regional coordination of insecticide applications, but their effectiveness is limited by low grower participation in some areas. Models predict that reducing ACP populations during critical times can decrease HLB spread, but even 100% CHMA participation would not likely slow HLB progression because refugia for vector and pathogen exist in abandoned groves and backyard trees. Other management methods include use of reflective mulch to repel ACP, thermotherapy for small trees in groves or greenhouses, and manipulation of citrus nutrition and irrigation to enhance tree health. Although peer-reviewed literature on these practices is scant, the underlying logic is that stressed trees are more vulnerable to HLB.

Citrus Genetics, Breeding, and Biotechnology

Many generations of breeding and selection are required to develop new citrus cultivars having specific qualities. The long juvenility period for citrus grown from seed and the need for large land areas to grow and evaluate trees add to the challenges of conventional breeding. Genetic engineering can potentially circumvent some of these obstacles, but the introduction of genes into citrus (genetic transformation) is specific to genus and species and is limited to relatively few genotypes.

HLB RESEARCH AND DEVELOPMENT EFFORTS FUNDED BY CRDF

Eradication of HLB-infected and nearby trees in Florida eliminated one-tenth of citrus production capacity by 2008 and the disease was estimated to have caused the loss of thousands of jobs and over $1 billion in grower revenue. CRDF has strived to halt or reverse industry losses by targeting research on all disease system components. A summary of research efforts supported by CRDF and other state and federal agencies is provided below. The broad, diverse research portfolio was the central focus of the committee's review.

HLB Pathogen, Vector, and Hosts

Research on HLB Causal or Associated Bacteria

CRDF-funded projects included efforts to culture CLas *in vitro* and to culture *Liberibacter crescens* as a CLas proxy system. Genome sequencing for CLas, CLam, and CLaf was funded, as were studies to identify HLB cooperative or antagonistic microbes in the citrus microbiome. Other research focused on microbial products for ACP management and a phage-suppressing protein from an ACP endosymbiont for manipulating CLas behavior. The National Institute of Food and Agriculture Specialty Crop Research Initiative (NIFA-SCRI) supported projects to culture CLas; identify CLas-secreted proteins for targeting by plant proteases; and characterize roles of genetic regulators in CLas persistence in citrus. The Citrus Research Board (CRB) funded identification of CLas-secreted proteins for developing bacterial inhibition strategies. The Foundation for Food and Agriculture and Southern Gardens Citrus are funding the development of a root-based bioassay for culturing studies and antimicrobial screening.

Research on HLB Vector: Asian Citrus Psyllid

CRDF funded projects to characterize ACP dispersal and reproduction behavior, including measuring psyllid responses to abiotic factors to optimize trapping, predicting ACP attack, and evaluating seasonality and frequency of dispersal. NIFA funds research on the proteome and transcriptome of ACP alimentary canal, midgut, and salivary glands with and without CLas and the development of methods to generate vector-incompetent transgenic ACP. NIFA supports work toward the completion of the ACP genome. CRDF and NIFA funded the construction of complementary DNA (cDNA) libraries from CLas-infected and CLas-free ACP to characterize gene expression profiles. Several federal agencies, with CRB, supported construction of transcriptomes of egg, nymph, and adult ACP. NIFA supported annotation to complement ACP genome sequencing by the National Institutes of Health National Center for Research Resources, U.S. Department of Agriculture (USDA) Agricultural Research Service, and CRB.

Research on HLB Vector–Host Interactions

CRDF funded projects to screen citrus germplasm for traits affecting ACP attractiveness, quantify impacts of citrus species and flushing on ACP traits, and identify ACP overwintering habitats and alternative hosts. Other studies were designed to understand Cleopatra mandarin and trifoliate orange resistance to ACP and CLas, respectively; to identify semiochemicals impacting psyllid–host interactions; and to examine plant structures interfering with ACP feeding. Also studied were attractants that influence ACP flight, color preferences, and the effect of host genus on ACP; signals produced by ACP; ACP responses to volatiles produced on ACP-damaged foliage or released by CLas-infected citrus; and impacts of ACP experience on recognition of host stimuli. Host plant flush availability was evaluated, as were movements between managed and abandoned groves, geographic barriers, and wind direction. The effects of abiotic factors on psyllid movement, feeding, infection status, fecundity, and population density were assessed. NIFA funds research on ACP responses to CLas infection. CRB supported studies of ACP responses to volatiles produced by flushing shoots, identification of volatiles for surveillance and management, odor coding in ACP, and mitogenome analysis to identify region of origin for ACP in California.

Research on HLB Host: Citrus

CRDF supported research on citrus genetic engineering, gene discovery, and genetic mapping. No source of high-level resistance to HLB is known, but genetically engineered (GE) resistance is under development with genes from citrus and noncitrus sources. Transgenic rootstocks have been produced using juvenile tissues, and there are efforts to transform mature tissues. Several projects attempted to create transgenic citrus expressing antimicrobial proteins, disease resistance genes, anti-CLas antibodies, regeneration and transformation-associated genes, or promoters targeting specific tissues. Efforts to edit clustered regularly interspersed short palindromic repeats (or CRISPR) were also funded by CRDF. Projects on HLB tolerance/resistance gene discovery through differential expression in susceptible and tolerant genotypes were supported, as was the use of a *Citrus tristeza virus* (CTV) vector to deliver and express resistance transgenes in citrus. CRDF supports a citrus transformation facility (Lake Alfred, Florida), which provides services and resources for testing gene or promoter efficacy, and the Picos Farm (Fort Pierce, Florida), a site critical for long-term testing of HLB resistance and tree performance, and for developing data necessary for regulatory approval. NIFA and CRB fund projects to identify candidate resistance genes in resistant or tolerant citrus relatives for gene editing to produce non-GE HLB-resistant citrus cultivars.

Research on HLB Pathogen–Vector Interactions

CRDF-funded projects aimed to characterize and interrupt CLas movement within, and interactions with, ACP. Other efforts are to identify molecules interfering with ACP transmission, evaluate the ability of other bacteria to trigger ACP immune responses, identify ACP gut receptors for key toxins, identify quorum-sensing compounds that disrupt CLas communication and biofilms, and induce the CLas phage lytic phase within ACP. CTV delivery of ribonucleic acid interference (RNAi) to target ACP, enhancement of transgene expression in the CTV vector, and RNAi strategies that target genes linked to ACP survival or CLas transmission were investigated. The NuPsyllid project aimed to generate a GE ACP, unable to transmit CLas, to displace wild ACP populations. The USDA Animal and Plant Health Inspection Service Multi-Agency Coordination (MAC) group supports projects focused on ACP management and the use of thermotherapy to reduce ACP acquisition and transmission of CLas. NIFA and CRB funded study of the genetics of ACP transmission competency and characterization of ACP proteins regulating CLas movement and survival in the insect. Projects to integrate and curate omics data on ACP, citrus, and CLas and to use these data, along with *de novo* generated genomic, proteomic, and metabolomics data, to discover molecules that can block pathogenesis and transmission pathways were also funded. CRB funds projects to investigate transmission efficiencies among ACP populations, identify proteins involved in ACP–CLas interactions, and characterize factors influencing transmission efficiency.

Research on HLB Host–Pathogen Interactions

CRDF funded projects aimed at identifying host genes highly upregulated in resistant versus susceptible citrus varieties; identifying and mapping HLB resistance gene(s) in *Poncirus*; curating genomic sequences of CLas, ACP, and citrus; and bioinformatically analyzing proteins of each species to predict their interactions. Other CRDF projects investigated host gene–pathogen effector interactions, putative host target proteins, and enzyme activity in HLB pathogenesis. NIFA-SCRI funded efforts to identify and characterize the roles of Liberibacter spp. effectors in HLB pathogenesis and to identify candidate host genes for HLB resistance or tolerance. CRB supports research to identify and characterize CLas small RNAs, messenger RNAs, and citrus targets.

HLB Management

Research on Bacterial Control

CRDF has supported research on the use of bactericidal or bacteriostatic chemicals or antibiotics, thermotherapy, biocontrol using CLas bacteriophage, nutritional and microbiome enhancements, and induction of citrus defense responses. Research foci included expression in *Arabidopsis* of CLas signaling and defense marker genes, and evaluation of drug-like molecules for gene induction, using a *Ca.* Liberibacter psyllaurous proxy system. Research also aimed to create *Arabidopsis* mutants lacking specific defense mechanisms to determine the basis for disease tolerance, investigate suppression of host defenses by CLas salicylate hydroxylase, and determine effects of CLas flagellin, a protein involved in host defense induction. CRDF funded the evaluation of zinc-based formulations, nonmetal antibiotics, and tetracycline derivatives against CLas. Hundreds of chemical formulations were tested for activity against *L. crescens*. Work to enhance delivery of antibacterial compounds into the plant, to evaluate their movement *in planta*, and to optimize methods of field assessment were also supported, as were projects on thermotherapy, alone or with chemo- and nutrient therapy, against HLB. The use of a psyllid repressor protein as a phage cycle regulator, and CLas phage peroxidase and phages from *Xanthomonas axonopodis* pv. *citri* for HLB therapy, are being investigated. The impact of citrus microbial or phytobiome communities on tree health and HLB resistance was examined.

Research on Insect Control (Chemical and Biological)

Major research themes have included insecticide testing against all psyllid stages, integration of insecticides into integrated pest management programs, documentation of approaches for organic orchards, examination of attractant and repellent compounds, and optimization of ACP sampling protocols for justifying pesticide use. Also supported was evaluation of indigenous ACP predators and parasites, including the wasp *Tamarixia radiata* and entomopathogens. Chemical and biological control programs supported by CRDF were complemented with funding from the MAC program and CRB.

Research on Cultural Control

CRDF funded projects investigating effects of nutritional supplements on plant growth and development and on root and soil health, as well as use of high-density plantings, mulches, and psyllid-proof enclosures to control ACP. MAC has supported research on cultural management, such as removing abandoned groves, reducing irrigation water pH, applying thermotherapy or mulches, and intensive grove management.

Research on Diagnostics

No current CRDF-funded projects directly address CLas diagnostics, but a past project investigated optical sensing to screen seedlings for HLB resistance. CRB has funded research on metabolomics and proteomic biomarkers in CLas-infected trees and conventional detection methods. MAC funded research on canine detection and antibody-based early detection, overlapping with a NIFA-supported effort to identify CLas-secreted effector molecules. NIFA funded projects to use yeast biosensors and loop-mediated isothermal amplification (or LAMP) technology to detect CLas in ACP. Development of technologies for detecting infection prior to symptom appearance, funded by other state and federal agencies, relies upon postinfection spectral reflectance changes, changes in plant-produced volatiles released, or changes in metabolic, proteomic, RNA, and microbial population profiles due to infection.

The analysis of the CRDF research portfolio indicated that CRDF has not directly funded research on economic and sociological factors associated with citrus production and HLB management and their impacts on decision making by growers, processors, and the public. These factors influence the likelihood of implementation and success of future HLB management approaches, as shown by studies, funded by other agencies, on grower participation in CHMAs as well as surveys on grower willingness to plant GE citrus and consumer willingness to purchase GE products.

NOTABLE OUTCOMES, PITFALLS, AND FUTURE DIRECTIONS

Key research findings and recommended future research are listed here to highlight areas in which the committee found that progress had been made and to point to research efforts the committee believes should be continued or initiated.

Biology and Ecology of the HLB Causal/ Associated Bacteria and Its Insect Vector

Key Research Findings

- Numerous CLas genomes worldwide were sequenced, revealing mutation patterns and potential control targets and allowing for comparative genomic studies.
- CLas-killing bacteriophages were characterized and factors that suppress them were identified.
- *Liberibacter crescens*, a culturable CLas relative, was developed as a proxy system to study pathogen–host interactions.
- ACP biology and reproductive behavior were characterized extensively.
- ACP genome and transcriptome annotation led to discoveries that can reveal control points at the vector–pathogen interface.
- Emerging biotechnologies, such as RNAi, offer mechanisms to achieve new, sustainable, and environmentally friendly ACP management.
- Elucidation of ACP seasonal activity patterns and abundance have facilitated effective targeting and timing for management efforts.

Recommendations for Future Research

- Sequence additional CLas isolates, monitoring for changes that have altered or could alter virulence or the efficacy of control strategies.

- Continue to study bacteriophage-suppressing factors and essential CLas-encoded proteins produced in or secreted by the plant to identify control targets.
- As more CLas proteins or compounds are confirmed as essential and produced in or secreted by the plant, shift the research focus to identifying plant-compatible strategies to inactivate or recognize them.
- Develop protocols for ACP DNA recovery from traps to facilitate evaluation of population structure, pesticide resistance, and other biological characteristics.

Interactions of HLB Pathogen, Vector, and Host

Key Research Findings

- Genome sequencing of *Ca*. Liberibacter strains allowed identification of candidate effector genes that may be involved in pathogen–plant interactions.
- Evidence was found for disease-promoting roles for at least five CLas virulence factors that are potential control targets.
- Resistant or tolerant citrus varieties highly upregulate host genes, including some that suppress plant immunity mechanisms and cause metabolic dysfunction, in response to *Ca*. Liberibacter infection.
- Nymphal stages of ACP acquire CLas quickly and are important in CLas transmission and dissemination.
- CLas replicates in ACP and is vertically transmitted at low levels.
- Mechanisms and pathways of circulative transmission of CLas in ACP were elucidated.
- CLas alters citrus and ACP biology, impacting ACP dispersal and mating behavior and the attractiveness of trees to ACP.

Recommendations for Future Research

- Characterize additional CLas effectors and identify and functionally analyze host targets, applying new knowledge to novel HLB management tools.
- Identify and characterize new critical citrus genes or gene products that are targets of CLas.
- Seek new resistance genes, in citrus or other species, that counteract CLas effectors.
- Identify new molecules that interfere with the CLas life cycle, leading to titer reductions in citrus or ACP.
- Identify new molecules that hamper ACP transmission of CLas.

- Explore strategies for physical protection of trees against ACP, including repellents, mulches, and screens.

HLB Management

Key Research Findings

Bacterial Control

- New approaches developed for bacterial control (nanoparticle or nanoemulsion formulations, addition of adjuvants, use of chemical mixtures, combining chemical treatments with thermotherapy, or triggering host plant defense mechanisms) can enhance treatment effectiveness and minimize the amount of active antibacterial chemical needed.

Vector Control

- Vector management remains important in Florida, as repeated inoculations speed disease development and increase symptom severity.
- Nearly all insecticides available, and many in development, were evaluated against ACP in Florida.
- Pesticide resistance management plans were developed and are in use in some area-wide programs.
- The benefits of indigenous ACP predators and parasites were evaluated and quantified.
- The imported psyllid parasite *Tamarixia radiata* was reared and released throughout Florida; its impact has been greatest in urban settings and abandoned orchards.

Host Resistance: Breeding

- HLB susceptibility varies with citrus cultivar and rootstock.
- HLB-tolerant citrus rootstock and scion genotypes and HLB-resistant citrus relatives were identified, providing material for breeding and a temporary production bridge for the industry.
- Potentially useful levels of resistance were identified in citrus relatives for incorporation into breeding programs.

Host Resistance: Genetic Engineering

- Numerous transgenic citrus expressing genes that may confer HLB resistance were produced and are being tested.

- Genomes of a number of citrus and citrus relatives were sequenced, yielding information on host responses to HLB and processes that may be manipulated for HLB management.

Cultural Control

- Optimizing fertilization and irrigation can result in short-term tree health improvement and reduce HLB impacts on fruit yield but has no curing effect, is expensive, and is likely unsustainable.
- Increasing citrus density in orchards can decrease the rate of HLB spread.
- Multiple inoculations of infected trees exacerbate HLB impact on fruit yield and quality.

Diagnostics

- Molecular and serological diagnostic technologies for CLas are ultrasensitive, but their use for epidemiological and regulatory applications are hampered by uneven pathogen distribution in the tree.
- No single diagnostic method will be sufficient to identify recently infected trees.
- Detection of infection prior to symptom development is possible through detection of changes in host metabolites and volatiles.

Recommendations for Future Research

- Determine the most effective bacterial targeting strategies for transgenic plant development and support their development in citrus.
- Explore novel chemical therapies; chemical genetics approaches can help in identifying key candidates. Look at chemicals that act as regulators or intermediates in host defense responses.
- Implement field testing to determine actual impacts of chemical applications on disease severity and fruit production and develop predictive disease impact models to support decision making about this management approach.
- Focus, in Florida, on managing HLB as a chronic problem in which incremental improvements in control have value; even minor improvements should be considered.
- Evaluate the effects of new cultural management approaches (high-density plantings and nutritional supplementation) on tree infection, fruit production, and pathogen acquisition/transmission by ACP.

- Explore the effectiveness of new pesticidal chemicals, particularly those providing ACP repellency or having minimal effects on biocontrol agents, on HLB incidence.
- Investigate new parasites/predators of ACP as possible psyllid biocontrol agents; the parasite currently established in Florida is useful but provides inadequate control on its own.
- Continue to explore the use of biomarkers associated with HLB-diseased trees as tools for early diagnosis, comparing these to volatile organic compounds.

OVERARCHING FINDINGS, CONCLUSIONS, AND RECOMMENDATIONS

To help achieve progress in finding a viable solution to HLB, the committee provided overarching findings, conclusions, and recommendations (directed at all agencies that fund HLB research unless CRDF is specifically mentioned) related to other factors that can affect the adoption of HLB management practices, tracking research progress, fostering communication among researchers, and coordination among all funders of HLB research. Detailed recommendations are presented in Chapters 3 and 4.

Finding 3.1: CRDF support for HLB research is responsive to several recommendations contained in the 2010 NRC report, particularly to 8 of the 11 near- to intermediate-term (NI) research and technology recommendations NI-1, NI-4, NI-5, NI-6, NI-7, NI-8, NI-9, and NI-10, and long-term recommendations L-1, L-2, and L-3 (see full text of the 2010 NRC recommendations in Chapter 1, Table 1-1).

Finding 3.2: Other agencies are funding efforts to address recommendations NI-2 and NI-11.

Finding 3.3: ***Recommendation NI-3: Establish citrus orchard test plots for evaluation of new scouting and therapeutic methods*** remains to be addressed.
Conclusion 3.3: Although CRDF supports Picos Farm, a secure transgenic field test site in Florida, additional sites are needed for assessment and validation of scouting and therapeutic approaches for HLB.
Recommendation 3.3: CRDF should consider establishing an infrastructure enhancement project to assess field testing needs for all citrus disease and insect research and validation activities and to design plans to meet those needs by enhancing current field test sites, establishing new field test sites, and/or developing collaborations with citrus growers to use production orchards for testing.

Finding 3.4: A single breakthrough discovery for managing HLB in Florida in the future is unlikely, since intensive research efforts over almost 20 years have not led to this result.
Conclusion 3.4: Finding the best combinations of control strategies suited to different environmental and growing conditions, vector and pathogen pressure, tree cultivars, and tree health would help growers in Florida and in other states where HLB is not yet a chronic problem but soon could be, especially if HLB infection is not detected quickly and if there is reluctance to remove inoculum sources.
Recommendation 3.4: Consider specific funding for the development of sets of management approaches that can be combined in different ways, optimized, and validated for use in different locations and conditions.

Finding 3.5: Plant pathologists, sociologists, and economists are using modeling to assess the complex interactions that characterize HLB; however, no CRDF funding has directly supported research on economic and sociological factors that impact decision making and behaviors of growers, processors, and the public and can influence the adoption and success of future HLB management efforts.
Conclusion 3.5: Greater investment and researcher involvement are needed to develop and apply modeling technologies for analysis and prediction of the effects of economic and sociological factors on the acceptance and application of HLB management practices.
Recommendation 3.5.1: CRDF should consider adding these research areas to its funding portfolio.
Recommendation 3.5.2: CRDF should consider creating centralized, researcher-accessible databases to support sociological and economic modeling of HLB-related research outcomes and application projections. It should support systems approaches for field testing combinations of the most promising developments in replicated field studies, emphasizing the need to collect research data sufficient to inform and support model training and applications to effectively predict cost–benefit ratios of all HLB management strategies.

Finding 3.6: Recent and growing interest in research using genetic modification to develop HLB-resistant citrus, and concerns about stakeholder acceptance of these technologies, indicate that expanded efforts in educational outreach to growers, processors, and consumers could facilitate eventual deployment of new citrus lines. Data from previous advertising strategies directed at adjusting consumer attitudes about citrus consumption have demonstrated that such communication with the public can change behaviors.
Conclusion 3.6: Further research is needed to assess the level of current stakeholder understanding of genetic modification technology, and the fac-

tors that influence their willingness to purchase genetically modified (GM) foods.
Recommendation 3.6: CRDF should consider funding research to assess consumer attitudes toward genetic modification technologies for producing HLB-resistant citrus cultivars and their willingness to consume GM citrus, as well as how targeted advertising campaigns could be effective outreach strategies.

Finding 4.1: Although research supported by CRDF and other agencies has advanced our knowledge of HLB since 2010, the disease remains an intractable threat to the Florida citrus industry and has progressed from an acute to a chronic disease present throughout the state.

Finding 4.2: A number of technical obstacles have been addressed by funded projects but continue to represent significant barriers to research progress and the generation of HLB solutions.

Conclusion 4.1: Citrus growers, particularly in Florida, still need short-term solutions for the industry to remain viable while researchers continue to generate longer-term approaches for managing HLB.
Recommendation 4.1: Continue support for both basic and applied research and both short- and long-term research efforts.

Conclusion 4.2: Longer-term HLB solutions are likely to involve citrus variety improvement, derived primarily from new molecular techniques.
Recommendation 4.2: Continue to support the development and application of gene modification, including gene editing, focusing on targets mediating molecular interactions among plant, bacteria, and vector.

Conclusion 4.3: HLB research is hampered by the lack of standardized methods and parameters for measuring, evaluating, and analyzing factors including vector transmission rates, fruit yield, plant tolerance/resistance, citrus variety performance, antibacterial compound effectiveness, and diagnostic assay evaluation. Inconsistency in experimental designs, sampling methods, and field investigations limit the ability to compare findings and use previous research as a springboard for further exploration.
Recommendation 4.3: Support the development of community-accepted standards for the conduct, evaluation, and assessment of research to facilitate comparisons of research results across teams and institutions.

Finding 4.3: Novel approaches to foster communication, collaboration, and innovation among HLB researchers and representatives of funding agencies and the citrus industry may advance progress and facilitate solutions to HLB.

Finding 4.4: There are inconsistencies in the format, content, and frequency of CRDF-funded research progress reporting by researchers, as well as in the inclusion of specific outcomes, impacts, and products.
Conclusion 4.4: Improved reporting consistency is needed to reduce constraints in reviewing research progress and delays in applying new information to HLB solutions.
Recommendation 4.4: CRDF should develop a standardized format, procedure, and timeline for mandatory reporting of midterm project progress and final reports, to include publications and presentations, outcomes, practical applications, and impacts. CRDF should consider hiring a staff person to review and analyze HLB research findings annually.

Finding 4.5: More timely publication of research results in refereed scientific journals and trade journals would facilitate communication among the research community and between researchers and growers and support research assessment efforts.

Finding 4.6.1: Engaging with a disease that threatens the survival of an industry and requires a short-term and sustainable solution could benefit from a nonacademic research model or approach.
Finding 4.6.2: Despite the commendable efforts of multiple funding agencies to coordinate funding and encourage appropriate interstate, interagency, and interdisciplinary collaborations, decisions about research funding priorities and allocations occur largely within the domain of each agency.
Conclusion 4.6: The committee concludes that the current system of research prioritization and funding, accomplished primarily within each relevant funding agency, is not optimally efficient and has not led to the development of an overarching master plan for HLB research and its translation to management solutions.
Recommendation 4.6: CRDF should consider working, together with representatives of other agencies at the national and state levels, to create an overarching HLB research advisory panel to develop a fresh systems approach to HLB research prioritization and the strategic distribution of resources for research leading to effective HLB management.

1

Introduction

CITRUS GREENING

Huanglongbing (HLB), also known as citrus greening, has gained renown as the most serious threat to citrus worldwide. First observed more than a hundred years ago in Asia, the disease is a challenging threat due to its complexity, its destructiveness, the susceptibility of most commercial citrus species and cultivars regardless of rootstock, and the lack of an effective method for suppressing its causal agents or its insect vectors.

HLB is associated with three species of the genus Liberibacter: *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter africanus, and *Candidatus* Liberibacter americanus. All *Ca*. Liberibacter species that cause disease in citrus are transmitted by *Diaphorina citri* (Kuwayama), the Asian citrus psyllid (ACP), and *Trioza erytreae* (del Guerco), the African citrus psyllid. The pomelo psyllid, *Cacopsylla* (*Psylla*) *citrisuga* Yang & Li, was found to be a carrier of CLas that may potentially transmit CLas to healthy citrus (Cen et al., 2012). Trees infected with HLB have blotchy mottling of leaves, shoot stunting, and gradual dieback of branches. They produce small, deformed fruits with bitter juice, which is unmarketable. Infected trees do not die right away; they can remain in a steady state of decline for several years. If not removed from the grove and destroyed, infected trees can serve as sources of inoculum. For a detailed account of HLB history, etiology, biology, epidemiology, detection, geographical distribution, and control methods, the reader is referred to *Huanglongbing: A Destructive, Newly-Emerging, Century-Old Disease of Citrus* by Bové (2006) and *Citrus Huanglongbing: The Pathogen and Its Impact* by Gottwald et al. (2007).

HLB may have originated in southern China,[1] where yellowing and leaf mottle on citrus trees were reported by Reinking in 1919. However, there are reports that symptoms of HLB were observed in India in the 1700s and 1800s, and that by 1912 it had become a severe problem in Bombay province (Capoor, 1963). By the 1970s, HLB had devastated many citrus-growing regions in Asia and Africa. At that time, ACP had been established in the Western Hemisphere for several decades, having been first reported in Brazil in 1942 (da Costa Lima, 1942). By 1998, it was found in south Florida and was soon thereafter widespread throughout the state (Halbert and Manjunath, 2004). In 2004, HLB was first detected in the Americas, in the São Paulo state of Brazil. In 2005, HLB was detected in Miami-Dade County in Florida. HLB has since been reported in six other states in the United States: California, Georgia, Hawaii, Louisiana, South Carolina, and Texas. The disease is now widespread in all citrus-growing counties of Florida, having been reported in the Florida Panhandle, among the last remaining disease-free regions, in 2016 (Iriarte et al., 2017). Between 2000 and 2014, citrus acreage in the state declined from roughly 750,000 acres (303,514 hectares) to 476,000 acres (192,630 hectares), and production volume has declined by 58% since 2005. In Florida alone, HLB has caused a cumulative loss of $2.994 billion in grower revenues over the 2006–2007 to 2013–2014 period, an average of $374 million per year (Hodges et al., 2014).

CITRUS GREENING RESEARCH

As the HLB epidemic raged in 2008, Florida citrus growers began allocating funds for HLB research in hopes of finding short-, medium-, and long-term solutions. This effort created the Citrus Research and Development Foundation (CRDF), an organization with oversight responsibility for HLB research and development efforts in Florida. By the summer of 2010 CRDF was managing a portfolio of about 124 projects worth $14.3 million, which aimed to address the research and technology recommendations in the National Research Council (NRC) 2010 report *Strategic Planning for the Florida Citrus Industry: Addressing Citrus Greening Disease* (see Table 1-1). Projects funded during that time were focused on the following areas: (1) interrupting the breeding/feeding of psyllids, (2) vector management, (3) creating disease-resistant plants produced through

[1] This widely accepted assumption has been mentioned in literature; however, evidence that HLB did not originate in China has been presented by Beattie et al. (2008) in their paper "On the Origins of Citrus, Huanglongbing, *Diaphorina citri* and *Trioza erytreae*" available at https://www.plantmanagementnetwork.org/proceedings/irchlb/2008/presentations/IRCHLB.K.2.pdf. Accessed May 4, 2018.

TABLE 1-1 NRC (2010) Research and Technology Recommendations

Near to Intermediate Term	Long Term
NI-1. Improve insecticide-based management of ACP.	L-1. Support the development of transgenic HLB-resistant and ACP-resistant citrus.
NI-2. Support searches for biomarkers that may be exploited to detect CLas-infected citrus.	L-2. Support development and testing of bactericides, therapeutics, or systemic acquired resistance activators.
NI-3. Establish citrus orchard test plots for evaluation of new scouting and therapeutic methods.	L-3. Support analysis of ACP behavior, ACP–plant interactions, and ecology to enhance the knowledge base available for new ACP management strategies.
NI-4. Accelerate the sequencing, assembly, annotation, and exploitation of a sweet orange genome to provide a powerful tool for all future citrus improvement research.	L-4. Explore possible control strategies based on release of modified psyllid males.
NI-5. Support development of HLB model systems.	
NI-6. Exploit the CLas genome sequence for new strategies of HLB mitigation.	
NI-7. Support research aimed at developing alternative ACP management strategies.	
NI-8. Support small-scale studies on the feasibility of alternative horticultural systems suited to endemic HLB.	
NI-9. Support demonstration of RNA interference effects for possible suppression of ACP.	
NI-10. Develop *in vitro* culture techniques for CLas to facilitate experimental manipulation of the bacterium for insights into gene function.	
NI-11. Sequence, assemble, and annotate the ACP genome to provide basis for new approaches to ACP management.	

traditional breeding methods or genetic engineering, and (4) control of the HLB bacteria and improvement of the host response (CRDF, 2010). Between 2010 and 2017, CRDF invested $124 million in 398 citrus projects, of which about 90% are focused on HLB.[2] The categories of the currently funded CRDF projects, established by the CRDF's Research Management Committee, are presented in Table 1-2.

[2] H.W. Browning, COO, CRDF, personal communication, June 13, 2017.

TABLE 1-2 HLB Research Areas Funded by CRDF, MAC Group, NIFA CDRE, and CRB

Citrus Research and Development Foundation (CRDF)	Multi-Agency Coordination (MAC) Group
Currently Funded Research Areas	**2014 Research Areas**
1. Consequences of HLB infection	1. Early detection
2. *Candidatus* Liberibacter asiaticus (CLas) culture, genomics, molecular biology, and Koch's postulates	2. Sustainability (through management practices)
	3. Therapies for infected trees
3. Citrus response to infection: symptoms, defense, CLas spread in the plant, systemic acquired resistance	4. Vector control
	2015 Research Areas
4. HLB pathogen and disease detection	1. Psyllid management
5. HLB epidemiology and mitigation of HLB cultural practices	2. Presymptomatic HLB detection
	3. Therapies to protect existing trees
6. ACP monitoring and behavior, cultivation and relationship to CLas	4. Sustainability of new plantings
7. ACP chemical, biological, or biochemical management, chemical attractants and repellants	5. Inoculum management
	2016 Research Areas
	1. Early detection
8. ACP trapping and repelling plants	2. Sustainability (through management practices)
9. Citrus genomics and transcriptomics	
10. Conventional citrus breeding for resistance	3. Therapies for HLB-infected trees
11. Transgenic and viral/bacterial vector resistance to HLB mediation of citrus	4. Vector control
12. Model systems, including chemical screening	
13. Unclassified	
14. Product development	

SOURCES: MAC 2014 and 2015 Research Areas (USDA, 2017a); MAC 2016 Research Areas (USDA APHIS, 2016); NIFA CDRE RFA (USDA NIFA, 2017); CRB Research Areas Funded/2017 Priorities (Schulz, 2017).

National Institute of Food and Agriculture (NIFA) Citrus Disease Research and Extension (CDRE) (Fiscal Year [FY] 2017)	Citrus Research Board (CRB)
NIFA CDRE Priority Research Areas (FY 2017)	**Research Areas Funded Since 2008**
1. Development of therapies to kill or suppress CLas within trees or prevent CLas infection of healthy trees	1. HLB-resistant/tolerant scions and rootstocks
	2. Early detection technologies (EDTs)
2. Development of tolerance or resistance to HLB in cultivars commercially important in all citrus production regions with a focus on delivery of new cultivars (scion and rootstock) using all available plant-improvement strategies	3. Vector control
	4. Antimicrobial therapies
	5. Biocontrol (integrated pest management)
3. Systems for delivering new or currently available therapies into the phloem of citrus trees	6. Citrus Clonal Protection Program
	2017 Research Priorities
	1. Field implementation of EDTs
4. Development of techniques or substrates that permit CLas to be produced in artificial culture	2. Use of EDTs within a systems approach to rapidly identify infected trees
5. Development of methodologies that allow for the early detection of CLas in nonsymptomatic citrus plants and in *Diaphorina citri* (ACP), the insect vector of the pathogen	3. Breeding of new rootstock and scion varieties to provide resistance/tolerance to CLas
	4. Alternative methods of culturing the CLas bacterium
6. Development of pre- and postharvest tools to maximize citrus fruit quality for use as fresh fruit or processed products	5. Further development of bactericides

The U.S. Department of Agriculture (USDA) also has been supporting HLB research, providing more than $380 million between fiscal years (FY) 2009 and 2015. Since 2015 it has allocated an additional $43.6 million (for 14 HLB projects) through the National Institute of Food and Agriculture (NIFA) Specialty Crop Research Initiative (SCRI) Citrus Disease Research and Extension (CDRE) program (USDA, 2016). The CDRE program's regional and national priorities address these four general categories: (1) the HLB pathogen, (2) the HLB insect vector, (3) citrus orchard production systems, and (4) nonagricultural citrus tree owners. In January 2017, NIFA funded four grants worth $13.6 million through its CDRE program. In April 2017, the agency announced the availability of $21.8 million for HLB research, also through their CDRE program. The most recent HLB-related request for applications (RFA) issued by NIFA requested that applicants address the six areas of highest priority for delivery of solutions in the near or intermediate timeframe, that were identified by the Citrus Disease Subcommittee of the National Agricultural Research, Extension, Education, and Economics (NAREEE) Advisory Board. According to the RFA, these priority areas (see Table 1-2) are to be addressed "through the integration of research and extension activities that use systems-based, trans-disciplinary approaches."

The USDA Agricultural Research Service (ARS) is also actively involved in HLB research. In 2017, the ARS annual expenditures on HLB-related research exceeded $21 million. Collaborative investigations are ongoing at seven ARS locations in California, Florida, Maryland, and New York, and with scientists at several universities. Areas of study include breeding for resistance to HLB, impact of HLB on flavor quality, vector control and transmission disruption, early detection, therapeutic treatments via thermotherapy, and antimicrobial sprays.[3]

In December 2013, the HLB Multi-Agency Coordination (MAC) Group[4] was formed by then–agriculture secretary Thomas Vilsack to promote coordination across federal and state agencies and industry in order to rapidly deliver near-term solutions to help citrus growers maintain grove productivity until long-term solutions could be developed. The MAC Group awarded $20 million to 31 projects in FY 2014–2015 and $3 million to six projects in FY 2016–2017 (USDA, 2017a). The focus areas of projects funded by the MAC Group in 2014 and 2015 are listed in Table 1-2. As of June 2017, the MAC Group has awarded about $25.5 million in projects;

[3] G. Wisler, USDA-ARS (retired), personal communication, January 25, 2018.

[4] The HLB MAC Group is composed of representatives from USDA Animal Plant Health Inspection Service (APHIS), USDA NIFA, USDA Agricultural Research Service, Environmental Protection Agency, State Departments of Agriculture from Arizona, California, Florida, and Texas, and the citrus industry.

an additional $5.5 million was received by the MAC Group from Congress for FY 2017–2018, which brings the total amount of MAC funding for HLB research to $31 million.[5]

The USDA Animal and Plant Health Inspection Service (APHIS) Citrus Health Response Program (CHRP) provides funding for the administration of domestic regulations, pest surveys, coordinated area-wide suppression of ACP, and other initiatives in partnership with state regulatory agencies and the citrus industry in Florida, California, Texas, and Arizona (USDA, 2017b). From FY 2014 to FY 2016, however, CHRP also invested about $1.6 million on six research projects that were focused on HLB detection, HLB management practices, and ACP biocontrol (CRDF, 2016).

California citrus growers, through the Citrus Research Board (CRB), have invested $33 million in ACP and HLB research since 2008. HLB projects funded since 2008 fall into six categories (see Table 1-2). The 2017 CRB HLB research priorities (Schulz, 2017) are also listed in Table 1-2. To date, CRB has funded about 97 HLB research projects and has co-funded about 5 projects with CRDF.[6]

Texas growers also fund citrus research through the Texas Citrus Producers Board (TCPB) but not to the same extent as California and Florida growers. From 2015 to 2016, TCPB invested about $193,000 on research related to HLB and citrus production/plant improvement (USDA, 2017b).

STUDY ORIGIN/RATIONALE

In late 2016, CRDF contacted the Board on Agriculture and Natural Resources of the National Academies of Sciences, Engineering, and Medicine to request an independent review of the portfolio of research projects that have been or continue to be supported by the CRDF. This request was made to identify ways to retool HLB research—which, despite significantly increasing understanding of the factors involved in HLB, has produced no major breakthroughs in controlling the disease—and accelerate the development of durable tools and strategies that could help abate the damage caused by HLB and prevent the possible collapse of the Florida citrus industry.

STATEMENT OF TASK

This study was carried out by the Committee on a Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting a Ravaging Disease in

[5] A. McMellen-Brannigan, APHIS, personal communication, June 29, 2017.
[6] G. Schulz, President, CRB, personal communication, June 19, 2017.

accordance with the statement of task in Box 1-1. The committee members' biographical information is given in Appendix A of this report.

COMMITTEE'S APPROACH

This review of the HLB research and development efforts was conducted using information from various sources, including CRDF (which provided publicly accessible information on past and current projects), principal investigators of past and current projects supported by CRDF, HLB researchers who were invited to speak at committee open sessions and webinars, refereed journal articles, and nonrefereed publications (e.g., trade magazines, extension materials, and meeting or conference proceedings).

BOX 1-1
Statement of Task

An ad hoc committee will conduct an assessment of citrus greening research efforts supported by the Citrus Research and Development Foundation (CRDF) from 2010 to 2016 to identify ways to retool citrus greening research to accelerate the development of durable tools and strategies that could help abate the damage caused by citrus greening and prevent the total collapse of the citrus industry.

The committee will examine the state of knowledge on citrus greening and review the portfolio of research projects that have been or continue to be supported by CRDF to determine

1. if the research efforts are in line with the research and technology recommendations in the 2010 National Research Council report,
2. research areas where progress has been achieved/not achieved,
3. research areas where efforts should be continued/discontinued,
4. research areas where more focus is needed or in which efforts need to be expanded and intensified,
5. research areas where efforts need to be integrated,
6. other promising research avenues to pursue, and
7. applicable research techniques/approaches from other scientific disciplines for consideration.

The committee will also look at the portfolio in light of research efforts that are currently being funded by the California Citrus Research Board and federal agencies and institutions and examine opportunities for synergy. The committee will prepare a report describing what is currently known about citrus greening: the disease, its causal organism, its vector, and the vector–pathogen–plant relationship; what knowledge is needed to improve disease control; and the committee's conclusions with respect to a viable research strategy.

The committee held four meetings and three webinars between March and December 2017. At CRDF's request, the first committee meeting was held in conjunction with the 5th International Research Conference on Huanglongbing (IRCHLB) in Orlando, Florida. At this meeting, the committee members had the opportunity to listen to oral presentations at the IRCHLB. The committee also talked with CRDF representatives, received clarification on the committee's charge, and learned about HLB research supported by USDA APHIS, the HLB MAC Group, and California's CRB from some of the invited speakers.

The committee's second, third, and fourth meetings were held in May (Irvine, California), July (Washington, D.C.), and November (Washington, D.C.), respectively. Public forums were held during the open sessions of the second, third, and fourth meetings on the following topics: (1) citrus breeding and transformation for HLB resistance, (2) ACP control and management, and (3) cultural practices to keep HLB-infected trees productive. Webinars were held in August, September, and October on the following topics: (1) HLB diagnostics and detection, (2) CLas and bacterial control, and (3) economic and sociological impacts of HLB and HLB management strategies. The speakers and agendas for the forums and webinars are provided in Appendix B.

To aid its analysis of what is currently known about HLB and the research efforts, progress, and pitfalls, the committee classified the various research projects (funded by CRDF and other agencies) into these categories: HLB causal (or associated) bacteria biology and ecology, HLB insect vector biology and ecology, HLB plant hosts, host–pathogen interactions, pathogen–vector interactions, vector–host interactions, diagnostics, and management (bacterial control, insect control, and cultural control).

Part of the committee's charge was to determine which research areas should be continued or discontinued by highlighting notable outcomes in each of these research areas; the committee addressed this charge by highlighting notable outcomes in each area, reviewing factors that hamper or present significant challenges (pitfalls) to the work, and providing suggestions of possible areas for future research (Chapter 4). However, the committee was unable to define further the extent of research progress or to comment more specifically on the selection of research areas to be continued or discontinued because information available to it on research outcomes, applications, and impacts was insufficient to do so in many cases, particularly for recent projects. A number of projects have resulted in no, or relatively few, publications in peer-reviewed journals, and midterm and final research progress reporting to CRDF was inconsistent in both compliance and the amount of detail provided.

SCOPE AND ORGANIZATION OF THE REPORT

This report consists of four chapters. This chapter provides the context for this study. Chapter 2 provides key information about the disease that reflects the current understanding of the factors that influence its occurrence, severity, and incalcitrance to effective management. Chapter 3 examines the HLB research areas that have received or continue to receive support from CRDF and the HLB research efforts that are being funded by other agencies (state and federal) and overlaps or duplications with CRDF-funded research efforts. Chapter 4 presents significant research findings and progress, as well as issues of concern (pitfalls), from the past decade of CRDF-funded HLB research as well as from input to the committee by researchers, growers, and other stakeholders. Chapter 4 also includes possible future research directions for CRDF consideration, as well as overall major findings, conclusions, and recommendations of the committee.

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2

Current Knowledge on Huanglongbing (HLB) and the Interactions of the Pathogen, Vector, and Host

Research on citrus huanglongbing (HLB) and its causal agents, vectors, and hosts has been going on for many decades, and while controlling HLB remains a major challenge, much has been learned about this disease. Although not a comprehensive review of what is known about HLB since its first reported occurrence in the early 1900s, this chapter provides key information about the disease that reflects the current understanding of the factors that influence its occurrence, severity, and incalcitrance to effective management.

HLB: THE CAUSAL (OR ASSOCIATED) BACTERIA

Candidatus Liberibacter asiaticus

Candidatus Liberibacter asiaticus (CLas), the gram-negative, walled bacterium to which citrus greening (HLB) in the United States is attributed, is a member of the family Rhizobiaceae[1] in the phylum Proteobacteria. Two closely related bacteria also believed to cause HLB, *Ca*. Liberibacter africanus (CLaf) and *Ca*. Liberibacter americanus (CLam), have been characterized from other geographical locations and named for the continent on which they were first found. All three species are nonculturable and phloem restricted *in planta*, leading to the "*Candidatus*" taxonomic status.

[1] A family of Proteobacteria having a range of impacts on plants, some fixing nitrogen after becoming established inside root nodules of legumes, others causing plant disease by genetically engineering the plant host.

Genetic and Morphological Characteristics

Understanding the plant-colonizing and disease-inducing mechanisms of CLas has been hampered by the inability to grow it in the laboratory, and much of what is known or hypothesized has been deduced from multiple genome sequences of the three HLB-associated bacteria. A critical feature for both survival and pathogenicity of the bacteria is the ability to secrete cytoplasmically synthesized enzymes and virulence factors through the cell plasmalemma (a plasma membrane that bounds a cell, especially one immediately within the wall of a plant cell).

Most of the known CLas strains harbor two nearly identical prophages, SC1 and SC2 (Zhang et al., 2011). SC1 replicates, produces phage particles, and becomes lytic in the plant host (but not in the psyllid vector), while SC2 lacks lysis-associated genes and exists as a replicative excision plasmid (a small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently). The CLas phage SC2 encodes two putative peroxidases believed to contribute to host defense responses by scavenging reactive oxygen species (Zhang et al., 2011) and downregulating genes involved in H_2O_2-mediated defense signaling in plants (Jain et al., 2015), thereby suppressing host symptom development.

The genome of CLas, at 1.23–1.15 Mb (Duan et al., 2009; Zheng et al., 2014), is significantly smaller in size and has fewer genes and lower guanine and cytosine content than related culturable bacteria. Sequence annotation indicated that these features are reflective of the loss of a number of functions during the microbe's adaptation to its intracellular parasitic lifestyle within the host plant and insect vector. Several genes related to DNA and excision repair are lacking, and the species has only a small subset of sigma factors (bacterial transcription initiation-related proteins that enable specific binding of RNA polymerase to gene promoters) involved in transcription (Hartung et al., 2011). Some metabolic enzymes, including those required for purine and pyrimidine[2] metabolism, also have been lost (Hartung et al., 2011).

Disease Development and Symptoms

Because noncultivability of CLas and its HLB-associated relatives prevents the completion of Koch's postulates, the question remains whether these bacteria are solely responsible for HLB or whether they are components of a disease complex (Sechler et al., 2009). In fact, phytoplasmas have also been associated with the disease in Brazil (Teixeira et al., 2008)

[2] A purine is a heterocyclic aromatic organic compound consisting of a pyrimidine ring and an imidazole ring, whereas a pyrimidine is an aromatic heterocyclic organic compound containing two nitrogen atoms; both are components of nucleic acids.

and China (Chen et al., 2009), frequently in mixed infections with *Ca.* Liberibacter spp. What role, if any, other microbes play in the disease remains to be clarified.

Initial tree symptoms are frequently the appearance of yellow shoots and blotchy, mottled leaves, sometimes with vein yellowing. Symptoms may differ on opposite halves of a leaf. As the bacteria translocate and colonize the plant systemically the canopy progressively becomes chlorotic, tree growth slows, leaves remain smaller than normal, and leaf tips become necrotic (Timmer et al., 2000; da Graça and Korsten, 2004; Halbert and Manjunath, 2004; Bové, 2006; Gottwald et al., 2007). Often, parts of the tree remain healthy or symptomless such that the disease has a sectored appearance. Leaves can thicken and veins enlarge and appear corky. Later, yellow blotches appear between veins that remain green, similar to zinc deficiency,[3] and leaves may drop as twig ends become necrotic (Gottwald et al., 2007).

Fruit from diseased trees fails to achieve full size, may be asymmetrical and have areas on the surface that remain green, and is marked by bitter taste. Seeds may be aborted. As the disease progresses fruit yield and quality decline (Timmer et al., 2000; Halbert and Manjunath, 2004; Bové, 2006), eventually falling below an economically tolerable level. All species of the family Rutaceae are potential hosts. Historically, the most susceptible are sweet oranges (*Citrus sinensis* L. Osb.), tangelos (*Citrus* × *tangelo*), and mandarins (*C. reticulata* Blanco). Moderately susceptible hosts have included grapefruits (*C. paradisi* Macf.), lemons (*Citrus* × *limon*), Rangpur lime (*Citrus* × *limonia* Osb.), calamondins (*X Citrofortunella microcarpa*), and pomelos (*C. maxima*). Mexican limes or key limes (*Citrus* × *aurantifolia* Swingle) and trifoliate orange (*C. trifoliata* or *Poncirus trifoliata*) may be even more tolerant. Noncitrus Rutaceae species, such as *Murraya paniculata*, also serve as hosts of the HLB-associated pathogens (Timmer et al., 2000; Appel, 2004; da Graça and Korsten, 2004; Halbert and Manjunath, 2004; Lopes et al., 2005; Bové, 2006). The symptoms of HLB caused by the American (CLam) and African (CLaf) bacteria are very similar to those caused by CLas, but environmental optima differ, CLaf developing under cool temperatures (20°C–25°C) and CLam under a wider temperature range (20°C–35°C).

After inoculation into the citrus phloem sieve tubes by the psyllid vector, CLas population levels increase rapidly, peaking at approximately 200 days postinoculation and reaching about 10^8 cells per gram of plant tissue (estimated by copies of 16S ribosomal DNA) (Coletta-Filho et al., 2014). However, infected trees remain asymptomatic through the early disease

[3] Zinc deficiency symptoms include reduced leaf size, narrow leaves, yellow mottled on green background, and decreased overall fruit yield.

stages, and early symptoms are difficult to recognize because of their mildness and resemblance to other conditions.

HLB: THE PSYLLID VECTOR

Asian Citrus Psyllid

Geographic Distribution and Invasion

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), originally from India or other parts of Asia,[4] has been recognized as an invader in North America and Latin America for several decades. In 1998, it was initially discovered in the Caribbean Basin, on the island of Guadeloupe (Étienne et al., 1998), and in South Florida (Halbert, 1998; Halbert et al., 2002). Since then, populations have spread widely among islands and other countries in the Caribbean Basin, including the Bahamas, the Caymans, Cuba, Dominican Republic, Jamaica, Mexico, Puerto Rico, and Venezuela (Halbert and Nuñez, 2004). In the continental United States its range now extends from South Carolina to Florida in the south; and from Georgia westward to Alabama, Arizona, Louisiana, Mississippi, southern California, and Texas (USDA APHIS, 2017). It has also been found in the Hawaiian Islands, on Hawaii, Kanai, Lanai, Maui, Molokai, and Oahu (Conant et al., 2009). In the insect's native range (e.g., Asia), ACP infests native citrus but rarely if ever causes direct damage as a result of feeding. Importantly, the insect affects citrus as a result of its capacity to persistently transmit different species of HLB-associated bacterial pathogens classified as *Ca.* Liberibacter spp. (Yang et al., 2006). Two separate ACP introductions (founding events) have likely occurred in the Americas, one each in South and North America, whereas North America and Hawaii appear to share a similar source of invasion. In most of the areas where the ACP has become established, it has spread rapidly in residential and commercial plantings through both natural and human-assisted transport. Across nearly all of Florida, populations of ACP are well established, and now active populations have been detected in many areas of California. Since the initial introductions, recent phylogeographic[5] investigations of the ACP mitochondrial cytochrome oxidase subunit I gene have revealed variations in psyllid populations collected in the Americas and the Pacific region (Boykin et al.,

[4] The origin of ACP is discussed at length by Beattie et al. (2008) in their paper "On the Origins of Citrus, Huanglongbing, *Diaphorina citri* and *Trioza erytreae*" available at https://www.plantmanagementnetwork.org/proceedings/irchlb/2008/presentations/IRCHLB.K.2.pdf. Accessed May 4, 2018.

[5] Pertaining to the study of the historical processes responsible for geographic distributions of individuals by considering population genetics.

2012). Specifically, 23 ACP haplotypes[6] were identified, and these fell into two groups: from South America (group 1) and from North America and Hawaii (group 2).

Psyllid Biology: Description, Communication, Life Cycle, and Reproduction

Psyllids, or jumping plant lice, have been classified historically as members of the family Psyllidae (a family composed of homopterous insects), although recently the group has been recognized as a superfamily comprising eight families, including the Liviidae, to which *D. citri* belongs (Burckhardt and Ouvrard, 2012). Similar in size to aphids, psyllids are typically more host specific. They commonly infest new flush growth or tender leaves. Consistent with their common name, the jumping plant lice are generally more active than aphids, readily propelling themselves into the air by rapid movements of their hind legs (Burrows, 2012) or by taking flight if disturbed. Psyllid antennae usually possess 10 segments and lack abdominal cornicles, which are unique to the more sedentary aphids. Adult ACPs are approximately 3.5 mm in length and have mottled brown bodies. Forewings of the adults are broader distally and mottled, with a brown band that encircles the outer half. Antennae have black tips with two small, light brown spots on the middle segment of the flagella. Older adults are covered with a whitish waxy secretion, giving the adult insect a dusty appearance (Martin et al., 2012). Adult ACPs are oviparous and eggs are very small, elongate, and thicker at the base and tapered toward the distal end (Martin et al., 2012).

Like other psyllid species, the ACP depends on multimodal signaling for mate finding and courtship (Stockton et al., 2017a). Depending on plant species, volatiles from intact or psyllid-damaged foliage can act as long-distance attractants for male and/or female adults searching for host trees and populations of conspecifics (Gharaei et al., 2014). Males rapidly vibrate their wings to send substrate-borne signals to females, who respond in kind (Wenninger et al., 2008) in a behavior called duetting (Mankin et al., 2013). After this exchange of vibrational signals, cuticular hydrocarbons produced by females appear to function as short-range orientation cues for males. Which cuticular constituents are responsible for male attraction remains unclear, but dodecanoic acid, which is present in cuticle extracts, was attractive to males in laboratory bioassays. Moreover, traps baited with dodecanoic acid in the field attracted more males at the highest concentration tested than did traps without the compound (Mann et al., 2012a).

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[6] A haplotype (haploid genotype) is a particular combination of markers (alleles) at multiple locations on a chromosome, used in assessing relatedness among individuals.

Experience can modify behavioral responses to chemical signals; prior to sexual maturity, which typically is achieved 3 to 4 days posteclosion (emergence from the pupal case or hatching from the egg), both males and females appear to engage in "practice courtship and copulation behaviors" (Stockton et al., 2017a); as well, after successfully mating, males are more responsive to female odors. Females are capable of mating with multiple males, but the presence of multiple males over a period of weeks may reduce oviposition success (Wenninger et al., 2008). During mating males can transmit CLas to uninfected females (Mann et al., 2012a).

Female ACPs require multiple matings throughout their adult life to maintain viable eggs and begin to lay eggs as soon as 1 day after mating. Eggs are laid on the emergent tips of new citrus shoots (e.g., "feather flush" growth) on and between unfurling leaves, and adult females may lay more than 800 eggs during their lives (Liu and Tsai, 2000). Newly laid eggs are oriented with the long axis vertical to the leaf surface; they are initially pale in color, later turning yellow and finally orange before hatching. Eggs hatch within 48 to 96 hours. Nymphs are significantly smaller than adults, and the five nymphal instar stages are typically completed in 11 to 15 days. Instars are initially light yellow in early stages, transitioning to yellowish orange in later stages. There are no abdominal spots but the wing pads are large, and filaments are confined to the apical plate of the abdomen. The total life cycle requires between 15 and 47 days, depending upon environmental conditions, and newly emerged adults can live for several months (Grafton-Cardwell et al., 2013).

Dispersal and Seasonal Phenology

There is no known ACP diapause, but activity of adult populations, as measured by sticky cards and beat samples, is considered low in winter months and prior to spring flush in citrus. Estimated through field-based life history investigations, the ACP has 9 to 10 generations per year, whereas in field cage evaluations up to 16 generations have been recorded (Hall et al., 2008; Hall and Hentz, 2010). The nymphs, which are nearly always found on new growth, move in a slow, steady manner when disturbed. The adults can jump or leap greater distances when disturbed and may even fly short distances. Adults are usually found in aggregations on lower leaf surfaces with their heads often pressed against the abaxial (lower) surface of new leaves. The time of year of greatest psyllid activity corresponds with the periods of new flush growth in citrus, and some variation in this activity is concomitantly associated with variation in citrus varietal flush patterns (Hall and Hentz, 2010; Khan et al., 2014). Investigations of disease progress in citrus have made inferences about the timing and distance of ACP dispersal that suggest that adult flights can regularly occur over 25- to 50-m distances. In portions of Southeast Asia, dispersal of nearly 500 km has been suggested, resulting from major synoptic weather patterns (Khan et al., 2014; Lewis-Rosenblum et al., 2015). Protein marking has revealed that adults can regularly move distances approaching 100 m in 72 hours and that movement into managed citrus groves in Florida often resulted from invasion from adjacent abandoned citrus in Florida (Kobori et al., 2011; Hall and Hentz, 2014).

Once dispersing adults have located suitable host plants for feeding or oviposition, trivial plant-to-plant movements appear to constitute most of their activity. Based upon preliminary trapping data, no obvious seasonal movement patterns have been described except for elevated activity around the spring flush of citrus foliage. Both managed and abandoned citrus plots are infested by adult ACPs, but flight patterns of these insects are not obviously related to either discrete edaphic (of, produced by, or influenced by the soil) or abiotic sets of conditions (e.g., wind, short-wave radiation, temperature, and humidity) (Patt and Sétamou, 2010; Hall and Hentz, 2011). Laboratory-based flight mill tests suggest that adult ACPs are able to sustain continuous flight for estimated distances of nearly 1.2 km (Arakawa and Miyamoto, 2007). The majority of adult ACPs, however, have far shorter flight times and their dispersal consists of short-duration, tree-to-tree flights. Although temporal patterns of these short-duration flights are consistent in local citrus-producing regions, no flight phenology predictions have been generated to describe patterns of movement, migration, or capture over larger landscapes. However, the elevated activity in captures may be due to seasonal variation in the timing of spring flushing in citrus, which can also vary regionally. The lack of consistency in adult ACP movement patterns underlies the need for vigilance in monitoring and surveillance of these mobile pests.

Microbial Associates and Interactions with the Asian Citrus Psyllid

The microbiome of ACP has recently been investigated by analysis of adults, nymphs, eggs, and cell cultures. Kolora et al. (2015) found evidence of 10 distinct types of bacteria, and 4 species of bacteria were detected in cell cultures with 2 new types associated with psyllids. Kruse et al. (2017) reported that ACP has three well-described bacterial endosymbionts, *Candidatus* Profftella armatura, *Candidatus* Carsonella ruddii, and *Wolbachia* spp., as well as other internal, extracellular bacteria. Bacterial symbionts of sap-sucking insects generally function to compensate for phloem that is nutritionally poor, specifically providing amino acids that cannot be synthesized by the host. While the prevalence of each endosymbiont in the ACP is positively correlated, their localization and function within the insect vary. Both Profftella and Carsonella are localized in collections of cells called the bacteriome, while *Wolbachia* is found in somatic and reproductive tissues in addition to the bacteriome (Dobson et al., 1999; Hosokawa et al., 2010).

HLB: THE RUTACEOUS HOSTS

Citrus Origin and Taxonomy

Citrus species are widely believed to be native to subtropical and tropical regions of Asia and the Malay Archipelago.[7] Citrus spread from these areas of origin to regions across the globe having tropical and subtropical climates (Webber, 1967). Ancient Roman literature describes citrus culture, and people of the Mediterranean region have continued to cultivate citrus trees, particularly mandarins, sweet orange, and lemons, producing fruits for local consumption and for export (Webber, 1967). Spanish conquistadors brought citrus to Florida sometime between 1513, when Ponce de León first landed in Florida, and 1565, when the city of St. Augustine was established (Webber, 1967).

The genus *Citrus* belongs to the family Rutaceae, which contains about 160 genera and 1,800 species. Around 7 million years ago, citrus progenitors diverged into two main genera, *Citrus* and *Poncirus* (Webber, 1967; Pfeil and Crisp, 2008). Phylogenetic and gene-sequencing studies (Scora, 1975; Barrett and Rhodes, 1976; Wu et al., 2014) indicate that most cultivated citrus, sweet oranges, grapefruit, lemons, limes (*Citrus* × *aurantiifolia*), and tangerines (*C. reticulata*), resulted from hybridization that occurred over several thousand years between three citrus species—*Citrus reticulata* (mandarins), *C. maxima* (pomelos), and *C. medica* (citrons)—and possibly a fourth species as yet unidentified. *Poncirus trifoliata* is native to northern China and Korea, and, while cross-compatible with citrus species, *P. trifoliata* differs from the latter in having deciduous compound leaves and pubescent fruit. It is significantly more cold hardy than citrus species and can be cultivated in U.S. Department of Agriculture (USDA) Plant Hardiness Zone 6.

Citrus Biology and Propagation

Citrus trees are broad-leafed evergreens adapted to sunny, humid environments having fertile soil and adequate rainfall or irrigation. Trees flower in the spring, and fruit begin to ripen in the fall or early winter months, depending on the cultivar. Citrus fruits are good sources of vitamin C

[7] The possible origin of citrus is explored by Beattie et al. (2008) in the paper "On the Origins of Citrus, Huanglongbing, *Diaphorina citri* and *Trioza erytreae*" available at https://www.plantmanagementnetwork.org/proceedings/irchlb/2008/presentations/IRCHLB.K.2.pdf. Accessed May 4, 2018.

and flavonoids. The vitamin C content depends on the species, variety, and mode of cultivation (Duarte et al., 2010). The flavonoids include various flavanones and, in lower concentrations, flavones and flavonols (Benavente-Garcia et al., 1997). Citrus fruits are nonclimacteric; i.e., they ripen without ethylene or respiration bursts. Respiration slowly declines during fruit development and ethylene release by the mature fruit is extremely low (Aharoni, 1968; Eaks, 1970; Goldschmidt et al., 1993). Fruits of early varieties of citrus can meet legal maturity standards before the peel attains the characteristic varietal color and therefore require degreening to enhance coloration. This is accomplished by exposure of fruit to ethylene gas, which destroys chlorophyll and allows the yellow or orange rind pigments to predominate (Ritenour et al., 2015).

Citrus species are generally not cold hardy. Mandarins (*C. reticulata*) tend to be the hardiest of the common *Citrus* species and can withstand short periods as cold as –10°C (14°F), but commercial production requires that temperatures do not fall below –2°C (28°F). Citrus fruits are less tolerant to freezing conditions than citrus trees or foliage. Freeze damage to fruit is influenced by variety, minimum temperature, and duration of temperatures (Oswalt and Hurner, 2012). Freeze injury in fruit is characterized by the appearance of water-soaked areas on the segment membranes with the juice sacs or vesicles in injured areas subsequently becoming dry and collapsed. Fruit damaged by freeze may drop quickly or over time. Freeze injury in citrus leaves, caused by ice formation in the intercellular spaces, is indicated by the appearance of water-soaked areas on the leaf surface. Ice formation in wood may result in bark splits, which, if extensive, may cause limbs to die and later break off many years after the freezing event (Zekri et al., 2016).

Citrus cultivars are propagated vegetatively by grafting to maintain the integrity of the desired cultivar. Rootstocks are selected for disease and nematode resistance, soil adaptation, resistance to flooding and drought, cold hardiness, and effects on tree size (Castle et al., 2016). Rootstocks may be seed propagated. For example, rough lemon, sour orange, *P. trifoliata*, and hybrids are polyembryonic and therefore can be seed propagated true to type (Castle et al., 2016).

Citrus Diseases and Insect Pests

In addition to HLB and ACP, there are a number of other diseases and pests that affect citrus. Other diseases that occur in citrus cultivated in Florida include citrus canker, which is a leaf, fruit, and stem blemishing disease caused by the bacterium *Xanthomonas citri* subsp. *citri* (Dewdney and Graham, 2017); fungal diseases that include *Phytophthora* foot and root rots, and brown rot of fruit, which can be caused by *Phytophthora* *nicotianae* or *P. palmivora* (Graham and Dewdney, 2017; Graham et al., 2017), citrus black spot, caused by *Guignardia citricarpa* (Dewdney et al., 2017), *Alternaria* brown spot, caused by *Alternaria alternata* (Dewdney, 2017), and postbloom fruit drop, caused by *Colletotrichum acutatum* (Peres and Dewdney, 2017); viroid[8] diseases that include exocortis and cachexia (Roberts and Brlansky, 2017); *Citrus tristeza virus* (CTV), which is a major cause of the decline and eventual death of trees on sour orange rootstocks (Roberts et al., 2017); and citrus blight, a disease of unknown etiology that causes trees to decline and become unproductive and affects all major rootstocks and seedlings to varying degrees (Futch et al., 2017).

Insects that affect citrus in Florida include the citrus leafminer (*Phyllocnistis citrella*) (Hunsberger et al., 2006); several scale insects such as the snow scale (*Unaspis citri*), the Florida red scale (*Chrysomphalus aonidium*), and the purple scale (*Lepidosaphes beckii*); several whitefly species such as the citrus whitefly (*Dialeurodes citri*), the cloudy-winged whitefly (*D. citrifollii*), the woolly whitefly (*Aleurothrixus floccosus*), and the citrus blackfly (*A. woglumi*); the citrus mealybug (*Planococous citri*); three species of aphids: the green citrus aphid (*Aphis spiraecola*), the cotton or melon aphid (*A. gossypii*), and the brown citrus aphid (*Toxoptera citricida*), which is the vector of CTV (Stansly and Rogers, 2017); three species of root weevils: Diaprepes root weevil (*Diaprepes abbreviatus)*, the blue-green citrus root weevil *Pachnaeus litus*, and the Northern citrus root weevil *Pachnaeus opalus* (Duncan et al., 2017a).

Species of mites that attack citrus include the pink citrus mite (*Aculops pelekassi*), the citrus rust mite (*Phyllocoptruta oleivora*), and the Texas citrus mite, *Eutetranychus banksi* (Rogers and Stansly, 2017).

Nematode species of major economic importance in Florida citrus include *Tylenchulus semipenetrans*, which causes “slow decline” of citrus, and the burrowing nematode *Radopholus similis*, which causes the “spreading decline” of citrus. Nematodes of limited economic importance include the sting nematode *Belonolaimus longicaudatus*, and two species of lesion nematode, *Pratylenchus coffeae* and *P. brachyurus* (Duncan et al., 2017b). For more information on Florida citrus pests and diseases, see the *2017–2018 Florida Citrus Production Guide* (Rogers et al., 2017).

Citrus Production

For many years the United States was the third largest citrus producer in the world, after China and Brazil; however, Food and Agriculture Organization (FAO) data for 2015 (FAO, 2017a) indicate that the United States

[8] An infectious entity affecting plants, smaller than a virus and consisting only of nucleic acid without a protein coat.

is currently behind India in total citrus production. It now ranks fourth and is followed by Mexico and Spain (FAO, 2017b, Table 1, pp. 14–15). While citrus is produced by several countries around the world, orange juice production occurs mainly in Brazil and Florida, with Brazil as the largest orange juice exporter (UNCTAD, 2010).

The major citrus-producing states in the United States are Florida, California, Arizona, and Texas; smaller quantities of citrus are grown in other Sunbelt states[9] and in Hawaii. During the 2016–2017 season in the United States, citrus was grown on 711,000 acres (288,000 hectares) with a total fruit yield of 7.8 million tons. Approximately half of the U.S. citrus produced is sold fresh, and the remainder is sold processed, mostly as juice, with California producing 85% of the fresh and Florida 77% of the processed citrus (USDA NASS, 2017).

Genetics, Breeding, and Biotechnology

The genetic complexity of the genus *Citrus* results in high levels of heterozygosity, and consequently many generations of breeding and selection are required to develop new cultivars having the combinations of genes that produce the desired fruit and juice quality, productivity, and stress and disease resistance traits that are required for the commercial fresh or processing markets. The long juvenility period (3 to 7 or more years) for citrus grown from seed, as is necessary in a breeding program, along with the large areas of land required to grow and evaluate trees, add to the challenges of citrus breeding. Breeding can be hampered also by the peculiar biology of some citrus types or cultivars; nucellar embryony or polyembryony is a condition in which embryos are produced from the seed nucellus, which is derived from the mother tree (Kepiro and Roose, 2007). Since these nucellar embryos grow more rapidly than the hybrid embryo produced from cross pollination, they crowd out the hybrid embryo, which does not develop further. In this case, even though a hybridization was made to produce a new genetic combination, the result is simply a reproduction of the original mother tree and provides no genetic advancement. While a great deal of breeding has been carried out in the United States and other major citrus-growing areas, most of the commercial cultivars currently grown were selected in the 19th century (Purseglove, 1968).

Genetic improvement through genetic engineering offers the potential of circumventing some of the major hurdles affecting conventional

[9] U.S. Sunbelt states include Alabama, Arizona, Florida, Georgia, Louisiana, Mississippi, New Mexico, South Carolina, Texas, roughly two-thirds of California (up to Greater Sacramento), and parts of Arkansas, Nevada, and North Carolina.

breeding and allows for the targeted improvement of existing cultivars, avoiding random assortment of genes and loss of the genetic integrity of the desired cultivar. Genetic engineering relies on transformation, which is the ability to insert a single gene or multiple genes into plant cells and to regenerate whole plants from the transformed cells. Transformation of citrus is specific to genus, species, and genotype and is limited to relatively few genotypes (Donmez et al., 2013). It has generally been undertaken with nucellar embryos, which are produced from the parent tree and are not hybrid. While these explants reproduce the original source tree, the time to fruiting is extended due to juvenility. Transformation rates are lower when using explants from mature trees but the time to fruiting for transgenic plants is reduced (Marutani-Hert et al., 2012). Commercially important cultivars that can be transformed include "Pineapple," "Hamlin" and "Valencia" oranges, "Rio Red" grapefruit, "Eureka" lemon, and "Sucarri" sweet orange (Hu et al., 2016). Nevertheless, rates of transformation are quite low for many genotypes, generally less than 1% transformed plants per total number of explants (Hu et al., 2016). An alternative to the "conventional" genetic engineering technologies is the use of viral-based vectors for the expression of transgenes in citrus plants. This technology does not require the regeneration of plants from genetically transformed tissues but rather the infection of plants with an otherwise symptomless virus expressing a gene(s) for resistance to HLB (Folimonov et al., 2007; Agüero et al., 2012). Expression of the resistance factor may be translocated to all plant parts depending upon the virus, and infection may be initiated through grafting in the nursery or field. Application of gene-editing technologies in citrus should allow for genetic improvement of cultivars by deleting or adding a small number of base pairs in an existing gene that is responsible for a particular trait to be improved (Jia and Wang, 2014; Peng et al., 2017). Gene editing may also be used to add genes from the same species or other organisms as practiced through genetic engineering. Transformation is required for most gene-editing technologies.

To utilize many biotechnological improvement techniques, the identification of genes and gene function is required. The genomes of several citrus cultivars and species have been sequenced[10] (Xu et al., 2013; Wu et al., 2014), 9 and the identification of genes is under way, a process that must take into account the large and complex genomes of citrus species and cultivars.

[10] Citrus genome database URL: https://www.citrusgenomedb.org. Accessed December 18, 2017.

HOST PLANT–PATHOGEN INTERACTIONS AND HOST PLANT DEFENSE MECHANISMS

The molecular interactions between host plants and pathogens determine whether the host will be susceptible, tolerant, or resistant. In the case of HLB, citrus host susceptibility is associated with the development of a variety of symptoms associated with HLB, including reduced fruit production and quality. Study of these interactions provides the ground for basic understanding of the disease and presents targets for control remedies (Wang and Trivedi, 2013; da Graça et al., 2016; Wang et al., 2017). After inoculation of phloem sieve tubes in the citrus host during feeding of the ACP, *Ca.* Liberibacters exploit phloem cellular processes for nutrient acquisition. Annotation of pathogen genomes has revealed hints about how these host nutrients are utilized, although experimental evidence for the dependence of those genes on host nutrients for pathogen viability have yet to be obtained. *Ca.* Liberibacter genomes carry genes encoding transporters and enzymes for metabolism of nucleotides, carbohydrates, amino acids, and lipids derived from host cells (Duan et al., 2009; Lin et al., 2011; Wulff et al., 2014).

Plant defense responses to pathogens are triggered by recognition of conserved "pathogen-associated molecular patterns" (PAMPs) by plant receptors, resulting in a partial reduction of pathogen growth. If the pathogen secretes virulence proteins that are recognized by resistance (R) proteins in the plant, a much stronger form of resistance will occur to arrest pathogen growth completely, making R genes the optimal source of crop disease resistance. Concerted screening efforts have not found evidence of R genes to HLB in any variety of citrus. However, there is substantial variation in the disease tolerance of different citrus varieties and rootstocks; "tolerance" is the ability of a plant to keep growing and producing fruit in the presence of infection. Tolerant citrus varieties have been found to accumulate a greatly reduced pathogen load compared with susceptible varieties (Albrecht and Bowman, 2012) and show less severe symptoms (Fan et al., 2012).

Known HLB-tolerant varieties have been a focus of citrus hybridization efforts, and study of tolerance mechanisms may lead to better strategies for screening and breeding for tolerance. Tolerance may be related to preformed pathogen barriers (such as structural and chemical defenses) or an increased ability to actively mount an effective PAMP-triggered immunity (PTI). However, compared with some other well-studied gram-negative bacterium–plant systems, basic knowledge about the interactions between *Ca.* Liberibacters and their hosts has lagged, largely due to the unculturability of the pathogen *in vitro* (Wang et al., 2017; Blaustein et al., 2018). CLas induces a variety of host hormonal and defense gene changes consistent with PTI (Kim et al., 2009; Pitino et al., 2017), and genomic analysis indicates the presence of typical PAMP elements (Duan et al., 2009). One CLas-derived PAMP, CLas-flg22, is sufficient to trigger defenses in citrus and tobacco (Zou et al., 2012; Shi et al., 2018). CLas-flg22 is a conserved N-terminal 22–amino acid domain that triggers defense gene activation and physiological responses (callose deposition) when expressed in *Nicotiana benthamiana* cells; the activation of many citrus defense genes by flg22 is stronger in tolerant than in susceptible cultivars (Zou et al., 2012; Shi et al., 2018). Treatment of citrus with either CLas or CLas-flg22 caused distinct defense gene expression responses in tolerant and susceptible orange and grapefruit varieties, allowing the identification of candidate tolerance-associated genes (Wang et al., 2016; Shi et al., 2018). However, some defense mechanisms could actually harm the plant, resulting in phytotoxicity and symptom development or causing emission of volatile signals that attract ACP (Mann et al., 2012b; Pitino et al., 2017).

Plant defense mechanisms are typically suppressed by pathogen virulence proteins, and CLas appears to suppress defense responses in citrus (Nwugo et al., 2013a). Study of predicted pathogen virulence genes, revealed through genome sequencing, has provided clues to important components of the citrus defense response. Two predicted CLas-secreted proteins are very effective degraders of reactive oxygen species and salicylic acid, respectively, suggesting that these are critical defense signals in citrus, as they are in other plants (Jain et al., 2015; Li et al., 2017).

Citrus–*Ca*. Liberibacter interactions also involve effector proteins of bacterial origin that are predicted to modulate host cellular functions for the benefit of *Ca*. Liberibacter CLas multiplication and colonization of host phloem cells (Wang et al., 2017). Generally, microbial effectors play an important role in bacterial pathogenesis by restricting the host defense or interfering with host developmental processes in ways that benefit the pathogen (Jones and Dangl, 2006). *Ca*. Liberibacter genome sequences revealed the presence of substrate proteins derived from type I secretion systems (T1SSs)[11] and general secretory systems (Sec) in some *Ca*. Liberibacters, but genes for other secretion systems commonly found in other plant pathogenic bacteria are absent from the *Ca*. Liberibacter genomes sequenced to date (Duan et al., 2009; Lin et al., 2011; Fagen et al., 2014; Wulff et al., 2014). T1SS effector genes encoding serralysin and hemolysin have been identified from CLas isolates, but their role in host interactions remains to be experimentally determined (Duan et al., 2009). More than a hundred genes encoding substrates associated with Sec (i.e., containing a signal peptide for the Sec system) have been predicted for CLas strains (Prasad et al., 2016). Some predicted Sec-dependent effectors (SDEs) have

[11] T1SS is a simple gram-negative bacterial secretion system that moves cytoplasmic molecules across all layers of the cell envelope and the periplasm in a single step.

been confirmed experimentally as substrates of Sec (Cong et al., 2012; Pitino et al., 2016). Although the role of SDEs in relation to HLB remains unclear, there is evidence that some could play an important virulence role. For example, Las5315 has been shown to be localized in the chloroplast and to induce callose deposition and trigger host cell death when expressed transiently in *N. benthamiana* (Pitino et al., 2016). Their role could be deciphered by *in planta* transient or transgenic expression in a plant, perhaps *N. benthamiana* or citrus.

Most importantly, the host plant responds to bacterial infection also at the transcriptional level. Citrus or citrus relatives, whether tolerant or susceptible to HLB, reprogram their transcriptomic network in response to *Ca*. Liberibacter infection. Response levels are generally stronger at earlier stages than later ones, and stronger in tolerant cultivars than in susceptible ones (Kim et al., 2009; Albrecht and Bowman, 2012; Fan et al., 2012; Martinelli et al., 2012, 2013; Aritua et al., 2013; Mafra et al., 2013; Yan et al., 2013; Zhao et al., 2013; Zheng and Zhao, 2013; Rawat et al., 2015, 2017; Xu et al., 2015; Fu et al., 2016; Wang et al., 2016; Zhong et al., 2016; Hu et al., 2017; Yu et al., 2017). The transcriptomic responses involve upregulated genes that can be categorized into a range of biological processes with which they are associated, such as those related to defense networks, photosynthesis and metabolism, hormone-mediated signaling networks, cell wall metabolism, and reduction/oxidation processes. Most defense or stress-response genes are upregulated in all genotypes of host plants at an early stage of infection (Mafra et al., 2013; Martinelli et al., 2013), but some, such as those involved in the mitogen-activated protein kinase (MAPK) signaling pathway, activation of peroxidases, Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and POD4, and nucleotide binding site–leucine-rich repeat (NBS-LRR) type genes, are differentially upregulated in tolerant genotypes (Fan et al., 2012; Nwugo et al., 2013b; Hu et al., 2017; Yu et al., 2017).

Genes related to callose deposition in the phloem of infected plants (both susceptible and tolerant cultivars) are upregulated along with genes involved in starch biosynthesis, while genes involved in starch degradation are downregulated more strongly in susceptible than in tolerant genotypes (Boava et al., 2017). These findings are consistent with the hypothesis that callose deposition at the sieve pores interferes with the transport of photosynthetic products in phloem, enhancing the symptoms of HLB (Kim et al., 2009; Koh et al., 2012; Martinelli et al., 2013; Boava et al., 2017).

Certain genes reflecting the transcriptomic responses of host plants to *Ca*. Liberibacter infection can be confirmed at the proteomic level. For example, defense-related chitinase, miraculin-like proteins, Cu/Zn-SOD, and lipoxygenase are upregulated in CLas-infected sweet orange plant leaves (Fan et al., 2011). Evidence of upregulation of radical ion detoxification proteins (e.g., glutathione-S-transferases) for tolerance to CLas has also been reported (Martinelli et al., 2016).

Finally, host–pathogen interactions in HLB also include host metabolic responses that may result from the manipulation of metabolic pathways by the pathogen for its benefit, or may result from host cellular function for defense reaction. One of the most obvious host responses to *Ca*. Liberibacter infection is an increase in total amino acid abundance, indicating benefits to both pathogen and host defense (Killiny and Hijaz, 2016; Killiny and Nehela, 2017). Other metabolites, such as terpenoids, appearing at higher levels in tolerant than in susceptible genotypes, may play an important antibacterial role, restricting pathogen growth, while other metabolites, present at lower levels, may restrict pathogen nutrient acquisition (Hijaz et al., 2013, 2016; Albrecht et al., 2016; Killiny and Nehela, 2017).

PATHOGEN–VECTOR INTERACTIONS

CLas is dependent upon the ACP to move between plant hosts and perhaps to contribute to its long-term survivability, since the bacterium replicates to some extent in the insect (Inoue et al., 2009; Ammar et al., 2016). Because CLas impacts ACP fitness it is a pathogen of both plant and insect. There is some evidence that CLas evolved first as an insect pathogen or symbiont, later becoming a plant pathogen (Gottwald, 2010). In the ACP, CLas must first be ingested and then translocated into the gut tissues. Subsequently the bacteria can replicate and systemically infect the insect, ultimately moving to the salivary tissues from which they can be expelled with salivary secretions into other host plants (Ammar et al., 2011; Kruse et al., 2017).

As early as the mid-1960s, it was reported that an as-yet-unknown agent associated with HLB was transmissible by psyllids (da Graça, 1991). By the late 1960s, ACP was confirmed as a principal vector of the greening agent. Long before ACP and CLas were identified in the United States, the general characteristics of CLas transmission by ACP were well described, e.g., the duration of acquisition, latent, retention, and inoculation periods (da Graça, 1991). Also well described were the propensity of late instar and adult ACP to acquire CLas on young flush tissue and the attraction of ACP to the yellow-green color of HLB-infected trees. Although early reports suggested that ACP nymphs were not important in the transmission of CLas (da Graça, 1991), more recent studies have shown that early instar nymphs acquire CLas more efficiently than adults and that transmission efficiency is higher in ACP reared on CLas-infected plants than in adults fed on CLas-infected plants (Inoue et al., 2009; Canale et al., 2017), although long feeding times by adults tend to negate these transmission efficiency differences (Pelz-Stelinski et al., 2010).

The multitude of studies on transmission of CLas by ACP done over the past five decades support the conclusion that the general parameters of transmission are similar to expectations for most arthropod-borne pathogens that circulate in their vectors; i.e., longer acquisition and inoculation times facilitate transmission efficiency, and there is an optimal latent period measured in days between acquisition and transmission (Grafton-Cardwell et al., 2013). Higher pathogen titer in the source tissue will enhance acquisition and transmission efficiency, and higher numbers of the bacteria inoculated to a host in a susceptible state will facilitate infection efficiency (Ukuda-Hosokawa et al., 2015). Details of CLas infection of ACP have remained elusive. Longer acquisition times result in higher CLas titer in insects, but maintenance of "infected" ACP on plants that are not hosts of CLas result in conflicting results that bacterial titers can either decrease (Pelz-Stelinski et al., 2010) or increase over time (Ammar et al., 2016). This discrepancy may be due to the age of the psyllids used in the study. Other studies also suggest that CLas replication may occur in nymphs but not in adults (Inoue et al., 2009); this phenomenon may be related to the immune response in ACP, which appears to be suppressed in nymphs (Ramsey et al., 2017). Recent work has shown that several CLas genes are expressed differentially when the bacteria are associated with plants versus when they are associated with ACP. While the bacteria may not replicate efficiently in all ages of psyllids, it is clear that the bacteria survive for long periods of time in the insect and can be vertically transmitted, albeit at a low frequency (Mann et al., 2011).

VECTOR–HOST INTERACTIONS

As its common name suggests, ACP is native to Asia, but both scientific and common names are misleading with respect to its host plant use patterns. Although all known host plant species belong to the subfamily Aurantioideae in the family Rutaceae, ACP infests species in at least 10 genera in the family in addition to *Citrus* (Grafton-Cardwell et al., 2013). Not all genera reported as host plants are equally suitable for oviposition, development, and adult feeding and reproduction, however. Outside the Aurantioideae, white sapote (*Casimiroa edulis*), in the subfamily Toddalioideae, was rejected by all ACP life stages in free-choice field studies (Westbrook et al., 2011). Studies of host plant preference and suitability both within the genus *Citrus* and across the Rutaceae have confirmed a strong link to leaf flush, irrespective of species or genotype (Ruan et al., 2015; Hall and Hentz, 2016). For example, orange jasmine (*Murraya paniculata*) flushes continuously and, relative to commercial citrus, supports more rapid ACP population growth (Tsai and Liu, 2000). Among plants that are distinctly less preferred is *P. trifoliata* (hardy or trifoliate orange), possibly due to reduced rates of oviposition. Of the native Rutaceae species potentially at risk of colonization by ACP in its North American nonindigenous range, *Choisya ternata, C. arizonica*, and *Helietta parvifolia* all support ACP growth and reproduction (Sétamou et al., 2016) and may serve as potential host plants. By contrast, six other native rutaceous species failed to support oviposition, nymphal development, or adult survival.

Multimodal sensory inputs are important in host finding by the ACP. With respect to chemosensory stimuli, the preference for newly flushed leaves is mediated at least in part by responses to volatile signals. As in other psyllids (Park and Hardie, 2002), the olfactory system of ACP is relatively simple; glomeruli are absent from the antennal lobes. Perception of host plant odors is associated primarily with the olfactory neurons innervating the rhinarial plates[12] on adult antennae. More than three dozen volatile odorants from a diversity of chemical classes have been characterized from *Citrus* and *Bergera* species that are suitable hosts for development. Among possible attractive odorants, monoterpenes, esters, and aldehydes elicited strong activation of olfactory neurons (Coutinho-Abreu et al., 2014), as did acetic and formic acid, both of which are breakdown products of terpenoids (George et al., 2016). Olfaction also plays a role in behavior related to host plant rejection. Repellency of guava (*Psidium guajava*), a nonhost, is associated with the production of volatiles, including β-caryophyllene (Zaka et al., 2015; Alquézar et al., 2017). After settling, ACP assesses host plant suitability using gustatory cues. Mixtures of formic acid, acetic acid, and *p*-cymene can stimulate probing and salivary sheath formation (Lapointe et al., 2016).

Visual cues also influence host plant choice by ACP. The day-flying adults are positively phototactic and are attracted to yellow sticky traps (Sétamou et al., 2016). Walking behavior is elicited by wavelengths in the short-wave range of the spectrum and by vertically oriented polarized white light. Responses to UV light may account for dispersal out of the canopy and settling on foliage for feeding when flushing foliage is less abundant (Paris et al., 2017). Responses to visual cues can change in the presence of either olfactory or gustatory chemical stimuli (Patt et al., 2011), although under field conditions in urban environments in California combining olfactory and color stimuli did not enhance capture rates over traps lacking any lures (Godfrey et al., 2013). To some extent, host plant location and assessment by adult ACP are influenced by previous developmental and adult experience. Under certain circumstances, ovipositing females display a preference for the host plant species on which they develop but these preferences can shift after exposure to an alternative host plant 1 or 2 days later (Stockton et al., 2016, 2017b).

[12] The lower part of the clypeus, which is a plate on the anterior median aspect of the head.

Adult ACPs are attracted to yellow and yellowish green colors that mimic the reflectance spectra of their rutaceaous host plants. The recent identification of methyl salicylate (MeSA) as an attractive host plant volatile (Mann et al., 2012a) has been investigated as a means of improving the deployment of sticky traps for monitoring ACP, but this approach has met with only limited utility in the development of attract-and-kill technologies because of the widespread distribution of diseased trees in selected areas (Yan et al., 2014). CLas infection of the citrus tree further induces the release of MeSA, which renders infected plants more attractive than uninfected plants. However, infected trees are less suitable than uninfected plants as hosts for development of ACP, and the tendency of psyllids to leave infected plants shortly after pathogen acquisition may promote the spread of the pathogen.

DIAGNOSTICS

Accurate, sensitive, and cost-effective means of detecting CLas infections in both citrus trees and ACP are of paramount importance to the management of HLB. There is an extensive literature describing various techniques to detect CLas in plant and insect tissues (Valdes et al., 2016). Because CLas has not yet been cultured outside its hosts, there are no methods to enrich the bacteria from selected host tissues to facilitate detection by molecular, serological, or other means. A quantitative polymerase chain reaction (qPCR)-based assay (Li et al., 2006) for amplifying CLas 16S RNA has become the standard assay accepted by many laboratories and, more importantly, by regulatory agencies to provide an initial determination of CLas infection. This is followed by conventional PCR assays and DNA sequencing for final verification. Although many reports have been published in the past decade on other methods to detect CLas in plant and insect tissues (Valdes et al., 2016; Warghane et al., 2017), none of the mechanistically similar technologies (e.g., digital PCR, immunoblots, LAMP, or loop-mediated isothermal amplification, CANARY or Cellular Analysis and Notification of Antigen Risks and Yields) have proven to be more sensitive than qPCR. Furthermore, since qPCR can detect as little as one copy of bacterial DNA the issue for CLas detection is not the sensitivity of bacterial detection but rather the uneven spatial and temporal distribution of the pathogen in trees and insects (Tatineni et al., 2008; Li et al., 2009; Kunta et al., 2014; Louzada et al., 2016).

Further exacerbating the problems with the conventional diagnostics mentioned above is that there is a long latent period between inoculation of the plant and multiplication of the bacteria to detectable levels (Manjunath et al., 2008; Gottwald, 2010; Chiyaka et al., 2012). There are perhaps some parallels with movement and distribution of phloem-limited viruses, but it is unclear if any studies have addressed this question. Several studies have shown that conventional detection of CLas in trees lags behind the time of inoculation by several weeks to months depending on the size and type of tree (Bové, 2006; Gottwald, 2010). More importantly, the tree is a source of bacteria for ACP vectors long before CLas can be detected.

HLB MANAGEMENT

The two basic management strategies for any vector-borne pathogen transmitted in a circulative manner are (1) to reduce the number of vectors available to transmit the pathogen and (2) to reduce the amount of inoculum available to the vectors. While considerable effort has gone into controlling ACP it seems that less effort has been devoted to the removal of inoculum sources, i.e., infected trees, even though research indicates that removal of infected trees can slow epidemics. The effectiveness at slowing and managing the incidence of HLB as well as that of other diseases having similar etiologies, using these two basic strategies, is well documented and supported (Belasque et al., 2010; Bassanezi et al., 2013a,b; Ayres et al., 2015; Bergamin Filho et al., 2016). This strategy, however, works only if the disease epidemics are in the early stages, i.e., when the level of inoculum is relatively low and identification and removal of infected plants does not compromise the ability of the producer to realize an acceptable level of economic return. This strategy may still be important in California, and possibly in Texas and Arizona, where the reported level of HLB in commercial citrus orchards is still low. However, optimization of the strategy will depend on the validation and acceptance of early detection technologies so that infected trees can be identified and removed prior to becoming sources of inoculum for ACP. Infected tree removal and vector control were the primary eradication strategies early on in Florida, prior to the realization that significant numbers of trees were infected and serving as sources of inoculum while still asymptomatic. Currently, the incidence of HLB in Florida citrus has reached or is approaching 100% and removal of inoculum on individual groves is no longer an option.

Vector control remains an important component of HLB management in Florida, although any type of effective HLB management will depend on area-wide implementation as well as cooperative and sustained participation by all citrus production operations. Citrus Health Management Areas (CHMAs) were established in Florida with the goal of regional participation in insecticide applications for ACP control. These have been only somewhat effective due to incomplete grower participation in some areas (Singerman et al., 2017). A CHMA-like approach has been effective in Brazil, but a component of their effectiveness has been continuous removal of infected trees and complete eradication of abandoned groves and alternative hosts (Belasque et al., 2010). Timely and effective disposition of abandoned citrus groves and infected backyard trees in urban areas near commercial citrus production areas has not been a practice in Florida, and these pathogen sources continue to add to the disease pressure on all Florida citrus (Tiwari et al., 2010). Models predict that reducing ACP populations during critical times can reduce spread (Lee et al., 2015), but even with 100% participation in CHMAs, ACP population management in managed groves alone would be unlikely to significantly slow HLB progression due to the refugia for ACP and CLas provided by abandoned groves and backyard trees. ACP repellents can also slow and prevent transmission of CLas by immigrating insects. One promising strategy for protecting young transplants is the use of reflective mulch (Croxton and Stansly, 2014), which has been used in the management of several insect-borne plant viruses in other crop systems (Budnik et al., 1996; Abou-Jawdah et al., 2000; Antignus, 2000). An additional benefit of using reflective mulch as ground cover was increased citrus tree growth rate (Croxton and Stansly, 2014).

Other cultural control efforts have focused on the use of thermotherapy to reduce or eliminate CLas infection in trees. While thermotherapy has been successful at reducing CLas titer in small trees growing in the greenhouse (Fan et al., 2016), results with mature trees in commercial grove settings have been mixed (Yang et al., 2016; Doud et al., 2017).

Historically, Florida citrus groves have not been intensively managed with respect to nutrition and water, such that trees are continually exposed to some level of stress. Attacks of insects and pathogens, either direct or opportunistic, contribute to additional stress that affects fruit yields and quality (Ashraf et al., 2014). A growing trend in Florida is the manipulation of nutrition and irrigation regimes to reduce the effects of HLB on tree health, fruit production, and fruit quality. Although there is little peer-reviewed literature to support these actions by many growers (Kadyampakeni et al., 2015; Morgan et al., 2016; Hamido et al., 2017; Plotto et al., 2017), the general sense is that trees under any type of stress are less able to resist CLas infection and ACP infestation. If stress can be reduced by ensuring optimal irrigation and nutrition, the trees may show less severe disease symptoms, including milder effects on fruit production and yield. While some growers have reported milder disease symptoms, increased fruit yields, and improved fruit quality with fertigation, controlled research findings have been mixed (Gottwald et al., 2012; Stansly et al., 2014; Kadyampakeni et al., 2015; Morgan et al., 2016; Hamido et al., 2017; Plotto et al., 2017; Rouse et al., 2017; Tansey et al., 2017). The fact that effects of optimal nutrition and water are apparent only after 2 to 3 years of ideal management may explain why some of the early, shorter studies found no benefits (Plotto et al., 2017).

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3

HLB Research and Development Efforts

In 2009, at the request of Florida Department of Citrus, the National Academies appointed the National Research Council (NRC) Committee on Strategic Planning for the Florida Citrus Industry: Addressing the Citrus Greening Disease (huanglongbing). This committee released its report, *Strategic Planning for the Florida Citrus Industry: Addressing Citrus Greening Disease,* in early 2010. Included in this report were organizational, informational, and research and technology recommendations. Among the organizational recommendations was to ***identify one organization and empower it to have oversight responsibility over huanglongbing (HLB) research and development efforts (Recommendation O-2)***. The Citrus Research and Development Foundation (CRDF), a nonprofit corporation that was created through the initiative of the Florida citrus industry in 2009, currently serves as the organization in Florida with oversight responsibility for HLB research and commercial product development and delivery.

This chapter examines the HLB research areas that have received or continue to receive support from CRDF[1] and discusses whether these research efforts are in line with the recommendations in the NRC 2010 report (notable outcomes from HLB research and pitfalls are discussed in Chapter 4 of this report). This chapter also examines the HLB research efforts that are being funded by other agencies[2] (state and federal) and overlaps or

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[1] Information on CRDF-funded projects (project number, principal investigator, funding duration, funding amount, and project objectives), can be found in Appendix D of this report.

[2] Information on projects funded by other agencies is available online at http://citrusrdf.org/wp-content/uploads/2012/10/US-Citrus-Research-Inventory.pdf. Accessed June 8, 2018.

duplications with CRDF-funded research efforts. The chapter ends with a section addressing sociological and economic factors that influence grower decision making with respect to citrus cultivation and disease management, as well as market and consumer acceptance of fruit and juice produced in various ways. These factors were not mentioned specifically in the 2010 report, but the committee deems them to be critical to the success of any future HLB management efforts and, indeed, to the health of the citrus industry in Florida.

HLB PATHOGEN, VECTOR, AND HOSTS

HLB Causal or Associated Bacteria

CRDF-Funded Research

CRDF has funded 26 projects, totaling $8.6 million, with at least one major objective related to understanding the biology of *Candidatus* Liberibacter asiaticus (CLas). CLas-focused projects address several major themes, including efforts to grow the bacterium in culture, characterize its associated bacteriophages and other biocontrol microbiota, understand its genetic diversity and distribution, and identify essential proteins as control targets. Several projects address more than one of these themes. The largest number support efforts to isolate and culture CLas *in vitro* (Projects #048, 162, 306, 407, 307, 336, 418, 769, and 15-027, totaling about $2.9 million). Investigators of five past (#535, 726L, 711, 803, and 15-009) and one current (#15-042) CRDF project (totaling about $2.5 million) have studied the properties of bacteriophages or bacteria that coexist with CLas in order to identify ways that these may be used for bacterial control. Relevant projects include studies of bacteriophage viruses encoded in the CLas genome and of antagonistic microbes found in citrus phloem. Two past (#045 and 15-008) and one current project (#16-001), totaling $979,000, have aimed to understand the patterns of multiplication and movement of CLas within the plant host, or to develop improved tools for pathogen tracking in the plant. Four completed projects (#125, 065, 123, and 162), totaling $1.4 million, focused on the genetic or genomic characterization of CLas to understand its diversity and distribution, identify virulence factors or targets, and generate public resources for genome sharing and analysis. Five projects (#424, 15-028, 805, 15-017, and 711), totaling $1.7 million, have aimed to use bacterial proteins to develop or optimize CLas inhibitory strategies.

Research Toward Culturing CLas. In addition to evaluating a wide array of culture media formulations, researchers have worked to mimic aspects of the insect and plant environment through the addition of insect cells to the culture or the use of phloem-mimicking flow chambers, and through profiling the molecular components of host, vector, and pathogen to identify potential metabolic needs of CLas. Researchers have also optimized methods for obtaining CLas starter culture from plant or insect tissues having the largest titers of bacteria. These efforts are in line with the NRC 2010 recommendation ***NI-10: Develop* in vitro *culture techniques for CLas to facilitate experimental manipulation of the bacterium for insights into gene function.***

One project funded by CRDF focused on culturing the nonpathogenic, plant-associated bacterium *Liberibacter crescens* to provide the closest-available proxy system to CLas for use in experimentation (Fagen et al., 2014). This effort is in line with the 2010 recommendation ***NI-5: Support development of HLB model systems.***

Genome Sequencing and Bioinformatics Analysis. The completion of the CLas genome (Duan et al., 2009) in 2009 (with funding from the Florida Citrus Production Research Advisory Council, the CRDF's precursor agency) was later followed by the completion of the genome of other HLB-causing species *Candidatus* L. americanum (CLam) and *Ca.* L. africanum (CLaf) (Wulff et al., 2014), and other Liberibacter species that cause diseases in other plant species. Genomic comparison among these and with genomes of related free-living species, including a culturable nonpathogenic Liberibacter strain, have been performed toward identification of essential pathogenesis genes (Hartung et al., 2011; Kuykendall et al., 2012; Leonard et al., 2012). These efforts are in line with the 2010 recommendations ***NI-5: Support development of HLB model systems*** and ***NI-6: Exploit the CLas genome sequence for new strategies of HLB mitigation.***

Functional Comparative Genomics and the Identification of Infection-Associated Genes and Proteins. CRDF funded projects (#123, 733, and 314) aimed to curate the genomic sequences of HLB pathogen, vector, and host; perform comparative bioinformatics analysis of proteins among the genomes of each species; predict interactions among proteins from different genomes; and provide a user-friendly website to the community[3] (Cong et al., 2012). Another CRDF project (#123) focuses on bioinformatics efforts in Liberibacter genome analysis and extending to bioinformatics characterization of vector and hosts.[4] For many of the virulence-associated proteins identified through genomics, translational work has begun to identify targeting strategies. Small-molecule screens aim to find inhibitors of specific virulence factors, such as the master regulator LdtR and the CLas salicylic acid–degrading enzyme (Pagliai et al., 2014). Other research projects (#805, 314, and 424) have sought to identify and characterize other secreted CLas

[3] See http://prodata.swmed.edu/citrusgreening. Accessed June 8, 2018.
[4] See https://www.citrusgreening.org. Accessed June 8, 2018.

proteins in order to find or engineer proteins that may bind or degrade them. These efforts are in line with the 2010 NRC recommendation ***NI-6: Exploit the CLas genome sequence for new strategies of HLB mitigation.***

Understanding Antagonistic Phage and Bacteria. More than 6% of the sequenced CLas genome is composed of two bacteriophages (antibacterial viruses) that may exist as lytic (bacteria-killing state) or lysogenic phages (repressed state) (Zhang et al., 2011). Phage-associated sequences are highly variable among CLas isolates (Zhang et al., 2011; Wang et al., 2012); research has been aimed at using this variability to understand the diversity, evolution, and geographic origin of CLas populations (Zhou et al., 2011; Zheng et al., 2017). Phage could also play a critical functional role in limiting CLas growth in different host contexts. These efforts are in line with the 2010 NRC recommendation ***NI-6: Exploit the CLas genome sequence for new strategies of HLB mitigation***.

Impacts of the Citrus Microbiome. CLas is among thousands of bacterial species present in citrus phloem and in psyllid guts (Fagen et al., 2012). Bacterial communities could affect HLB development and transmission through nutrient competition or cooperation, chemical antibiosis or growth promotion, triggering plant defense, or activating regulatory signals in CLas; alternatively, CLas effects on other bacteria might increase stress on the plant. CRDF-funded projects (#916 and 15042) have sought to characterize or manipulate the citrus microbiome to understand the role of cooperative or antagonistic microbes in HLB. CRDF-funded researchers helped form a Citrus Microbiome Consortium in 2015, aimed at coordinating microbial community exploration in citrus tissues, including the effects of variety, diseases, and management on the microbiome (Wang et al., 2015). Other research has focused on the role of individual microbial species for disease-controlling properties. Microbial species that have been tested include 327 citrus-inhabiting bacteria (Riera et al., 2017) and *Burkholderia* spp. (Zhang et al., 2017). A current project is focused on optimizing the effectiveness of a form of *Bacillus thuringiensis* (Bt) toxin against the Asian citrus psyllid (ACP) (#711) (see also the sections Pathogen–Vector Interactions, page 82, and Insect Control, page 90). A phage-suppressing protein derived from the endosymbiont *Wolbachia* is also being investigated to determine its role in CLas behavior (Jain et al., 2017). These efforts are in line with the 2010 NRC recommendation ***NI-7: Support research aimed at developing alternative ACP management strategies.***

Research Funded by Other Agencies

In 2016, the National Institute of Food and Agriculture (NIFA) Specialty Crop Research Initiative (SCRI) awarded funding to three different projects related to the biology of CLas. Two projects (#2016-70016-24824 and 2016-70016-248441) with $2.1 million and $4 million funding, respectively, aim to identify possible new culturing strategies that will increase the span of viability through comprehensive analyses of phloem sap and insect gut composition, the effect of the physical environment, the role of CLas chemical signals, and several other factors. The third NIFA project (#2016-70016-24781), a $3.3 million effort, aims to identify CLas-secreted proteins as candidates for targeting by plant-expressed proteases.

The NIFA Plant-Biotic Interactions program awarded $863,000 to Project #2017-03060,[5] which aims to characterize the role of the master regulator LdtR in the persistence of CLas in citrus. A current project (#5300-160) funded by the California Citrus Research Board (CRB) aims to identify the core secreted proteins of CLas in order to develop bacterial inhibition and diagnostics strategies. Finally, K. Mandadi at Texas A&M was awarded the Foundation for Food and Agriculture New Innovator Award in 2017, receiving $600,000 from the Foundation for Food and Agriculture Research and Southern Gardens Citrus to develop a root-based bioassay for culturing studies and antimicrobial screening.[6]

HLB Vector: Asian Citrus Psyllid

CRDF-Funded Research

CRDF has previously funded 15 projects, totaling $5.9 million, that have at least one major objective related to understanding the biology and ecology of ACP. These are in line with the 2010 recommendation ***L-3: Support analysis of ACP behavior, ACP–plant interactions, and ecology to enhance the knowledge base available for new ACP management strategies.***

Dispersal Dynamics of ACP. One previously funded project (#214) evaluated the dispersal dynamics of ACP to determine the distances traveled by adults, the seasonal phenology of these movements, and cues for movement of psyllids into and out of citrus groves. In this study, a protein-marking technique was employed to investigate dispersal dynamics using marked individuals from abandoned citrus groves to adjacent managed citrus (Lewis-Rosenblum et al., 2015). Additional studies, based on the specific color-morphs of *Diaphorina citri*, were carried out to determine patterns of flight initiation and duration using flight mills (Martini et al., 2014a). Paris et al. (2017) similarly investigated how calling behavior, mate seeking, and phototaxis were affected by changes in atmospheric pres-

[5] Project information available at https://www.nsf.gov/bio/pubs/awards/IOS_awards_list.htm. Accessed January 8, 2018.

[6] Announcement of award available at https://foundationfar.org/new-innovator/kranthi-mandadi/. Accessed January 8, 2018.

sure. Another project (#15-024) aimed to improve the understanding of ACP flight and dispersal behavior, and more specifically how temperature, humidity, barometric pressure, and wind speed affect the probability that ACP initiate and perform long-distance flights.

Infection Status and Psyllid Dispersal. One past study investigated the influence of CLas infection status and how it influences ACP dispersal behavior, flight capacity, and sexual attraction (Martini et al., 2015a).

Effects of Host Variety and Nutrition on Vector Fitness or Behavior. The influence of citrus variety and nutrition on ACP biology, fitness, and behavior was also investigated (Tsagkarakis and Rogers, 2012; Project #176). Moreover, the effect of different plant growth regulators on citrus tree vegetative growth and subsequent impact on ACP fitness were also evaluated in greenhouse and growth chamber experiments.

Increasing the Efficiency of ACP Monitoring/Trapping. To learn more about the visual cues that influence ACP attraction and repellency, light-emitting diodes, emitting light of wavelengths within the insect visible spectrum (Paris et al., 2015), were designed to study the responses of ACP to variations in light and light quality. Mankin et al. (2013; Project #567) investigated the possibility of increasing trapping efficiencies by attracting male ACP using vibrational communications produced by females. A micro-controller platform was evaluated for its capacity to regulate vibrational sensing and output devices and has subsequently been programmed to send mimics of different *D. citri* signals to a buzzer or calling device.

Disrupting ACP Feeding Mechanisms. Specific compounds that inhibit the formation of salivary sheaths and/or degrade hemipteran style sheaths (Project #330; Appl. No. US20160316762 A1[7]) were investigated for direct topical delivery to plants by spraying or root applications. These compounds and delivery methods were designed to prevent and/or reduce the transmission of vascular tissue–associated hemipteran vector-borne pathogens to plants. Specific investigations focused on the characterization of the ACP feeding process and testing of compounds that block or inhibit psyllid feeding as a mechanism for disease control.

Research Funded by Other Agencies

U.S. Department of Agriculture (USDA) NIFA funding supported research on ACP behavioral responses to the presence of CLas in trees. CLas infection leads to changes in the host's odor, which influences attraction of noninoculative vectors and repellency of inoculative individuals. Shatters et al.[8] (2015–2017) compared the proteome and transcriptome of

[7] See https://www.google.com/patents/WO2016176061A1. Accessed December 20, 2017.
[8] See https://www.ars.usda.gov/research/project/?accnNo=427147. Accessed January 9, 2018.

ACP alimentary canal and midgut regions, as well as salivary glands with and without the CLas infection. Another USDA NIFA project (Handler et al.[9]; 2015–2017) focused on the development of efficient transformation protocols to create vector-incompetent, transgenic ACP to replace vector-competent strains in the field.

Hunter et al.[10] (2015–2017) conducted deep sequencing runs to assist in the completion of the ACP genome, releasing this assembly on two websites for free access by the research community. The transcriptome dataset was used to promote the Innocentive Challenge, whose aim is to produce and screen genetic sequences for RNA-interference (RNAi) products that work against psyllids. These efforts are in line with the 2010 recommendation ***NI-11: Sequence, assemble, and annotate the ACP genome to provide a basis for new approaches to ACP management.***

Transcriptome assemblies and other sequence data are available at websites of the International Asian Citrus Psyllid Genome Consortium[11] and the National Center for Biotechnology Information.[12] Bioinformatic and annotation resources for genomes of ACP and other HLB components have been assembled in a NIFA-funded comprehensive, publicly available resource, CitrusGreening Solutions.[13] Investigations of insect gustation (act or sensation of tasting) have examined the function of ACP sugar receptors and identified compounds that enhance or inhibit their activity, testing whether the presence of these compounds alters feeding behavior. USDA Animal and Plant Health Inspection Service (APHIS) Multi-Agency Coordination (MAC) Group funding (2016–2017) was focused primarily on pathogen detection in ACP in discrete, hot-spot locations, along with the development of attract-and-kill procedures based upon vector host location cues.

HLB Hosts: Citrus

CRDF-Funded Research

During the early rounds of funding CRDF provided approximately $11.5 million in support of research and development (R&D) in citrus transformation (genetic engineering) and has continued to support efforts in gene discovery and genetic mapping[14] (for example, Projects #071 and 356), in line with the 2010 recommendation ***L-1: Support the development of transgenic HLB-resistant and ACP-resistant citrus*** and ***NI-4: Accelerate***

[9] See https://www.ars.usda.gov/research/project/?accnNo=430081. Accessed January 9, 2018.
[10] See https://www.ars.usda.gov/research/project/?accnNo=429787. Accessed January 9, 2018.
[11] See http://psyllid.org/download. Accessed December 20, 2017.
[12] See http://www.ncbi.nlm.nih.gov/bioproject/29447. Accessed December 20, 2017.
[13] See www.citrusgreening.org. Accessed January 24, 2018.
[14] Identifying the respective loci of genes on a genome, and the distances between them.

the sequencing, assembly, annotation, and exploitation of a sweet orange genome to provide a powerful tool for all future citrus improvement research.

Citrus Transformation. While varying levels of tolerance to HLB have been noted in a variety of rootstock and fruiting cultivars, there appear to be no sources of high-level resistance that are viable in the sense that commercially acceptable fruiting citrus cultivars could be produced in time to sustain the Florida citrus industry. Currently, the most promising approach is the development of high-level resistance through genetic engineering using genes from citrus-related (sexually compatible) or unrelated species or organisms (NRC, 2010). Transgenic rootstocks, sweet oranges (*Citrus sinensis* (L.) Osb.), grapefruits (*C. paradisi* Macf.), mandarins (*C. reticulata* Blanco), limes (*Citrus* × *aurantiifolia*), and lemons (*C. limon* (L.) Osb.) have been produced using juvenile tissues with funding from CRDF (Dutt and Grosser, 2010; Orbović and Grosser, 2015). CRDF also funded efforts to transform mature tissues (Marutani-Hert et al., 2012; Orbović et al., 2015; Wu et al., 2015).

Transgene promoters have been characterized and used to target gene expression to particular tissues, most importantly the phloem, where CLas is located (Dutt et al., 2012; Benyon et al., 2013; Belknap et al., 2015), and methods are being developed for the production of transgenic plants without the use of antibiotic-resistance-selectable markers, utilizing a visual anthocyanin marker (Stover et al., 2013a; Dutt et al., 2016). Such a marker could be used alone or it could be used to select plants already containing another marker in order to retransform, or stack, transformation events.

Transgenic Plants. Several projects, together aimed at producing hundreds of independent lines of transgenic plants via genetic transformation, have received funding from CRDF. These projects focused on creating transgenic citrus plants that express a variety of antimicrobial proteins (AMPs) (Stover et al., 2013b; Dutt et al., 2015b), disease resistance genes or R genes isolated from citrus and other plant species (Lu et al., 2013; Dutt et al., 2015a,b), antibodies against CLas proteins (Project #606), genes to improve regeneration and transformation (Hu et al., 2016), and promoters targeting specific tissues (Dutt et al., 2012; Benyon et al., 2013; Belknap et al., 2015). RNA interference (RNAi) strategies have also been explored (Hajeri et al., 2014), in line with the 2010 recommendation ***NI-9: Support demonstration of RNA interference (RNAi) effects for possible suppression of ACP.*** Efforts to edit clustered regularly interspersed short palindromic repeats (or CRISPR) have also received CRDF funding (Jia et al., 2016, 2017; Project #922). There is potential for rootstock transmission of resistance to the scion, which would allow the scion itself to be nontransgenic.

Resources Required for Genetic Engineering. Genetic engineering involves gene identification, gene isolation, promoter selection, vector construction, and vector quality testing, sometimes in model plants such as *Arabidopsis* or tobacco. The test of the effectiveness of a gene or promoter relies on the production and testing of transgenic plants. Transformation is tedious and time consuming as all work must be done using sterile techniques, explants must be transferred onto fresh media multiple times, all putative transformants must be checked for the presence of transgenes and individual transgenic clones multiplied for inoculation tests, and shoots must be rooted and hardened off for moving from the culture room to the greenhouse for further testing. Yields of transformants are generally quite low. Not all laboratories have the trained personnel or facilities to undertake transformation but may have potentially useful genes to evaluate *in planta*. The Core Citrus Transformation Facility at the Citrus Research and Education Center in Lake Alfred, Florida, which receives funding from CRDF (Projects #579 and 15-033C), serves this purpose. Some laboratories that receive CRDF funding have their own transformation facilities, while others rely on an outside facility such as the one supported by CRDF.

In addition to the difficulty of transforming most citrus genotypes, rapid and reliable screening of transgenic plants is a significant obstacle to the development of HLB-resistant genetically engineered citrus. Projects #15-016C and 502, which aim to improve and speed the inoculation and HLB resistance screening process, have been supported by CRDF. These projects employed a high-throughput screening using ACP, which better simulates the natural route of infection than graft inoculation and thus would better identify trees having field resistance. CRDF has supported field testing at the Picos Farm in Fort Pierce, Florida (Project #15-039C). Such a site is critical for the long-term testing of HLB resistance, tree performance, and fruit quality and for developing additional data necessary for regulatory agencies on genotypes that will be submitted for approval (see also the section Vector–Host Interactions, page 76).

Genetic Engineering Traits. CRDF funding has enabled researchers to begin to investigate a large number of potentially useful disease resistance genes from citrus and other species. Promoters have been utilized to target transgene expression to phloem.

HLB Resistance and Immunity. A project aimed at testing transgenic trees for resistance or tolerance (Project #15-020) and those focused on HLB tolerance/resistance candidate gene discovery through differential expression of genes in infected susceptible and tolerant genotypes (for example, Project #724) have been supported by CRDF.

Citrus Tristeza Virus Gene Delivery. While the development of new highly HLB-resistant citrus cultivars through conventional breeding or genetic engineering would revitalize and stabilize the citrus industry, both approaches will take time, which a suffering industry can ill afford. CRDF is supporting (about $1.25 million) an innovative approach to provide a more rapid, if only temporary, route to resistance that could be applied quickly and provide the industry with HLB-resistant material at least until long-term solutions are available. This technology involves using a mild strain *Citrus tristeza virus* (CTV) vector to deliver and express resistance transgenes in existing citrus cultivars, even in mature trees that are already established in groves (El-Mohtar and Dawson, 2014). The CTV vector is phloem limited and therefore expresses the transgenes (AMPs) where CLas is located. CTV persists for at least 4 to 6 years in citrus trees (Folimonov et al., 2007; NRC, 2010). This technology, which is applied simply through grafting, may not only provide protection from infection but could cure infected trees or reduce symptoms. One previously funded project aimed to assess AMPs for activity against CLas and to develop transgenic trees with effective AMPs (Project #046). Delivery of RNAi by CTV to target ACP is another approach that was investigated (Hajeri et al., 2014). Maximizing expression of transgenes in the CTV vector is an issue and was also a focus of investigation (Project #516).

Research Funded by Other Agencies

A multifaceted project ($3.3 million; funded from 2015 to 2020) that aims to identify candidate HLB resistance genes in resistant or tolerant citrus relatives and utilize these for gene editing producing non-genetically modified HLB-resistant citrus cultivars is funded by NIFA (#2015-70016-23027). CRB also funded efforts to produce non-genetically engineered (non-genetically modified organism but engineered to contain resistance genes only from other edible plants) "consumer-friendly" HLB-resistant citrus (Project #5200-144).

Overlaps and Duplications

While there are no obvious overlaps or duplications of efforts and a number of CRDF-funded projects are multi-institutional, there appears to be a need for improved communication and coordination of citrus genetic improvement projects.

VECTOR–HOST INTERACTIONS

CRDF-Funded Research

By the end of 2017 CRDF had funded more than a dozen projects, 13 completed and 1 still active (#15-039C), that contain a substantial component addressing vector–host associations, generally in the context of developing new approaches or enhancing existing strategies for ACP management. These efforts are in line with the 2010 recommendation ***L-3: Support analysis of ACP behavior, ACP–plant interactions, and ecology to enhance the knowledge base available for new ACP management strategies***. In total, these projects represent $2.6 million of support from CRDF.

Host Range of ACP. Identifying the host range of ACP is vital to managing HLB. Finding resistant plant species or varieties can facilitate the characterization of potential genetic resistance mechanisms or phytochemicals that can function as repellents or deterrents. Moreover, in view of the presence of other native and introduced rutaceous species in Florida, characterizing the host range can pinpoint unmanaged host reservoirs where populations can build up and, via immigration, thwart efforts to control ACP in citrus groves.

Major efforts to find resistance to HLB have been focused on *Poncirus trifoliata* (hardy or trifoliate orange). *P. trifoliata* is closely related to *Citrus* and in fact is sometimes placed within the genus *Citrus*; it is genetically compatible with sweet orange and can hybridize to produce "citranges." CRDF projects have been aimed at characterizing the extent of trifoliate orange resistance and its underlying basis. In terms of current projects, Project #15-039C has as its objectives to establish the Picos Farm of the U.S. Horticultural Research Laboratory in Fort Pierce, Florida, as a secure experimental site for field testing citrus germplasm for resistance to HLB and ACP, and to make it available to the greater research community. Part of this project included testing of more than 1,000 *Poncirus* hybrid trees for resistance to facilitate gene mapping in a collaborative project with the USDA Agricultural Research Service (ARS), University of California, Riverside, and University of Florida.

With respect to completed projects, Project #315 set out to screen citrus germplasm for resistance to ACP with the ultimate goal of exploiting plant resistance as a management tactic. Plant trait targets for screening included kairomones,[15] color, phenology, and morphology. Although *Citrus* species are generally all capable of supporting ACP growth and development, host species can differ in their impact on ACP morphology and related life history traits; investigators quantified such differences. In a publication resulting from this project, Hall and Hentz (2016) compared flushing characteristics of nine rutaceous species and/or genotypes vulnerable to colonization by ACP: *Afraegle paniculata, Bergera koenigi, Citrus aurantiifolia*, *C. macrophylla, C. maxima*, *C. medica*, *C. reticulata*, *C. taiwanica*, and *Murraya paniculata*. Project #581/581-1 was focused on identifying overwintering habitats and alternative host plants of ACP and to assess the suitability of alternative hosts for oviposition and develop-

[15] Chemical substances emitted by an insect or plant that have an adaptive benefit, such as a stimulus for oviposition, to another species.

ment. This project yielded at least one publication, reporting on trapping of ACP in habitats without known rutaceous host plants (Martini et al., 2013). Project #538 was aimed at determining whether previously reported resistance of Cleopatra mandarin (*C. reshni*) seedlings to ACP (Tsagkarakis and Rogers, 2010) is genetically based and thus potentially useful in breeding for resistance in hybrid offspring of Cleopatra.

Semiochemical and Other Host-Associated Cues for Host Orientation, Assessment, and Acceptance. Both previous and ongoing studies have examined a multitude of sensory modalities used by ACP to find host plants; collectively, these studies indicate that multimodal cues interact and vary with context, including age, sex, and previous experience of the psyllid. Olfactory cues deriving from the host plant itself play a major role in ACP host orientation; as well, host orientation is influenced by chemical cues used to facilitate mate finding. With respect to phytochemicals, in most herbivore–plant interactions, volatile cues are primarily responsible for host attraction, whereas gustatory cues are important for host acceptance. CRDF-funded investigators set out to identify semiochemicals involved in psyllid host finding, host assessment, and host acceptance, with the ultimate goal of developing and optimizing multicomponent blends for use in monitoring and control.

One past project (#853) was designed to take advantage of ACP resistance exhibited by *P. trifoliata* in order to characterize phytochemicals that influence ACP behavior and physiology. The project's three major objectives were to identify host plant-produced volatile chemicals and leaf/plant metabolites that are attractive or repellent to adult ACP; to test preference and performance of ACP adults on susceptible and resistant host plants; and to evaluate ACP behavior in response to odorant and tastant blends.

For many herbivorous insects, particularly hemipterans, visual cues play a significant role in host orientation and attraction. One past project (#701/701-1), which was part of an effort to develop a push-pull system to increase the efficacy of ACP control through enhanced attraction, had among its objectives to characterize attractants including visual cues for inducing take-off propensity, flight duration, and color preferences. In this project, investigators compared the size and shape of ACP reared on four species of *Citrus* (*C. aurantiifolia*, *C. macrophylla*, *C. maxima*, and *C. taiwanica*) and two species in other rutaceous genera (*B. koenigii*, *M. paniculata*) to determine whether host plant identity influences dispersal potential through changes in wing aspect ratio (Paris et al., 2016).

Although the plant is an important source of semiochemical signals, other signals that influence vector behavior arise from the insect itself. This dimension of vector–host interactions was the focus of two successively funded projects: #15-024 and 766. The first of these (#15-024), which is ongoing, has the overarching goal of evaluating effects of abiotic factors on dispersal and flight capability of ACP. Specific objectives are to determine temperature and humidity effects on flight initiation thresholds, effects of wind speed on psyllid flight and directionality, and effects of barometric pressure changes on dispersal. The researchers also plan to develop a model to predict invasion risk based on abiotic factors. This project was a continuation of earlier funding to examine biotic factors affecting dispersal and flight capacity. Among biotic factors examined were contributions of female ACP-produced odors relative to mating state, the chemical composition of female-produced chemical cues, and impacts of male reproductive experience on responses. The second project (#766) focused on vector-influencing signals that arise from the insect itself and had the general objective of ascertaining the mechanistic basis for ACP acceptance of citrus hosts. Initial objectives of this project included characterizing differences in abiotic and biotic factors in resets, mature trees and trees having unusually high populations of CLas-infected ACP, or CLas-infected trees having high populations of ACP that are likely to be sources of inoculum. The goals were to define the biotic factors influencing ACP acceptance and performance, to measure ACP population size differences in resets and mature trees, to establish the impacts of time since HLB inoculation and proportion of infected trees on ACP host acceptance within groves, and to assess differences in ACP dispersal from resets and mature trees in the context of HLB transmission (Stelinski, 2016).

With respect to insect-derived attractants, there were efforts to examine the chemical composition of and behavioral responses to volatiles produced by female adult ACP in the presence and absence of volatiles of intact or ACP-damaged foliage (Mann et al., 2012). The behavioral responses of male and female *D. citri* to cuticular extracts in the laboratory and in the field and sex differences in the chemical composition of their cuticular extracts as well as effects of volatiles from insects themselves and from intact or insect-damaged plants on attractiveness were also been investigated (Mann et al., 2013; Martini et al., 2014b). There was also a study on the impacts of developmental and adult experience on learning to recognize olfactory and visual host plant stimuli (Stockton et al., 2016).

Microclimate Effects on Host–Vector Interactions. Inasmuch as the host plant is, for most insect herbivores, also its habitat for much of its life cycle, abiotic factors have a profound influence on population development. Abiotic environmental factors affect dispersal initiation and distances, while host plant dispersion patterns are important in determining dispersal success rates. CRDF funded projects aimed at characterizing psyllid responses to abiotic environmental factors with the ultimate goal of acquiring data useful in optimizing trapping and predicting risks of psyllid attack. A completed project examining abiotic factors was Project #214, which aimed to evaluate seasonality and frequency of psyllid dispersal. Among the host plant attributes evaluated was flush availability; abiotic factors evaluated included movement between managed and abandoned groves, geographic barriers, wind direction, and movement in the absence of severe weather. Another completed project was #600; objectives relevant to determining impacts of the abiotic environment were to assess effects of light quality, photoperiod, air flow, and temperature on psyllid movement; to evaluate physiological limits and life history traits including feeding behavior, infection status, fecundity, and population density; and to characterize seasonal patterns of ACP distribution and movement at different scales in the field.

As mentioned earlier, a component of Project #15-024 was to evaluate effects of abiotic factors on dispersal and flight capability of the ACP. Among the publications produced from this project, Martini et al. (2015b) documented the effects of windbreaks on dispersion patterns of mature and reset-replacement trees on ACP populations. Also mentioned earlier, Project #581 had objectives of identifying overwintering sites and alternative hosts, and describing effects of latitude and row orientation on psyllid density during winter (Pelz-Stelinski et al., 2017).

Influence of CLas on Insect–Plant Interactions. In many systems in which bacterial phytopathogens depend on a vector insect for transmission, pathogen-induced changes in the plant host affect the attractiveness and suitability of the host for the insect (Rid et al., 2016). This phenomenon was investigated in completed CRDF-funded projects, particularly with respect to the volatiles released by the host plant. Three completed projects examined the tritrophic interaction between CLas, ACP, and citrus, as mediated by host plant chemistry. One goal of Project #093 was to examine impacts of pathogen–vector interactions that influence vector specificity and vector competence. Project #334 was aimed at determining whether healthy ACP distinguish between healthy versus HLB-infected citrus plants, whether ACP infected with CLas behave differently relative to healthy and infected plants with respect to settling behavior in the laboratory and the field, and whether chemically mediated interactions vary in the presence of bacterial infection. Finally, Project #335 had as its goal to determine if methyl salicylate is repellent to ACP; researchers examined how CLas infection alters the volatile chemistry of citrus (including methyl salicylate) and the attractiveness to and settling behavior by ACP, and whether pathogen-induced volatiles attract parasitoids of ACP (Mann et al., 2012; Martini et al., 2014b).

Research Funded by Other Agencies

Of 16 NIFA SCRI grants supporting HLB research, evidently no funding was directed specifically toward elucidating the interaction between ACP and its host plants. CRDF partnered with USDA NIFA Coordinated

Agricultural Project Award No. 2012-51181-20086 and the Florida Citrus Advanced Technology Program, Contracts 21 and 510, on a project to construct complementary DNA (cDNA) libraries from CLas-infected and CLas-free ACP adults and nymphs to characterize gene expression profile with the goal to identify psyllid bacterial effectors that interact with factors required for circulative, propagative transmission of CLas (Vyas et al., 2015).

The National Institutes of Health (NIH) National Center for Research Resources (5P20RR016469) and the National Institute for General Medical Science (8P20GM103427), USDA ARS U.S. Horticultural Research Laboratory, Subtropical Insects Research Unit, Fort Pierce, Florida, and the CRB together supported a project to characterize the transcriptomes of egg, nymph, and adult ACP (Reese et al., 2014), although the focus was on identifying insecticide-resistance genes (some of which may actually contribute to phytochemical detoxification).

USDA NIFA grant 2015-70016-23028 supported annotation to complement ACP genome sequencing supported by NIH National Center for Research Resources (5P20RR016469), USDA ARS, and CRB (Saha et al., 2017). This sequencing effort resulted in annotation of 60 cytochrome P450 genes, some of which are likely to be involved in host–vector associations (e.g., detoxification of phytochemical resistance factors, CYP3 clan, degradation of volatile attractants, and CYP4 clan).

CRB has supported several projects on vector–host interactions, including studies of ACP responses to volatiles produced by flushing shoots (Patt and Sétamou, 2010), identification of volatiles for surveillance and management of ACP (Coutinho-Abreu et al., 2014a; Project #5500-186), odor coding in ACP underlying odor detection in ACP antennae (Coutinho-Abreu et al., 2014b; Project #5500-186), characterization of the ACP transcriptome (Reese et al., 2014), and collaborative work on mitogenome analysis to identify region of origin for ACP in California (Wu et al., 2016).

Overlaps and Duplications

With respect to characterizing interactions between ACP and its host plants, there was little overlap in objectives between projects funded by CRDF and other funding agencies. Although both agencies funded projects aimed at characterizing volatile attractants and repellents, there were differences in scope and experimental design that generated findings that were complementary rather than duplicative.

PATHOGEN–VECTOR INTERACTIONS

CRDF-Funded Research

The 2010 NRC report listed several gaps in understanding the ACP–CLas interaction affecting transmission and HLB epidemiology. These included a need to study the role of ACP nymphs in CLas transmission and HLB epidemiology, the effects of CLas acquisition and infection on ACP biology and fitness, and the potential for vertical transmission of CLas through ACP (NRC, 2010). Numerous investigators developed projects based on these recommendations, and CRDF funded several projects to investigate the various aspects of CLas transmission by ACP. As is often the case for plant pathogen transmission by insects, all of the measured parameters can vary widely depending on pathogen and insect populations, pathogen source materials, experimental design and methods, and environment. Nonetheless, important information has been generated that is being used to develop and modify HLB management recommendations.

Five projects funded by CRDF (totaling about $1.5 million) are directly and indirectly researching CLas–ACP interactions related to transmission. A majority of the transmission-related projects are aimed at identifying or developing molecules that could have direct or indirect effects on the ability of ACP to transmit CLas. Projects include studies on the effects of thermotherapy (see also the section HLB Management, Bacterial Control, page 86) on ACP transmission efficiency (#941C); the ability of other bacteria to initiate an immune response in ACP that would affect their ability to acquire and transmit CLas (#15-021); the identification of ACP gut receptors targeting the binding and uptake of Bt toxins (#711); the identification and expression of quorum sensing–related compounds that can disrupt bacterial cell-to-cell communication and biofilm formation in CLas populations (#15-017), thus resulting in less efficient survival and transmission of bacteria from ACP; and the manipulation of the CLas phage to induce a lytic phase in CLas harbored by the ACP to essentially cure the insect of the bacteria (#15-009). One CRDF-supported project relates to CLas having a single-component LuxR quorum-sensing mechanism and lacking the acyl-homoserine lactone (AHL) second component. AHL mimics produced in citrus in response to CLas infection may bind to LuxR and trigger cell aggregation and limit CLas movement in plants. ACP endosymbionts may also produce AHL mimics and allow CLas to form biofilms on the ACP gut. Feeding ACP on LuxR-expressing citrus results in a decrease in gut biofilm formation and a decrease in CLas titer in the insect, presumably because the LuxR from citrus binds the AHL mimic in ACP and disrupts the LuxR (bacteria)–AHL interaction. This continuing work (Project #15-017) is focused on developing resistance in citrus, although investigation of aspects related to acquisition and transmission of CLas from the AHL-expressing citrus plants is planned (Killiny et al., 2014, 2017). Several additional ongoing projects described in the Vector–Host Interactions section (page 76) are looking at ACP survival or deterrence of host finding or feeding. The outcomes from these projects may also provide ways to reduce transmission, although many of these projects are focused only on insect survival and do not incorporate CLas transmission.

Toward achieving the goals set out in the 2010 NRC report, CRDF and other agencies (mentioned below) have funded projects aimed at understanding the biological parameters affecting transmission efficiency (Pelz-Stelinski et al., 2010; Mann et al., 2011; Hall et al., 2012; Wang and Trivedi, 2013; Albrecht et al., 2014; Pelz-Stelinski, 2014; Coy and Stelinski, 2015; Martini et al., 2015a; Hall et al., 2016; Pelz-Stelinski and Killiny, 2016) and how the bacteria move within and interact with the ACP (Ammar et al., 2011a,b, 2016). In addition, they have funded studies to determine how different compounds may be used to prevent these interactions (Tiwari et al., 2011; Yan et al., 2013; Hoffmann et al., 2014; Pelz-Stelinski, 2014; Ramsey et al., 2015, 2017; Chu et al., 2016; Arp et al., 2017; Gill et al., 2017). A key component of the latter was the development of transgenic citrus expressing various compounds that may interfere with ACP feeding or ACP–CLas interactions. It also included the use of CTV vectors for transient expression (El-Shesheny et al., 2013; El-Mohtar and Dawson, 2014; Hajeri et al., 2014; Dawson et al., 2015) (see also the section Citrus Tristeza Virus Gene Delivery, page 75). Efforts in this research area are in line with the 2010 recommendation ***NI-9: Support demonstration of RNA interference [RNAi] effects for possible suppression of ACP.*** Several laboratories are currently identifying genes, proteins, and metabolites that are being tested for activity. Intellectual property concerns have kept many of the specifics out of this review. The expression and quantification of compounds either in transgenic plants or transiently using the CTV vector are not straightforward tasks, but progress is being made with promising, albeit inconclusive, results. It is appropriate to mention the recently ended 5-year, $9 million NuPsyllid project (#780nu, 781nu, 782nu, 783nu, 784nu, 785nu, 786nu, 787nu, 788nu, 789nu, 790nu, 791nu, 792nu, 793nu, 794nu, 795nu, 796nu, 797nu, 798nu, 799nu, 800nu, and 801nu) that was designed to generate a genetically modified ACP that would not be able to transmit CLas and could displace wild ACP populations.

Research Funded by Other Agencies

The USDA APHIS MAC program has many projects focused on the management of ACP, but because few of them integrate CLas transmission with vector control it is difficult to assess what ACP management strategies will have any meaningful effect on reducing HLB incidence. One MAC-funded project (#15-8130-0485-CA) investigated the use of thermotherapy to reduce the ability of ACP to acquire and transmit CLas; this type of work was also funded by CRDF (Project #941C).

Past CRDF funding has been instrumental in generating preliminary data and methods that have allowed larger, multi-institution and multidisciplinary projects to be developed and funded by other agencies. Most of these are fundamental studies aimed at medium- to long-term solutions to HLB. Relevant vector-pathogen projects funded by USDA NIFA include the use of cell-penetrating peptides to inhibit CLas and endosymbiont gene expression in ACP as a way of reducing CLas in the insects and reducing or disrupting transmission and/or insect fitness (Project #2016-70016-24781). This research appears to overlap with CRDF funding (Project #15-026).

NIFA (Project #2016-70016-24779) and CRB are funding research to characterize ACP populations differing in CLas transmission efficiency (Project #5300-163) that would enable a system to study the genetics of transmission competency in ACP and characterize ACP proteins regulating the movement and survival of CLas in the insect. Another NIFA-funded project (#2015-70016-23028) is focused on the much needed integration and curation of omics data becoming available for ACP, citrus, and CLas and to further use these data along with *de novo* generated genomic, proteomic, and metabolomics data to discover molecules that can block pathways related to pathogenesis and transmission.

CRB funded projects that aimed to discover an infectious CTV vector from a California CTV isolate (#5300-158), to discover ACP and CLas proteins that are involved in pathogen–vector interactions (#5300-155), and to characterize factors influencing transmission efficiency (#5500-203).

HOST–PATHOGEN INTERACTIONS

CRDF-Funded Research

CRDF has funded 16 projects, including 12 past projects (totaling $3.1 million) and four currently active projects (totaling about $1.7 million), with at least one objective that is directly related to host–pathogen interactions. The past projects were focused on determining the physiological response of host plants to HLB infection (#002 and 045), identifying host resistance or tolerance genes to HLB (#523 and 536), analyzing the genomes of the HLB pathogen and host to better understand their interactions (#733, 314, and 123), and studying pathogen effectors or host gene effectors to understand HLB and develop control strategies (#312, 750, 232, and 609).

The currently funded projects are focused on the application of knowledge acquired on the citrus microbiome (#15-042), host metabolomic response (#15-003), and effector and tool development for resistance and control of HLB (#15-028 and 16-005).

Host Physiological Responses to CLas Infection. Effort was made to understand the roles of plant callose and phloem proteins in symptom development and disease control by manipulating callose and phloem proteins in HLB (Koh et al., 2012; Aritua et al., 2013).

Identification of HLB Resistance Candidate Genes. Two projects aimed to identify host genes that are highly upregulated in resistant citrus compared to susceptible varieties through transcriptomic and informatics analyses (Folimonova et al., 2009; Nwugo et al., 2013; Pitino et al., 2016), and a study by Gmitter and colleagues (Rawat et al., 2015, 2017) was focused on identifying and mapping HLB resistance gene(s) in *Poncirus*.

Target Pathogen Effectors or Host Gene of Effectors for Understanding of HLB and Development of Control Strategies. Four past CRDF projects aimed to investigate the molecular interaction of host genes and pathogen effectors. One project (#312) involved development of a virus-based vector for gene expression in citrus plants. Using this system, putative effector genes of interest from Liberibacters were delivered into host cells using CTV for functional analysis (Zou et al., 2012). A second project (#750) focused on four CLas effectors and their host target proteins. It was hypothesized that the interaction of effectors and their targets would play important roles in pathogenesis of HLB. Tentative host target proteins were identified from yeast two-hybrid, or Y2H, screening, and exploration of their biological relevance is in progress. Two projects (#232 and 609) addressed the role of Liberibacter salicylate hydroxylase in HLB pathogenesis. The pathogen uses the enzyme to break down the host salicylic acid (SA) and its derivatives, messengers of the host defense system, leading to weakened resistance in host plants (Li et al., 2017).

Research Funded by Other Agencies

In 2016, NIFA SCRI funded two currently active projects (totaling about $7.3 million) with objectives directly related to host–pathogen interactions. One focuses on the identification of effectors in Liberibacter spp. and elucidation of their role in HLB pathogenesis (#2016-70016-24833), while another aims to identify host candidate genes for HLB resistance or tolerance (#2015-70016-23027).

CRB is currently funding two projects (totaling about $1.2 million) with objectives directly related to host–pathogen interactions. One project aims to identify and characterize small RNAs and mRNAs (#5300-131), and the other aims to identify and characterize citrus targets from CLas (#5300-160).

HLB MANAGEMENT

Bacterial Control

Despite aggressive research and development efforts, there are no fully effective strategies for field management of HLB in Florida citrus. Keeping citrus free of CLas is a goal not only in fruit production groves but also in seedling nurseries and germplasm-development greenhouses. Development of approaches directed at reducing or eliminating the pathogen are hampered by the phloem-restricted microbial habitat and the uncultivable nature of CLas, but researchers are exploring a variety of avenues to target the pathogen as a means of disease management.

CRDF-Funded Research

CRDF has supported a number of past research projects studying HLB management by targeting the bacterial pathogen; many, but not all, of these have resulted in publications in peer-reviewed journals and have served as a foundation for current research efforts, as noted below. Funding these projects is in line with the NRC recommendation ***L-2: Support development and testing of bactericides, therapeutics, or systemic acquired resistance (SAR) activators***. More recent projects aim to evaluate a variety of approaches, including the application of bactericidal or bacteriostatic chemical compounds or antibiotics (nine current projects), the use of high temperature (thermotherapy) to eliminate the pathogen from living tissues (two projects), biocontrol through triggering of the lytic cycle of bacteriophages native to CLas (two projects), nutritional strategies and microbiome enhancements (three projects), and understanding and inducing natural defense responses in the citrus host (four projects). Considering all of the funded research, past and present, related to bacteria-targeted disease management, CRDF has awarded just over $16,864,000 in grants.

Induction of Host Defense Gene Expression. Multiple factors and triggers for induction of genes encoding compounds related to host defense to bacterial invasion have been identified in citrus. These factors and inducers can be mediated not only by pathogens but also by beneficial microorganisms and a variety of chemical compounds. Their application can result in suppression of disease progress and reduction of disease severity and bacterial multiplication. One past project (#129) used the model plant *Arabidopsis* to investigate the effect of overexpressing the citrus gene CsNDR1 (an ortholog of an *Arabidopsis* gene that positively regulates SA accumulation) on the production of SA, which is a signaling molecule important for both basal and induced host defense responses in many plants, and the expression of the defense marker gene PR1 (Lu et al., 2013).

Another project (#609) evaluated several combinations of β-aminobutyric acid, 2,1,3-benzothiadiazole, 2,6-dichloroisonicotinic acid, ascorbic acid, and the glucose analog 1-deoxy-D-glucose for their effects on CLas replication in the host and HLB disease severity (Li et al., 2016). A chemical genomics approach was used in a proxy system involving the CLas-related bacterium *Ca.* Liberibacter psyllaurous, which causes zebra chip disease of potatoes and vein-greening disease of tomato (Prager and Trumble, 2018), in *Arabidopsis* (Patne et al., 2011; Project #326). In addition to screening many small, drug-like molecules to identify chemicals that induce plant tolerance or resistance against the pathogen, this project (#326) aimed to also create *Arabidopsis* mutants deficient in specific defense mechanisms to determine the basis for observed tolerance in this host (Roose et al., 2014). Project #15-018 investigated the role of CLas salicylate hydroxylase in suppressing host defense, while another (#572) explored the infiltration into citrus of flagellin protein (shown in other systems to be involved in pathogen-associated molecular pattern host defense induction) from both *Xanthomonas citri* pv. *citri* (*Xcc*, causal agent of citrus canker)[16] and CLas (Moore and Febres, 2014; Project #572).

Antibiotics and Chemotherapy. Although copper (Cu) bactericides have been used widely for controlling plant diseases, there are concerns about Cu accumulation in soil and leaching, as well as evidence of increasing microbial resistance (Young et al., 2017). Supplementation of bactericides with zinc (Zn) may help reduce the amount of Cu being released in the environment. One past project funded by CRDF (#15-037C) tested the effect of applying formulations designated "TSOL," containing chelated Zn and inert ingredients to help in-plant translocation, on the incidence of HLB. CRDF also funded the development of the zinc-based nanoparticle treatment, Zinkicide, which has undergone initial testing in the field (Project #907). A patent application[17] filed in 2013 is now listed as abandoned due to the absence of a statement of use; however, a current project[18] related to this formulation is now being supported through NIFA SCRI.

A number of nonmetal antibiotics, including sulfadimethoxine and sulfathiazole, cell-wall inhibitors in the β-lactam group (ampicillin, carbenicillin, penicillin, and cephalexin), and the transcriptional inhibitor rifampicin have also been tested for their efficacy in eliminating or suppressing CLas (Zhang et al., 2014; Projects #617C and 617-1). In another CRDF-funded project (#775C), several tetracycline derivatives that lack activity against human bacterial pathogens were tested against strains of Liberibacter.

[16] Currently the citrus canker pathogen is referred to as *Xanthomonas axonopodis* pv. *citri*.
[17] See https://www.trademarkia.com/zinkicide-86125365.html. Accessed December 20, 2017.
[18] Grant #2015-70016-23010; Project #FLAW-2014-10120 (2015-2020).

There has been a continual push to develop novel anti-CLas treatments, using both high-throughput and directed methods. Triplett et al. (2016; Project #782) tested 888 different chemical formulations for activity against *L. crescens* (a culturable proxy for CLas), including bacterial fermentation products obtained from both major and smaller startup companies. In an unusual approach to the identification of new antimicrobials having potential for HLB management, Pagliai et al. (2014, 2015; Project # 414) characterized a regulatory element containing a CLas transcriptional regulator (*ldt*R) and a transpeptidase (*ldtP*).

CRDF has also supported research in optimizing antibiotics and other antimicrobials for maximum efficacy. For all bactericidal treatments, the method of plant delivery is as critical as its chemistry; cost and damage issues associated with bactericidal injection and thermotherapy have obstructed their commercialization. One past project aimed to characterize the optimum parameters for trunk injection of oxytetracycline to achieve uniform antibiotic distribution *in planta,* and improve tree health, yield, and juice quality (Hu and Wang, 2016; Project #773). Two past projects focused on enhancing the efficacy of antimicrobials by combining them with adjuvants or delivering them as a nanoemulsion (Yang et al., 2015; Powell et al., 2016). "Soft" or nonclumping nanoparticles (SNPs) are being explored as vehicles for foliar application of natural biocides to infected trees (Moudgil et al., 2015; Projects #771 and 909). One past project (#15-031C) investigated the use of laser light in enhancing the uptake of foliar sprays (Etxeberria et al., 2016; Projects #818 and 15-031C). Other current CRDF projects are focused on the delivery mechanisms for agriculturally registered bactericides, essential oils, and other antimicrobials (Minter, 2017; Project #15-048C; Richardson and Shatters, 2017; Project #938C), their uptake and movement *in planta* (Tetard and Pelz-Stelinski, 2017; Project #16-017C), or their efficacy in the field (Richardson and Shatters, 2017; Project #938C).

A key point in the evaluation of antimicrobials is that their use may be enhanced when used in combination with other HLB management strategies in an integrated pest management (IPM) approach. For example, in one CRDF project (#910), Powell et al. (2016) aimed to determine the optimum combination of chemotherapy, thermotherapy, and nutrient therapy that can be registered for field citrus and control HLB.

A current CRDF project (Gabriel et al., 2017; #15-009) is investigating potential control of HLB using the putative CLas LexA-like (LC1) repressor protein, found in psyllids, as potentially a key phage lytic cycle regulator. Three protein repressors and target promoters are being screened for chemicals that might be used to activate the phage lytic cycle in CLas, in infected psyllids and citrus. Gabriel et al. (2017) also examined control of HLB using a CLas phage peroxidase, which degrades reactive oxygen species in the host defense, and a CLas lytic cycle activator, to enhance native citrus resistance to CLas. In an alternate approach, Gonzales (2016; Project #726) investigated phages from *Xanthomonas axonopodis* pv. *citri* for their HLB therapeutic potential.

Thermotherapy. CRDF has funded a number of research projects to explore the potential of thermotherapy for managing bacterial diseases such as HLB (Projects #910, 834, 586C, 586-1, and 941C). The impact of thermotherapy arises from two possible effects of elevated temperature: the direct effect of killing bacteria with heat and the indirect effect of triggering induction of a lytic phase in bacteriophages within the pathogen. In both cases it is necessary to identify an effective temperature that does not adversely affect the host plant. Both approaches have been effective in treating other bacterial diseases.

Host Plant Nutrition and Microbiome Approaches. The importance of both the entire plant-associated biotic community and the abiotic environment in shaping plant health has recently been reemphasized in the newly described phytobiome concept (Beattie et al., 2016), and how those principles apply to citrus and HLB was thoughtfully reviewed by Wang et al. (2017). All phytobiome species residing in plant-associated niches, external or internal, interact with one another and with the plant in beneficial, detrimental, and neutral ways that are now beginning to be appreciated, but these concepts may provide important clues not only to understanding complex diseases such as HLB but also to managing them. These principles have been explored in Projects #15-042, 916, and 608. This nontraditional approach to nutrient enhancement for citrus in the presence of HLB was reflected in the recent work of Zhang et al. (2017; Project #780C), who characterized the microbiomes of HLB-infected and healthy citrus trees and identified rhizosphere-to-rhizoplane-enriched taxonomic and functional properties associated with them. Another recent project (#15-042) addressed the effects of a number of beneficial bacteria on HLB disease index and CLas titers in the citrus host.

Research Funded by Other Agencies

Induction of Host Defense Gene Expression. The U.S. Department of Energy provided funding for a project that evaluated the application of epibrassinolide, a member of a group of essential plant-produced brassinosteroids, as a means of controlling HLB (Canales et al., 2016).

Nutritional Approaches. Most nutritional treatments for HLB management have focused on enhancement of tree health with the goals of extended productive lifespan and maintenance of fruit production and quality in HLB-infected trees (see also the section Cultural Control, page 91).

Insect Control (Chemical and Biological)

CRDF-Funded Research

Since the release of the NRC report in 2010, CRDF has funded at least 26 projects dealing specifically with chemical control and IPM for ACP, in line with the 2010 NRC recommendation ***NI-1: Improve insecticide-based management of Asian citrus psyllid.*** Essentially, every insecticide registered for use on citrus has been examined by researchers, and many insecticides in the development stage have been tested against various psyllid stages (Boina and Blumquist, 2015). Collectively, approximately $5,560,000 was spent on these projects since 2010. The major themes of the research have been insecticide testing against all stages of the psyllid (examples include Projects #447, 590, 603, and 091), integration of insecticides into IPM programs to minimize resistance development (such as Projects #15-024, 850, and 15-038C), documentation of approaches that could be used in organic orchards (Projects #711, 858, and 217), and examination of attractant and repellent compounds with potential for use as control materials (Projects #853 and 16-020C). As part of these goals, projects were funded to examine pesticide application strategies on young and mature trees and at different times of the year based on tree phenology (Projects #15-036C, 325, and 425).

Six additional projects funded by CRDF were designed to determine how to sample for ACP in order to determine if pesticide use is justified (#15-024, 214, 090, 701-1, 164, and 567). These were funded for just over $1,150,000, and a detailed description of sampling techniques can be found in Monzo et al. (2015). These projects examined many different sampling approaches and proposed psyllid population thresholds designed to indicate a need for pesticide applications on both young and mature trees.

Considerable funding has also been expended for psyllid suppression using biocontrol strategies, including eight projects funded by CRDF for approximately $1.9 million (including #711, 760-1, 212, and 434). These projects had three major foci. The first was to evaluate the potential of indigenous predators and parasites. Because of intensive pesticide applications in active commercial groves, these beneficial insects appeared to have the greatest potential for effective use in abandoned orchards and in urban/suburban citrus. A second approach was to rear and release imported parasites, particularly *Tamarixia radiata*. This was by far the biggest recipient of support, with well over 95% of the funding. The third focus was to investigate the use of entomopathogens in psyllid suppression.

Research Funded by Other Agencies

Additional funds of nearly $1.9 million for chemical control of ACP were provided by the USDA APHIS HLB MAC Group. CRB also funded projects on the use of pesticides for over $1 million, but this was not considered a duplication of funding because pesticides can have variable effects on a target insect depending on application strategy, crop, and environmental conditions.

The funding of the biological control programs supported by CRDF was supplemented by 10 projects and substantial funding from the MAC program (just over $4 million). This strategy is also being funded by CRB. Almost all of the additional funding from other agencies was designated for the parasite release program.

Overlaps and Duplications

Although funding for insecticide testing was provided by several agencies, this is a necessary duplication of effort. Insecticide efficacy can vary among cultivars, geographic locations, and environmental conditions. Thus, tests at multiple locations and on different cultivars are necessary and justified.

The biocontrol programs were well coordinated. Most of the predator and entomopathogen studies were conducted in Florida (with a small portion of the research occurring in Texas). The overlap between agencies for funding of the *Tamarixia* release projects was fairly minimal. Most of the CRDF funds were used to evaluate the effectiveness of the releases in Florida, while the CRB funding was used to support parasite releases in California. The much larger amounts of funding provided by the MAC program supported the Florida and California efforts by producing the large numbers of parasites needed for the releases in both states. Thus, these programs appeared to have good coordination with relatively little overlap.

Cultural Control

CRDF-Funded Research

CRDF has funded projects that aimed to investigate various aspects of cultural and on-farm management of HLB, CLas, and/or ACP. Studies were done to evaluate the effects of nutritional supplements on plant growth and development; these included the impacts of root and soil health (Projects #903, 15-013, 838, and 15-023), the use of endophytes to control CLas (Project #15-042), and the use of metallic mulches (Project #16-011C) and polymer films (Project #858) to repel ACP and improve tree growth.

Numerous CRDF-funded projects have investigated several potential cultural management strategies that could reduce the immigration of ACP, the inoculation efficiency of immigrating ACP, disease development in inoculated trees, and improvements in tree health and fruit production and quality (Projects #910, 903, 776C, 731,732, 943C, and 447). Advanced production systems for citrus have been partially supported by CRDF funding (Project #593) and have looked at integrating several production strategies, including high-density plantings, alternative irrigation and nutrient management, as well as using mulches and growing citrus in psyllid-proof enclosures (Campos-Herrera et al., 2013, 2014; Kadyampakeni et al., 2014, 2016; Ferrarezi et al., 2017a,b). High-density plantings are becoming more popular as growers replant in Florida, and the data generated by these studies are contributing to the enhanced citrus management systems being put in place by some growers. These efforts are in line with the 2010 recommendation ***NI-8: Support small-scale studies on the feasibility of alternative horticultural systems suited to endemic HLB.***

Research Funded by Other Agencies

The USDA APHIS MAC program has contributed in excess of $8 million to research on numerous management topics, including removal of abandoned groves, reducing pH of irrigation water, thermotherapy, integrated management, mulch, and intensive grove management.

Diagnostics

CRDF-Funded Research

There are no projects currently funded by CRDF that are explicitly researching new CLas diagnostics; however, there are several projects that could contribute valuable information to the development of next-generation diagnostics. A study (Project #15-003) comparing the metabolomic profiles of germplasm from citrus that is susceptible to CLas and citrus that may have some level of resistance or tolerance would be valuable for laboratories looking for metabolomic biomarkers for disease detection. Another project (#15-042) is focused on characterizing the phytobiomes and endophytic microbes from HLB survivor trees and HLB-infected trees to determine if the endophytic microbes from survivor trees could be used to manage HLB; results of this study can be used by researchers who are investigating microbial population changes as biomarkers for disease detection. There is also a project (#15-008) that aimed to improve the understanding of the movement of the pathogen in or between trees, and to elucidate the nature of signals from the pathogen. The focus of this work is not diagnostics; however, understanding pathogen movement and systemic signals from the pathogen could be helpful in developing next-generation diagnostics. These projects can contribute to ongoing efforts that are in line with the 2010 recommendation ***NI-2: Support searches for biomarkers that may be exploited to detect CLas-infected citrus.***

Past CRDF-funded projects with objectives related to CLas diagnostics include high-throughput screening of seedlings for resistance to HLB based on optical sensing (Project #880).

Research Funded by Other Agencies

Current CRB projects include research on metabolomics and proteomic biomarkers in trees related to CLas infection (Project #5300-150) and on improving detection using conventional methods, i.e., polymerase chain reaction-based amplification (for example, Project #5300-176). Canine detection is funded by the USDA, ARS, and USDA APHIS MAC program (Project #14-8130-0474-CA). MAC is also funding an antibody-based early detection project with apparent overlaps with a project funded by NIFA (Project #2016-2021) to identify CLas-secreted effector molecules that diffuse through the tree and can be detected by antibody-based diagnostics. Two additional NIFA-funded projects looked at CLas detection in ACP; one project was focused on the development of a yeast biosensor that can be used in the field to identify CLas-infected insects (#2016-70016-24779), and the other focused on the development of field assays using loop-mediated isothermal amplification (or LAMP) technology (#2015-70016-22992). These are subobjectives in larger projects aimed at understanding CLas transmission, ACP genetic variability, and ACP surveillance.

SOCIOLOGICAL AND ECONOMIC ASPECTS OF HLB AND ITS MANAGEMENT

The state of Florida has more acreage devoted to citrus production than any other U.S. state. In 2012 Salifu et al. (2012) estimated commercial production at just short of 600,000 acres (242,811 hectares) and annual proceeds at $9 billion, with about 76,000 jobs devoted to the Florida industry. However, since its detection in Florida in 2005, HLB has caused increasing economic losses each year to growers, processors, and the state as a whole (Roka et al., 2009; Muraro, 2012; Salifu et al., 2012; Court et al., 2017). These losses are incurred in a variety of ways and are felt by a variety of stakeholder groups.

The reactions and responses to the arrival and spread of HLB in Florida were influenced in a number of significant ways by the fact that in 2005, when HLB was first detected in Florida, growers and regulators were still occupied with containing a serious outbreak of citrus canker, a different disease caused by the bacterium *X. axonopodis* pv. *citri*. Canker, which had been found several times previously in Florida but successfully eradicated each time, was discovered again in 1996 near Miami (Gottwald et al., 2012). *X. axonopodis* pv. *citri,* disseminated primarily by wind, water, and the movement of infected plant parts, spread through substantial portions of the citrus-producing counties during the next 8 years. Because an eradication approach had been successful in past canker outbreaks it was implemented again, with targeted delimiting disease surveys and quarantines on the movement of citrus seedlings, buds, and fruit (USDA APHIS, 1999). A key feature of the canker eradication strategy was to rogue infected trees, including destruction (generally by burning) of trees that were symptomatic as well as all trees within a 1,900-ft. radius of a symptomatic tree (which may have already become infected but was not yet symptomatic). Although growers were compensated for approximately 60% of their evaluated losses, the impacts on the industry were heavy; losses to many growers were high and a number of farms were lost. Eradication costs rose above $200 million, and more than 10 million trees were destroyed, but the disease continued to spread (Gottwald et al., 2012). A series of hurricanes in 2004 further exacerbated the dissemination of *X. axonopodis* pv. *citri* within the state, and regulatory officials eventually amended their canker strategy; in 2006 USDA APHIS declared canker endemic and transitioned from eradication to disease management (USDA APHIS, 2017).

The arrival of HLB in Florida in 2005 was different in several important ways from that of canker in 1999. The ACP vector had been present in Florida since 1998 and by September 2000 it had been found in 31 counties (Halbert and Manjunath, 2004), making it extremely likely that if CLas ever made its way to Florida it would be impossible to eradicate. This expectation was indeed met after CLas arrived.

A traditional and generally successful approach to disease management for an insect-transmitted pathogen in a new location is to remove infected plants that could serve as reservoirs for the pathogen and practice strong insect control via chemical sprays and methods to discourage or prevent insect feeding. However, when HLB arrived in Florida the severe impacts of citrus canker—both the disease and the management strategies—were still fresh in the minds of growers and other industry personnel. Many growers had suffered financially from canker and had neither the funds nor the will to invest heavily in managing HLB. The disappointment and frustration that many growers felt over the efforts to manage canker influenced their willingness to accept new constraints, restrictions, regulations, and government involvement in the response to HLB. This reluctance continues today among some members of the citrus industry (Singerman, 2017) and must be factored into assessments of the potential success of any management strategies for HLB.

Thus, a comprehensive assessment of future research directions in support of HLB management and of the potential of new research findings to be accepted and implemented according to guidances developed for them would be incomplete without consideration of factors such as economics and sociology, which influence decision making and behaviors of growers, processors, and the public. Although the research findings described below emerged from projects funded by sources other than CRDF, their implications for future HLB research are critical.

Economic Impacts of HLB

Eradication of HLB-infected and nearby trees in Florida eliminated about 10% of the citrus production capacity by 2008 (Irey et al., 2008), and the disease is estimated to have led to the loss of 6,600 jobs and as much as $1.6 billion in grower revenue ($418 million/year) (Hodges and Spreen, 2012; Court et al., 2017). Between 2005 and 2012 the increased number of recommended sprays for psyllid control and other disease-related actions resulted in significantly higher production costs: cultural practices rose 107% (reaching, for example, $1,187/acre for Valencia), fertilizer costs were up 160%, and chemical sprays over 170% (Muraro, 2012). As a result, growers must realize 33 to 39% greater income just to break even financially. Today, most Florida citrus growers no longer remove infected trees (Muraro, 2012) and even abandon (or stop managing) entire orchards that have become unproductive due to HLB. These findings could serve to inform decision making by state and federal officials with respect to targeting disease reduction and prevention.

Economic Aspects of ACP Management and CHMAs

Pests are essentially “common property,” ignoring farm boundaries such that a pest problem for one grower is likely to be a problem for the neighboring farmers (Singerman et al., 2017). The establishment of 35 Citrus Health Management Areas (CHMAs) was an attempt to encourage neighboring citrus growers to share and coordinate HLB management actions, especially those targeting the psyllid vector, to more effectively manage pathogen dissemination and limit disease spread (Muraro, 2012; UF IFAS, 2018). The effectiveness of the CHMAs was evaluated by comparing the yields from blocks of trees managed within CHMAs having high levels of grower participation with those from blocks having lower participation (Singerman et al., 2017). Yields from the high-participation blocks were significantly higher than those from low-participation blocks in each year of the 3-year study, and the difference between the two grew each year from 28% (year 1) to 98% (year 3). However, the evidence indicating that higher levels of CHMA participation results in greater economic benefits has not led to proportional levels of grower participation in them (Singerman et al., 2017); rather, grower participation levels have actually fallen off over the years since CHMA establishment. In surveys of grower perspectives, the most important reason given for nonparticipation by CHMA members was the belief that neighboring farmers were not complying with the agreed-upon spray strategy, and the resulting doubt about whether an individual's own expense and effort would be effective in the absence of neighbor participation (Singerman et al., 2017). The second-most important reason given was that farmers preferred to spray on their own schedule, rather than following an area-wide schedule. Thus, "strategic uncertainty" has hindered the success of the CHMA approach. Singerman et al. (2017) made four recommendations for improving the success of CHMAs: (1) regulatory mandates for scheduled spraying, possibly with subsidies to farmers to defray a portion of chemical costs; (2) monitoring of sprays and consequences for noncompliance; (3) a process for in-kind transfers among growers and possible tax breaks; and (4) enhancement of communication and education among growers.

If CHMA programs continue, the use of newer ultra-low-volume sprayers, especially from aerial dissemination systems, should lower costs and enhance effectiveness (Muraro, 2012). However, the CHMA focus on pest control represents the more traditional formula for managing a vector-transmitted disease, a focus that many Florida citrus growers now question. Indeed, many growers are moving to new strategies having very different final objectives.

New Directions of HLB Management

Recent trends in HLB management in Florida have shifted from concerted efforts to stop the disease to actions intended to support and prolong the health and fruit production of HLB-infected trees (Roka et al., 2009; Muraro, 2012; Zansler, 2017). The idea is to live with the disease rather than eliminating it. One new approach is the use of advanced production systems, which have the potential to reduce the time to fruit production and to maximize and stabilize yields for up to 15 years using high-intensity cultural/agronomic approaches (Roka et al., 2009). Advanced production systems can include high-density planting (up to 360 trees/acre from the traditional 150) and a multifaceted approach to water and nutrient supplementation, including time-released fertilizers, computerized fertigation systems, hydroponic systems, and automated irrigation scheduling. Another new strategy, the Enhanced Foliar Nutritional Program (EFNP), involves the application of mixtures of foliar nutrients, generally as a replacement for traditionally applied fertilizers, in the attempt to compensate for nutrient losses caused by the blockage of conducting tissues in infected trees. When three different EFNP nutrient mixtures were applied to affected groves (Muraro, 2012), costs increased by $119 to 273/acre over that for traditional HLB management (tree removal). Irey et al. (2008) estimated that such new approaches to HLB management have increased production costs by about 40%.

Although some studies (not funded by CRDF) are under way to measure the impacts of the new nutritional/high-density approaches, it is still too early for the results of these analyses to be available. However, many Florida citrus growers have adopted some form of this strategy, and many believe that these approaches are working (Muraro, 2012), anecdotally reporting higher yield and more robust tree growth. The hope is that nurturing infected trees will allow them to remain productive much longer. Further, higher per-acre yields resulting from high-density planting (and proportionately higher grower revenues) should allow more frequent grove turnover as HLB symptoms become more severe. On the negative side, some researchers are concerned that the new emphasis on tree health in the face of HLB is leading many growers to reduce or eliminate their efforts to control the ACP, placing the two approaches in conflict with one another (Gottwald et al., 2012).

Any proposed new management approach will be practical only if the resulting yield and quality are such that grower application costs are more than compensated. For example, Roka et al. (2009) observed that advanced production systems will likely increase grower input costs by several thousand dollars per acre, so fruit yields must also increase to result in a favorable cost–benefit ratio; furthermore, market prices for citrus must be adequate to support that ratio.

Modeling

Because strategic uncertainty clearly influences grower behaviors in disease management (Singerman et al., 2017), it is critical to reduce their uncertainty by providing as much research-based evidence as possible for the benefits of various management strategies. Evidence should be based on realistic assessments of cost–benefit ratios of each approach as well as combinatorial approaches. Generally, a mixed set of approaches (i.e., insect control, removal of infected plants, and use of bactericides) will be more effective than a single one, but estimating cost–benefit ratios becomes more difficult and uncertain with each additional variable.

HLB is among the most complex of plant diseases, so it is among the most difficult to effectively manage. CLas, the associated pathogen, remains uncultivable, hampering many types of research into pathogen–host and vector–host interactions. Within the tree, it is restricted to the phloem sieve tubes and companion cells, through which it translocates erratically and unevenly throughout the tree. That fact and the fact that in-plant bacterial titers can remain very low for many months before symptom expression make sampling for disease detection frustrating and often misleading. On the host side, citrus is a perennial crop that is generally transplanted into the field as grafted rootstocks, so trees typically have two different genotypes. Young trees remain relatively unproductive until they are about 5 years old and then, if healthy, remain productive for about 20 to 25 years before production levels decline below profitability. The requirement of an insect vector for pathogen dissemination adds another layer of biological complexity, and elements of the psyllid's epidemiology (flight behavior, feeding and mating behaviors, and attraction to plant colors and volatiles) are strong influencers of disease epidemiology. Thus, there is a multitude of interactions among host, pathogen, and vector, as well as interactions of each of these with other members of the trophic level and with the physical environment (including climate, soil qualities, and agronomic practices).

Most scientific research, by its nature, is conducted within a limited set of parameters (controlled experimentation) to facilitate the attribution of research findings to the particular experimental variables employed. However, applicability of research findings to real-world problems requires a systems approach in which, ideally, all parameters pertaining to all of the participants (host, pathogen, and vector) should be considered. Because this level of inclusion is not possible, as many of the factors as possible should be incorporated. Modeling should be further developed as a systems approach to HLB management, in which it can be used in a myriad of applications, for example, to evaluate risk, to predict how best to incorporate HLB-tolerant groves in relation to susceptible neighboring fields, to provide guidance on how to deploy the CTV vector to express AMPs and RNAi and potential benefits of doing so, to help assess replant strategies, to compare the effectiveness of different single and combinatorial HLB management practices, and to inform economic decision making.

Genetically Modified HLB-Resistant Citrus

Most of the strategies discussed here for managing HLB in Florida are considered by growers, processors, and consumers to be stop-gap measures to preserve as much as possible of the state's citrus industry while awaiting the development and release of HLB-resistant cultivars that still retain desirable characteristics such as yield, flavor, size, and shelf life. Despite long-term intensive breeding programs, no such resistance is yet available. During the same period, however, significant advances have been made in crop improvement through genetic manipulations, including genetic engineering and CRISPR technology; these approaches are being explored by a number of researchers in Florida and elsewhere for the development of acceptable varieties having resistance or high levels of tolerance to HLB.

A critical question about whether genetic modification will provide the ultimate solution to HLB is whether the technology will be accepted by growers, processors, and consumers. Singerman and Useche (2017) surveyed[19] citrus growers from different production areas in Florida about their perceptions and concerns about the use of this technology. Fewer than half (40%) of respondents felt that they were reasonably well informed about genetic modification technology. Approximately 75% of growers indicated willingness to plant genetically modified (GM) trees if they were resistant, but 12% of these were concerned about consumer and processor acceptance, both necessary to assure their market. The most important specific concerns about GM HLB-resistant citrus in particular included, in order, (1) consumer acceptance, (2) processor acceptance, (3) production issues, (4) cost of acquiring and planting the new trees, (5) safety issues related to the origin of the added genes, and (6) environmental concerns (Singerman and Useche, 2017).

On the consumer side, House (2017) conducted three online surveys (2012, 2013, and 2016[20]) of the general public about their attitudes toward genetic modification technology. Consumers indicated concerns about its use, particularly in foods such as fruits and juices. Asked about hypothetical scenarios involving their attitude toward purchasing GM foods, a small proportion of respondents indicated that they would not purchase them at all. Most, however, were willing to purchase GM foods if they were priced at a significant discount. The amount of the discount required was influenced by factors such as (1) the type of food, (2) the country from which the technology originated (they were more likely to accept technology from their own country), (3) the type of institution from which the technology arose (academia, large industry, or government), and (4) the rationale for using genetic modification technology (“saving an industry” was partial justification but did not completely overcome their objections).

Ironically, the loss of a portion of consumers due to genetic modification concerns would add to an already-lowered consumption of citrus juices that is due in part to public concerns about its sugar content and in part to the decreased production of fruit for juice in Florida, which is outpacing the decline in consumption (Zansler, 2017).

[19] This survey was not funded by CRDF.
[20] These surveys were not funded by CRDF.

OVERALL FINDINGS, CONCLUSIONS, AND RECOMMENDATIONS

Finding 3.1: CRDF support for the multiple HLB research projects is responsive to several recommendations contained in the NRC 2010 report, particularly to 8 of the 11 near- to intermediate-term (NI) research and technology recommendations:

- ***NI-1: Improve insecticide-based management of Asian citrus psyllid***
- ***NI-4: Accelerate the sequencing, assembly, annotation, and exploitation of a sweet orange genome to provide a powerful tool for all future citrus improvement research***
- ***NI-5: Support development of HLB model systems***
- ***NI-6: Exploit the CLas genome sequence for new strategies of HLB mitigation***
- ***NI-7: Support research aimed at developing alternative ACP management strategies***
- ***NI-8: Support small-scale studies on the feasibility of alternative horticultural systems suited to endemic HLB***
- ***NI-9: Support demonstration of RNA interference (RNAi) effects for possible suppression of ACP***
- ***NI-10: Develop* in vitro *culture techniques for CLas to facilitate experimental manipulation of the bacterium for insights into gene function***

CRDF also funded research efforts that address three of the four 2010 NRC long-term research and technology recommendations: *L-1: Support the development of transgenic HLB-resistant and ACP-resistant citrus; L-2: Support development and testing of bactericides, therapeutics, or SAR activators;* and *L-3: Support analysis of ACP behavior, ACP–plant interactions, and ecology to enhance the knowledge base available for new ACP management strategies.*

Finding 3.2: Other agencies are funding efforts to address *Recommendation NI-11: Sequence, assemble, and annotate the ACP genome to provide a basis for new approaches to ACP management* as well as *NI-2: Support searches for biomarkers that may be exploited to detect CLas-infected citrus,* although there are CRDF-funded projects that could provide useful data leading to improved HLB detection.

Finding 3.3: *Recommendation NI-3: Establish citrus orchard test plots for evaluation of new scouting and therapeutic methods* remains to be addressed.

Conclusion 3.3: Although CRDF supports Picos Farm, a secure transgenic field test site in Florida, additional sites are needed for assessment and validation of scouting and therapeutic approaches for HLB management with significant impacts on Florida as well as California, Texas, and Arizona.
Recommendation 3.3: CRDF should consider establishing an infrastructure enhancement project to assess field testing needs for all citrus disease and insect research and validation activities and to design plans to meet those needs by enhancing current field test sites, establishing new field test sites, and/or developing collaborations with citrus growers to use production orchards for testing.

Finding 3.4: A single breakthrough discovery for managing HLB in Florida in the future is unlikely, since intensive research efforts over almost 20 years have not led to this result.

While it is possible that genetic engineering or gene editing will lead to the availability of HLB-resistant citrus cultivars, it is likely that such cultivars will also benefit from reduced inoculum loads, ACP control to reduce vector populations, and improved cultural management practices. Currently, each of the IPM strategies tested offers a partial solution, and combining these approaches is providing the best management solution available at present.

Conclusion 3.4: Finding the best combinations of control strategies suited to different environmental and growing conditions, vector and pathogen pressure, tree cultivars, and tree health would help growers in Florida and in other states where HLB is not yet a chronic problem but soon could be, especially if HLB infection is not detected quickly and if there is reluctance to remove inoculum sources.
Recommendation 3.4: Consider specific funding for the development of sets of management approaches that can be combined in different ways, optimized, and validated for use in different locations and conditions.

Finding 3.5: Plant pathologists, sociologists, and economists are using modeling to assess the complex interactions that characterize HLB; however, no CRDF funding has directly supported research on economic and sociological factors that impact decision making and behaviors of growers, processors, and the public and can influence the adoption and success of future HLB management efforts.
Conclusion 3.5: Greater investment and researcher involvement are needed to develop and apply modeling technologies for analysis and prediction of the effects of economic and sociological factors on the acceptance and application of HLB management practices.

Recommendation 3.5.1: CRDF should consider adding these research areas to its funding portfolio.

Recommendation 3.5.2: CRDF should consider creating centralized, researcher-accessible databases to support sociological and economic modeling of HLB-related research outcomes and application projections. It should support systems approaches for field testing combinations of the most promising developments in replicated field studies, emphasizing the need for collecting research data sufficient to inform and support model training and applications to effectively predict cost–benefit ratios of all HLB management strategies.

Finding 3.6: Recent and growing interest in research using genetic modification to develop HLB-resistant citrus, and concerns about stakeholder acceptance of these technologies, indicates that expanded efforts in educational outreach to growers, processors, and consumers could facilitate eventual deployment of new citrus lines. Data from previous advertising strategies directed at adjusting consumer attitudes about citrus consumption have demonstrated that such communication with the public can change behaviors.

Conclusion 3.6: Further research is needed to assess the level of current understanding of genetic modification technology, and the factors that influence their willingness to purchase genetically modified foods.

Recommendation 3.6: CRDF should consider funding research to assess consumer attitudes toward genetic modification technologies for producing HLB-resistant citrus cultivars and their willingness to consume genetically modified citrus, as well as how targeted advertising campaigns could be effective outreach strategies.

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4

Notable Outcomes, Pitfalls, and Future Directions

This chapter presents significant research findings and progress, as well as issues of concern, from the past decade of Citrus Research and Development Foundation (CRDF)-funded huanglongbing (HLB) research. For each research area, following the committee's statement of task (see Chapter 1), the committee highlighted the key research findings (see Boxes 4-1, 4-3, and 4-5), indicated which 2010 National Research Council (NRC) recommendations have been addressed, discussed notable outcomes, and reviewed factors that hamper or present challenges (pitfalls) to the work. The committee also provided recommendations and considerations for future research (see Boxes 4-2, 4-4, and 4-6 and Future Directions subsections) for each of the research areas. However, the committee was unable to define further the extent of research progress or to comment more specifically on the selection of research areas to be continued or discontinued because information available to it on research outcomes, applications, and impacts was insufficient to do so in many cases, particularly for recent projects. At the end of this chapter are the committee's overarching findings and its conclusions and recommendations for future research efforts in HLB management. The conclusions and recommendations are based upon the information available to the committee from peer-reviewed journal articles whenever possible; other sources included non-peer-reviewed publications, such as trade magazines, conference abstracts, presentations at forums of this committee, and progress reports and final reports from CRDF research.

BOX 4-1
Key Research Findings:
Biology and Ecology of the HLB Causal or Associated Pathogen and Its Insect Vector

- Numerous *Candidatus* Liberibacter asiaticus (CLas) genomes from around the world have been sequenced, revealing mutation patterns and conserved potential control targets, and are providing opportunities for comparative genomic studies.
- CLas-killing bacteriophages have been characterized, and factors that suppress them have been identified.
- *Liberibacter crescens*, a culturable relative of CLas, has been developed as a proxy system to study pathogen–host interactions.
- Asian citrus psyllid (ACP) biology and reproductive behavior have been characterized extensively.
- Annotation of the ACP genome and transcriptome has led to key discoveries that can be exploited to reveal control points at the vector–pathogen interface.
- Emerging biotechnologies, such as RNA interference using natural gene-based targeting, offer novel mechanisms to achieve new, sustainable, and environmentally friendly management of ACP and other citrus pests.
- Elucidation of patterns of ACP seasonal activity and abundance have facilitated effective targeting and timing for pest management efforts.

BOX 4-2
Recommendations for Future Research:
Biology and Ecology of the HLB Causal or Associated Pathogen and Its Insect Vector

- Continue to sequence diverse CLas isolates, monitoring for changes that have or could alter virulence or change the efficacy of control strategies.
- Continue to study bacteriophage-suppressing factors and essential CLas-encoded proteins that are produced or secreted in the plant in order to determine the most promising control targets.
- As more CLas proteins or compounds are confirmed to be essential and produced or secreted in the plant, the research focus should transition into identifying plant-compatible strategies to target them (i.e., through engineering) in ways that inactivate or recognize the factors.
- Develop protocols that allow recovery of ACP DNA from trap catches to facilitate evaluation of population structure, pesticide resistance, and other biological characteristics.

BOX 4-3
Key Research Findings:
Interactions of the HLB Pathogen, Vector, and Host

- Genome sequencing and analysis of Liberibacter strains led to the identification of candidate effector genes that may be involved in interactions with the host leading to HLB.
- Evidence has been found for a disease-promoting role of at least five CLas virulence factors, which are potential control targets.
- Resistant or tolerant citrus varieties highly upregulate host genes, including some involved in suppression of plant immunity mechanisms and metabolic dysfunction, in response to Liberibacter infection.
- Nymphal stages of ACP acquire CLas very quickly and are important in the transmission and dissemination of CLas.
- CLas replicates in ACP and is vertically transmitted at low levels.
- Mechanisms and pathways of circulative transmission of CLas in ACP have been elucidated.
- CLas alters the biology of both citrus and ACP, impacting disease development: infected ACP disperse earlier and farther than uninfected individuals, infected females are more attractive to males than uninfected females, and CLas infection alters leaf color and plant volatiles, rendering the plant more attractive to ACP.

BOX 4-4
Recommendations for Future Research:
Interactions of the HLB Pathogen, Vector, and Host

- Continue to characterize CLas effectors, including identification and functional analyses of their host targets, and apply new knowledge to the development of novel HLB management tools.
- Continue to identify and characterize critical genes or gene products in citrus that are targets of CLas.
- Continue to seek new resistance genes within citrus or in other species that counteract CLas effectors.
- Develop trapping protocols that allow recovery of ACP DNA, to facilitate evaluation of population structure, pesticide resistance, and other biological characteristics.
- Continue to identify new molecules that interfere with the CLas life cycle, leading to titer reductions in citrus or ACP hosts.
- Continue to identify new molecules that reduce the ability of CLas to be transmitted by ACP.
- Explore strategies for economically viable physical protection of trees against insect access, including ACP repellence formulations or mulches and screening structures.

BOX 4-5
Key Research Findings:
HLB Management

Bacterial Control

- New approaches developed for bacterial control (including nanoparticle or nanoemulsion formulations, the addition of adjuvants, the use of chemical mixtures, combining chemical treatments with other measures such as thermotherapy, or triggering the host plant's defense mechanisms rather than killing the pathogen through the use of bacteriophages or beneficial microbes) can enhance treatment effectiveness and minimize the amount of active anti-bacterial chemical needed.

Vector Control

- Efforts to control the vector remain important in Florida, as repeated inoculations increase the rate of disease development and the severity of symptoms.
- Nearly all available insecticides, and many insecticides in development, have been evaluated against ACP in Florida.
- Pesticide resistance management plans have been developed and are being used in some area-wide management programs.
- The benefits of indigenous predators and parasites of the psyllid have been evaluated and quantified.
- The imported psyllid parasite *Tamarixia radiata* has been reared in large numbers and released throughout Florida. The impact of this parasite has been greatest in urban settings and in abandoned orchards.

Host Resistance: Breeding

- HLB susceptibility varies with citrus cultivar and rootstock.
- Some new rootstocks having HLB tolerance have been identified.
- HLB-tolerant citrus rootstock and scion genotypes and HLB-resistant citrus relatives have been identified, providing material for further breeding as well

BIOLOGY AND ECOLOGY OF THE HLB CAUSAL OR ASSOCIATED PATHOGEN AND ITS INSECT VECTOR

Candidatus Liberibacter Species

Notable Research Outcomes

Genome Sequencing, Bioinformatics Analysis, and Genome-Enabled Distribution and Diversity Studies. The completion of the *Candidatus* Liberibacter asiaticus (CLas) genome in 2009 (Duan et al., 2009) was a major achievement that enabled new research directions in diagnostics, culturing, pathogenesis mechanisms, and bacterial control. Relevant to 2010

as a temporary production bridge for the industry as highly resistant materials are being developed.
- Potentially useful levels of resistance have been identified in citrus relatives and may be incorporated into breeding programs in the longer term.

Host Resistance: Genetic Engineering

- A large number of transgenic citrus expressing genes that may confer resistance to HLB have been produced and are being tested.
- The genomes of a number of citrus and citrus relatives have been sequenced. This genetic information will, in the long term, provide valuable information on the reaction of the host to HLB and key processes that may be manipulated to reduce or prevent HLB infection.

Cultural Control

- Optimizing tree nutrition and water can result in short-term benefits in citrus tree health and reduce the impact of HLB on fruit yield, but it has no curing effect and is expensive and likely unsustainable.
- Increasing plant density in citrus orchards can decrease the rate of HLB epidemic development.
- Multiple inoculations of infected trees result in increased disease impact on fruit yield and quality.

Diagnostics

- Molecular and serological diagnostic technologies for CLas are ultrasensitive but, on their own, are not ideal for epidemiological and regulatory purposes because of uneven pathogen distribution in the tree.
- No single diagnostic method will be sufficient to identify recently infected trees.
- Detection of infection prior to symptom development is possible through detection of changes in host metabolites and volatiles.

NRC report ***Recommendation NI-6: Exploit the CLas genome sequence for new strategies for HLB management***, genomic analysis has led to the identification of a variety of candidate proteins (i.e., virulence or other essential proteins) that could be targets for disease mitigation strategies. Many of these genes, some of which have significant effects on plants, have now been cloned and functionally characterized in other organisms. Sequencing of numerous CLas isolates in parallel revealed which candidate genes are highly conserved among strains, allowing researchers to screen out less desirable control targets. The genomes of the additional HLB-causing species *Candidatus* L. americanum and *Ca.* L. africanum (Wulff et al., 2014), and of Liberibacter species that cause other diseases were also sequenced. Genomic

BOX 4-6
Recommendations for Future Research:
HLB Management

- Evaluate which bacterial targeting strategies are most effective and feasible for transgenic plant development and support the development of a limited number of these in citrus.
- Continue to explore the use of novel chemical therapies; the use of chemical genetics approaches can help in identifying key candidates. Look particularly at chemicals that are involved as regulators or intermediates in key host plant defense response pathways.
- Implement field testing to determine actual impacts of chemical applications on disease severity and fruit production, and develop predictive disease impact models to support decision making with respect to this management approach.
- Focus, in Florida, on managing the disease as a chronic problem in which incremental improvements in control will continue to have value. Thus, even minor improvements that can be incorporated should be considered.
- Continue to evaluate the effects of cultural management approaches, including high-density plantings and nutritional supplementation, on tree infection, citrus production, and pathogen acquisition and transmission by ACP.
- Explore the effectiveness of new pesticidal chemicals, particularly those that provide repellency or have minimal effects on biocontrol agents, on the incidence of HLB.
- Investigate any new parasites/predators found on ACP as possible biocontrol agents for psyllids; the parasite currently established in Florida is useful but does not provide adequate control on its own.
- Continue to explore the use of biomarkers associated with HLB-diseased trees as tools for early diagnosis, comparing these to the detection of volatile organic compounds.
- Create a strong research effort to assess and model the nature and impacts of sociological and economic factors influencing decision making relevant to HLB management in the Florida citrus industry and among consumers and the general public.
- Assess diverse, multifaceted combinations of HLB management approaches under different environmental conditions and production regimes, with the objective of designing a flexible system of combinatorial recommendations tailored to the needs of individual growers in different citrus production areas.

comparisons among these, and between them and the genomes of related free-living species, including a culturable, nonpathogenic Liberibacter strain, have led to the identification of numerous essential functions likely lost in CLas, those retained, and a few properties predicted to be unique to CLas (Hartung et al., 2011; Kuykendall et al., 2012; Leonard et al., 2012).

The completion of the CLas genome led to the development of several variable genetic markers to improve global surveillance and expand knowledge of HLB epidemiology, allowing the rapid differentiation of CLas from different continents (Chen et al., 2010; Katoh et al., 2011; Islam et al., 2012). Advances in sequencing technology also provided a reference for sequencing of nine additional publicly available genomes[1] of CLas from infected citrus samples, and roughly 30 more isolate genome sequences are slated for publication in the near future (Duan, 2017). Comparative studies revealed thousands of points of genetic variation among sequenced CLas genomes, revealing new U.S. phylogenetic groupings as well as the presence of mixed CLas populations within a single host plant (Duan, 2017). This information will serve as a resource for tracking the basis and origin of future changes in the location, host range, and severity of HLB.

Functional Comparative Genomics and the Identification of Infection-Associated Genes and Proteins. CLas proteins required for host invasion could potentially be targeted for inactivation through targeted treatments. Analysis of the 1,136 predicted protein-coding genes in the CLas genome revealed numerous candidates for involvement in plant infection based on similarity to known pathogen proteins. Predicted virulence function of several CLas genes, including efficient scavengers of critical plant defense signals, was validated by expression analysis or study of gene function in other organisms (Jain et al., 2015; F. Li et al., 2017). The genome-enabled ability to analyze CLas gene expression in the plant and vector led to the identification of gene activation patterns and regulatory elements in the bacterium (Yan et al., 2013). A LuxR receptor of an unknown citrus signal increased CLas symptoms in transgenic citrus (Gabriel, 2017), and the LdtR transcriptional regulator was shown to be a master regulator of global gene expression, including that of the stress-response gene LotP (Pagliai et al., 2014, 2017; Loto et al., 2017). The genome also revealed that CLas likely has a functional Type II secretion system for transporting proteins from the bacterial cell to the extracellular environment. Sixteen genes encoding potential secreted proteins were identified based on predicted secretion signals,[2] and their subcellular localization was characterized in a tobacco leaf expression system (Pitino et al., 2016). One effector induced starch accumulation in tobacco cells, suggesting that this protein could cause the starch accumulation that accompanies symptoms in citrus (Duan, 2017). The protein also triggered cell death in tobacco, leading to the cloning of a tobacco resistance gene that represents the first plant gene encoding resistance to a CLas-secreted element (Duan, 2017).

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[1] National Center for Biotechnology Information (NCBI); https://www.ncbi.nlm.nih.gov. Accessed January 23, 2018.

[2] A secretion signal is a hydrophobic motif or peptide component at the N-terminus of a newly synthesized protein that directs it to the outer membrane for secretion.

Research Toward Culturing CLas. Many of the advances in understanding described above are still short of being confirmed because the obligate nature of CLas does not allow its phenotypes to be observed directly. The ability to culture CLas sustainably would be a major boon to long-term HLB research and control efforts, allowing dissection of pathogen behavior and facilitating work requiring plant and insect inoculations. Past culturing projects took an iterative trial-and-error approach, initially with plant- or insect-derived culture materials and later informed by improved models of CLas metabolic requirements. Groups have reported progress in the form of growth or prolonged viability of CLas using citrus-based media (Sechler et al., 2009; Parker et al., 2014) or co-cultivation with other bacteria (Davis et al., 2008), with viability lasting for up to 18 days. These outcomes provide a benchmark for progressive improvement.

The identification of the culturable *L. crescens* has provided an important model system for functional characterization of genes identified in CLas (***Recommendation NI-5: Support development of model systems***), and tobacco- and periwinkle-based experimental systems have also been extremely useful for bacterial characterization and proxy screening. *L. crescens* shares 75% genetic identity with CLas (Leonard et al., 2012), and the 76 *L. crescens*-specific genes essential for culturing are a focus of interest in current CLas culturing efforts (Lai et al., 2016). Other developments include the optimization of protocols to use insect biofilms as a source of culture inoculum and the use of microfluidic devices to replicate the phloem flow environment (Gabriel, 2017). Currently funded efforts to identify possible new medium ingredients that will increase the span of viability (stated goals of the two recently funded National Institute of Food and Agriculture [NIFA] projects) include comprehensive analyses of phloem sap and insect gut composition, the effect of the physical environment, the role of CLas chemical signals, and several other factors. Thus, culturing projects are designed to yield a wealth of biological information about the vector and pathogen, which may assist strategies other than culturing.

Understanding Antagonistic Phage and Bacteria. The discovery of a probable role of bacteriophage in CLas growth and fitness was an important research contribution suggesting another potential strategy for HLB mitigation. Phage dynamics could impact CLas growth in different host contexts; phage particles were observed microscopically in periwinkle (*Catharanthus roseus*) phloem, suggesting that they could confer bacterial lysis in this nonhost plant (Zhang et al., 2010). Accordingly, phage lytic genes that were suppressed in psyllid-associated CLas are activated only moderately in citrus-associated CLas, but highly activated in the nonhost periwinkle (Zhang et al., 2010; Fleites et al., 2014; Jain et al., 2015). Understanding the bacterial stresses that cause phage activation could be a route to activating a “self-destruct” mode in CLas or to suppressing it to enhance culturability. On the other hand, several researchers have investigated the potential for CLas phages to be used as a disease management approach (see section on Bacterial Control on page 149 in this chapter).

Impacts of the Citrus Microbiome. Whole-microbiome profiling studies have shown that levels of CLas and other bacteria abundant in the citrus microbiome fluctuate seasonally (Zhang et al., 2013a), that HLB infection is associated with an increase in xylem-inhabiting *Methylobacterium* spp. (Zhang et al., 2013b), and that HLB decreases the ability of citrus roots to recruit bacterial species from the rhizosphere (Zhang et al., 2017). HLB management treatments such as thermotherapy and antibiotics also exhibit significant effects on microbial composition (Zhang et al., 2013b; Yang et al., 2016). The effects of these changes on disease progression are still unknown. Other microbiome research has focused on the role of disease-controlling properties of citrus microbiome communities, or of particular microbial species, as potential approaches to HLB management. These efforts are described further in the Bacterial Control section of this chapter.

Pitfalls

Due to the continuing inability to culture CLas, the promising target candidate genes revealed by genomics have been characterized in an indirect manner through heterologous expression in other organisms. The 2010 NRC report recommended supporting research into *in vitro* culture techniques to allow completion of Koch's postulates and to facilitate genetic studies (***Recommendation NI-10: Develop* in vitro *culture techniques for CLas to facilitate experimental manipulation of the bacterium for insights into gene function***). While this area was an intensive focus of CRDF funding related to CLas, the goal of sustainable culturing has remained elusive. Genome-enabled metabolic reconstruction and comparison with culturable *Liberibacter* have shed light on numerous missing metabolic and culturability genes. In addition to the inability to synthesize critical substrates of primary metabolism, isolated CLas may also require its environment to detoxify methylglyoxal (the non-enzymatically produced byproduct of glycolysis), suppress internal phage, and provide physical and chemical regulatory signals (Project #FLAW-2015-10491[3]). The NIFA Specialty Crop Research Initiative (SCRI) has recently taken over most of the funding of CLas culturing efforts with two large multilaboratory projects.

[3] Project information available at https://citrusrdf.org/wp-content/uploads/2017/06/Cycle-2-2016-NIFA-SCRI-HLB-Project-full-abstracts-CRIS.pdf. Accessed January 23, 2018.

Future Directions

Understanding the bacterial processes occurring during host–vector interactions has progressed substantially for an unculturable bacterium, resulting in the identification of many known or predicted critical CLas genes. Multiple coordinated projects are now working to validate the function of these genes and to screen for or design interactors or suppressors. In the coming years, these projects could yield technological strategies for bacterial suppression through chemical controls or through citrus gene-editing/transgenic approaches. While any bacterial-targeting approach would ideally be multipronged to avoid evolution of bacterial resistance, the process of advancing each strategy through translation and field evaluation steps will be time consuming and expensive. Although this research is still in early stages, the research community could prepare to maximize funding efficiency by enhancing community coordination on selecting and advancing bacterial targeting strategies as they pass proof-of-concept stages. This process would benefit from the establishment of common benchmarks for evaluating and reporting research outcomes. Continued communication should take place with industry as to which strategy outputs would be preferable to growers and consumers (i.e., synthetic genes,[4] transgenes from other plants, or transgenes from other organisms), possibly through the publication in appropriate trade journals of articles describing research outcomes, targeted to growers and the lay public.

The research focus on genome sequencing (***Recommendation NI-6: Exploit the CLas genome sequence for new strategies for HLB management***) has provided not only an understanding of bacterial genes but also a picture of CLas evolution and community structure in hosts—knowledge important for selecting effective control strategies. Sequencing costs have fallen substantially since 2010 and continue to do so. While whole-genome sequencing is unlikely to yield additional returns in control targets, it will continue to be a cost-effective means of tracking the genetic changes in CLas and associated phages as the bacterium moves into new areas and evolves. Some sequencing efforts have also included taxonomic profiling of the citrus bacterial microbiome or "phytobiome" associated with HLB citrus, identifying some HLB-associated changes and isolating potential antibacterial microbes. The phytobiome is increasingly recognized to play an important role in plant disease (Beattie et al., 2016), and it is possible that other microbes provide signals needed for virulence and transmission of CLas. However, there are few examples of successful biological control against tree pathogens (Cazorla and Mercado-Blanco, 2016), and it is still unclear whether plant community profiling efforts could be translated realistically to HLB control.

[4] Genes constructed artificially from oligonucleotides by chemical means.

Several research groups have long worked toward the difficult goal of culturing CLas, supported by over $8 million of CRDF and NIFA SCRI funding. Having a singular end goal of maintenance in culture has led to some overlap in specific aims between groups. The absence of means for publishing negative data resulted in there being relatively few publications from these projects, which may have made it difficult for researchers to learn about the many culturing methods that were attempted but unsuccessful. The recent consolidation of funding by NIFA SCRI into two large culturing projects, both with rational and stepwise aims, goes far to address overlap and communication issues and should yield publishable data regardless of culturing outcome. Still, CLas metabolic, detoxification, and regulatory needs are now predicted to be much more complex than anticipated. Future CLas culturing projects might have complex outcomes; for example, culturing methods could require materials and equipment that would be so cost prohibitive as to greatly limit their use. Or, CLas may be obtainable in culture, but extremely difficult to manipulate genetically or to inoculate into vectors and hosts (a large part of the rationale for culturing). While current CLas culturing efforts will continue at peak levels until the end of their funding term (2020), the grower and research communities should evaluate whether to advocate for continued efforts if sustained culture is not attained by that time, taking into account the advances that have been made possible in the absence of culturing. The use of microbiome profiling of diseased and healthy citrus, while providing relevant basic information about the phytobiome, is at this time a developing science that is likely to offer greater returns in the future than it does at present.

HLB Vector: Asian Citrus Psyllid

Notable Research Outcomes

CRDF has supported fundamental investigations into the biology of the Asian citrus psyllid (ACP), especially as it has pertained to vector competence and management of the citrus greening pathogen. Moreover, CRDF has made efforts to implement the findings in terms of the 2010 NRC report (NRC, 2010), with particular emphasis on support of "near- and near- to intermediate-term" recommendations.

ACP Genome Sequencing and Applications of Sequence Information to HLB Management. Progress has been made to address ***Recommendation NI-11: Sequencing, assembly, and annotation of the ACP genome to provide a basis for new approaches to ACP management.*** Although support for this approach was only partially funded by CRDF, outcomes have been generated from a combination of these projects along with projects from U.S. Department of Agriculture (USDA) funding sources intended to provide information on which to base future strategies for ACP suppression of or interference with CLas transmission. The first genome draft of ACP was completed initially in 2011 (Hunter and Reese, 2014) and has more recently been amended through support from the Los Alamos National Laboratory, New Mexico, and USDA NIFA. The revised draft genome and transcriptome were assembled and submitted into the public domain at the National Center for Biotechnology Information (NCBI), and genomic resources are currently available from the Genome Sequencing Project[5] and the I5K arthropod genome project[6] and CitrusGreening Solutions.[7] The ACP transcriptome effort has identified over 25,600 predicted genes, supported by an additional 19,598 previous expressed sequence tags, and transcripts were identified for specific life stages, including adults, nymphs, and eggs. The NIFA-funded CitrusGreening Solutions initiative hosts a comprehensive, publicly available website[8] that provides a variety of genome resources for data integration, annotation, and other analyses for ACP as well as CLas and citrus.

RNA Interference (RNAi) for Possible Suppression of ACP. The availability of transcriptome data has aided in efforts to develop RNAi targets in ACP (***Recommendation NI-9: Support demonstration of RNA interference [RNAi] effects for possible suppression of ACP***), and several researchers have used these resources toward the development of strategies to suppress ACP populations using the *Citrus tristeza virus* (CTV) vector to deliver RNAi molecules into ACP (Hajeri et al., 2014). RNAi, a process in which double-stranded RNA (dsRNA) exerts a silencing effect on complementary messenger RNA (mRNA), has emerged as a promising research tool following functional genomic analyses, and transgenic citrus lines expressing RNAi for ACP control are in the developmental stages (Stover, 2015[9]).

Ease of delivery, high specificity of gene targets, and a lack of environmental persistence are among the benefits of RNAi approaches for crop protection. Recent functional genomic analyses of RNAi-related genes in the ACP genome showed an absence of sequences encoding a dsRNA-binding protein that functions as a cofactor of Dicer (Taning et al., 2016). Since RNAi is an efficient process in ACP after oral delivery of dsRNA, the absent R2D2 cofactor may not be necessary for this process in ACP (Taning et al., 2016). Presumably, a different dsRNA-binding cofactor may compensate for the absence of R2D2 in the ACP. Nevertheless, recent bioassays using both plant-launched systems and feeding delivery have shown that

[5] See http://www.ncbi.nlm.nih.gov/bioproject/PRJNA29447. Accessed June 8, 2018.
[6] See https://i5k.nal.usda.gov/Diaphorina_citri. Accessed June 8, 2018.
[7] See www.citrusgreening.org. Accessed June 8, 2018.
[8] See www.citrusgreening.org. Accessed June 8, 2018.
[9] Unpublished reports from CRDF project principal investigators (PIs) are available upon request from CRDF. Accessed June 8, 2018.

ACP is very sensitive to ingested dsRNA, demonstrating a strong RNAi response. Small doses of dsRNA (arginine kinase and superoxide dismutase), administered at the time of citrus flush, were sufficient to trigger the RNAi mechanism, causing significant suppression of the targeted transcript, and increasing psyllid mortality (Andrade and Hunter, 2017). Additional studies provide evidence of the functional RNAi machinery present in ACP, which could be further exploited through future research to develop RNAi-based management strategies for the control of ACP (Christiaens and Smagghe, 2014; Killiny et al., 2014a; Taning et al., 2016).

Dispersal Dynamics of the Asian Citrus Psyllid. Understanding of dispersal behavior has been enhanced by CRDF funding. Peak periods of ACP activity in Florida occurred during the spring and summer, whereas activity was lower during the cooler fall and winter seasons (Martini et al., 2014). As reported previously, adult ACPs dispersed relatively long distances (up to 2 km) and the number of immune-marked adults captured increased with "spring-flush" activity in managed groves. When patterns of flight initiation and duration were investigated, nearly one-third of blue ACP morphotypes (having green-blue abdomens) exhibited longer durations of flight, whereas fewer than 5% of gray morphotypes (having gray-brown abdomens) did so. Gray morphotype adults had shorter wings and smaller pronotal thoracic plates than did the blue morphotypes. Furthermore, ACP body size, wing width, and wing aspect ratio differed based on the host plant species on which they were reared, impacting dispersal (Paris et al., 2016). Finally, increases in ACP flight activity corresponded to times of increasing barometric pressure (Martini and Stelinski, 2017). As previously documented, increases in ACP activity and dispersal were also correlated with increasing temperature, but no relationships were observed with relative humidity, whether static or variable. Changes in pressure did influence the responsiveness of ACP, and the magnitude of these impacts corresponded to the degree of overall pressure swings (Paris et al., 2017). Cultural factors and practices, such as row orientation, significantly influenced psyllid density during the winter, and ACP abundance was higher when more than 20% of the surrounding landscape was urbanized (Pelz-Stelinski et al., 2017).

Infection Status and Psyllid Dispersal. When ACP were allowed to develop through immature stadia (stages between successive molts of an insect) on CLas-infected plants, short-distance dispersal of male ACP was greater than that of male counterparts reared on uninfected plants (Martini et al., 2015). The initiation and duration of flight by CLas-infected ACP occurred earlier and for longer time periods than for those of uninfected psyllids. Moreover, the titer of CLas, measured by quantitative polymerase chain reaction (qPCR), was greater in ACPs that performed long flights on the experimental flight mills than in presumably noninoculative psyllids, which flew for shorter periods. Finally, adult female psyllids containing higher titers of CLas were regarded as more attractive to male psyllids than females having lower CLas titers, suggesting that the bacterial pathogen may influence both movement and mate finding of the insect vector.

Determinants of Host Location and Vector Fitness. Increased levels of leaf nitrogen resulting from applications of plant growth regulators (PGRs) to container-grown citrus trees resulted in higher ACP reproductive rates, shorter development time, and larger body mass (Tsagkarakis et al., 2012). Psyllids reared on trees treated with selected PGRs exhibited significant reductions in fecundity, survivorship, and oviposition rates. In general, oviposition occurred later on PGR-treated trees than on untreated controls. The responses of ACP to the selected PGRs were apparently not influenced by access to suitable oviposition sites, nor by direct toxicity of the PGRs, but rather to induced plant biochemical changes that altered host plant quality. Analyses of leaf nutrients and photosynthesis showed no correlation between plant nutrient status or carbon assimilation with changes in psyllid behavior, although it was hypothesized that other changes could have occurred that were not detected in their whole-leaf analyses.

Increasing the Efficiency of ACP Monitoring. Investigations of psyllid vibrational communications in mate attraction (Mankin et al., 2013) revealed that broadcast mimic calls elicited strong female responses comparable to those elicited by a recorded male call, suggesting the potential to develop systems for increased trapping efficiency. Spectral and temporal patterns of calling pairs (duets) have been mimicked using computer-assisted vibrational tools. Other research relevant to increasing ACP monitoring effectiveness is provided in the section Vector–Host Interactions in this chapter.

Pitfalls

A recommendation of the 2010 NRC report was ***Recommendation NI-1a: Develop new methods to fine-tune field surveillance of ACP via more efficient and consistently applied trapping or other methods, to improve timing and targeting of insecticide applications***. CRDF has supported projects to investigate novel trapping techniques together with compounds that could be useful in enhancing trap capture efficiencies. Unfortunately, research has not yet led to standardization of trap selection and optimization of trapping approaches. Populations of ACP continue to be monitored with standard yellow sticky cards, which are not ideally suited for recovery of ACP DNA for CLas detection, and which continue to be collected and replaced according to protocols that differ in different regions of the United States.

Seasonal flight activity of the ACP has been investigated using yellow sticky traps placed in citrus groves and adjacent fallow areas (Hall and Hentz, 2011). These studies have demonstrated that flight can occur at any time of the year, but captures were more consistent during the spring flush of citrus. Except for a modest relationship with relative humidity, the activity of captured adult ACP could not be correlated with any abiotic factor or set of factors, including wind speed, sunlight, and air temperature. Trap color had no influence on the efficiency of ACP captured throughout the citrus production season. Comprehensive field trials over several weeks revealed that three-odor-lured yellow traps caught ~230% more ACP over time than solvent-control yellow traps placed on the same trees (Coutinho-Abreu et al., 2014). Unfortunately, the data from these individual investigations have revealed little about the principal factors that help to explain patterns of ACP capture.

Future Directions

Extensive ACP capture investigations have been supported by CRDF in the past decade. Additional formal efforts to standardize trap types across producers, government agencies, or crop consultants across broader geographic regions are probably not needed at this time, as the various yellow sticky panel traps now available capture adult ACP with similar efficiencies. However, new research is needed to improve the ability to extract DNA from trap samples for use in a number of research areas, and data management and informatics approaches could be used to capture additional value from data collected over years of trapping studies. It would be beneficial to collect and collate the extensive ACP capture data over several locations and years of investigation. Making these digital and georeferenced data more accessible to communities capable of utilizing advanced computational power and sophisticated algorithms for the analysis of large datasets would provide greater opportunities for predicting the complex behavior and patterns of capture of the ACP, especially in agricultural systems. If researchers and funding agencies are to contribute to realizing that promise, however, there is an immediate need to develop research methods that can resolve patterns from these large datasets to aid in limiting risk for producers. Agricultural informatics can be viewed as an offshoot of big data, whereby insights into integrated pest management (IPM) are realized by integrating multiple data streams to create a composite dataset for analysis. This approach can be particularly attractive for researchers wishing to investigate insect or disease management processes that occur at very large spatial or temporal scales not easily investigated through more traditional experimentation. Importantly, agricultural informatics methods are often best used in combination with hypothesis-driven experimentation, and together these can strengthen the potential to draw causal relationships (Boyd and Foody, 2011) as well as to lead to tangible management outcomes.

While researchers and grove managers consider patterns of citrus flush to be a primary cue that triggers ACP movement within groves, the interacting abiotic (or biotic) conditions that would help to describe actual population trends and inform the development of areawide management strategies have defied characterization to date. A more comprehensive ACP trap capture dataset across broad geographic regions in each year would be very important for observing long-term trends and examining their relationships to environmental factors. As discussed in the 2010 NRC report, surveillance should not be limited to commercial orchards but should also include urban landscapes and declining or abandoned groves to identify local ACP populations for treatment before they disperse.

Past and current research, described earlier (Notable Research Outcomes), has shown that novel pest management technologies, such as RNAi, have the potential to provide new strategies for ACP management (Meister and Tuschl, 2004), and research to develop such approaches should be continued.

INTERACTIONS OF THE HLB PATHOGEN, VECTOR, AND HOST

Vector–Host Interactions

Notable Research Outcomes

Host Range of ACP. In screening potential ACP rutaceous hosts, variation in suitability was documented and in some cases was determined to be a heritable trait. *Poncirus trifoliata* (hardy or trifoliate orange), which is resistant to ACP (Richardson and Hall, 2013; Hall et al., 2015), can hybridize with sweet orange to produce "citranges." Lower levels of attack by ACP on *P. trifoliata* appear to result from reduced oviposition. Unlike citrus, *P. trifoliata* is winter dormant and deciduous, lacking foliage through the winter. In early spring, while *P. trifoliata* remained in winter dormancy, *Citrus* and citrange hybrids were producing shoot flushes and supporting populations of ACP. Even after flush shoots were produced on *P. trifoliata* later in the spring, fewer eggs were laid on this species than on *Citrus* (Hall et al., 2017). Hybrid citrange cultivars tested, however, lacked resistance to ACP.

A number of native rutaceous host plants were ruled out as alternative hosts outside of managed groves and hence could be considered unlikely reservoirs for psyllid populations (Sétamou et al., 2016); in fact, no alternative hosts capable of maintaining ACP feeding or reproduction during leaf flushes were found, at least in central Florida. In contrast, abandoned groves were identified as important reservoirs for ACP and pathogen populations.

CRDF funding supported work showing that host plant identity influences ACP dispersal potential through changes in wing aspect ratio (Paris et al., 2016). Although all citrus hosts supported ACP development through adulthood, those reared on *Citrus taiwanica* were smaller and had shorter tibial leg segments, than adults reared on other host plants. Wing aspect ratios varied, with those of ACP reared on *Murraya paniculata* narrower than those of ACP reared on other hosts.

Host Orientation, Assessment, and Acceptance. Among the most notable achievements over the past decade with respect to vector–host interactions were characterizing ACP behavior and ACP–plant interactions and ecology (***Recommendation L-3: Support analysis of ACP behavior, ACP–plant interactions, and ecology to enhance the knowledge base available for new ACP management strategies***).

CRDF supported, in part or *in toto*, research that identified a diversity of signaling modalities associated with host finding and host assessment by ACP. Chemical cues that influence ACP orientation to citrus were investigated by George et al. (2016) and Lapointe et al. (2016), who described chemical blends eliciting probing. In terms of responses to visual signals, in both the field and laboratory, attraction to visual (color) cues varied with time of day, peaking in the afternoon; in the laboratory, ultraviolet (390 nm), green (525 nm), and yellow (590 nm) light elicited the strongest phototactic responses (Paris et al., 2015). The pronounced preference for yellow exhibited by ACP is consistent with orientation behavior of many other phytophagous insects, including other psyllids, and may relate to host leaf reflectance. Flush leaves from HLB-infected plants have higher reflectance in the green and yellow regions of the spectrum than do flush leaves from uninfected plants, which may facilitate orientation to diseased plants by ACP. To some extent, responses to visual stimuli, like responses to olfactory stimuli, are influenced by sex and experience of adults (Stockton et al., 2016).

CRDF-funded research also revealed that multiple forms of sensory stimuli associated with mate finding and reproduction influence host feeding. Documentation of the details of acoustic communication, particularly vibrational duetting, inspired new approaches to ACP trapping involving vibrational signals (Mankin et al., 2015), although they have not yet been field tested. In terms of olfactory stimuli associated with host finding, chemical signals mediating courtship and mating were found to originate in both the insect, in the form of cuticular hydrocarbon constituents, and the host plant. With respect to gustatory stimuli, the identification of phagostimulants that elicit probing behavior provide a foundation for developing attract-and-kill products, which have not yet been optimized for field use (George and Lapointe, 2017). Another important outcome of a range of studies was evidence of effects of experience on many behaviors involved in both mate and host finding and assessment. The finding that ACP nymphs acquire CLas quickly after probing (Ramsey et al., 2015; Pelz-Stelinski and

Killiny, 2016) has contributed to appreciation of this life stage as a significant factor in disease epidemiology.

Timing of Attack. Citrus leaves are particularly attractive to ACP during times of flush. Using protein-marked adults, Lewis-Rosenblum et al. (2015) identified leaf flush abundance and availability as primary factors driving dispersal and demonstrated that adult ACP can move 2,000 m or more in under 2 weeks to find new leaf flushes. Movement of marked individuals captured into a central test area of sweet orange trees increased in proportion to the density of emerging young leaves on the trees. The results confirm the threat presented by abandoned citrus groves in Florida by virtue of their status as reservoirs for ACP, which can disperse across long distances despite geographical barriers when leaves flush.

ACP nymphs settle and feed preferentially on the abaxial (lower) side of young leaves on the sides of the midrib, whereas adults settle and feed on adaxial (upper) and abaxial surfaces of either young or old leaves (Ammar et al., 2013). The feeding preference of nymphs is consistent with constraints imposed by morphology; phloem tissue located in young leaves and on the sides, rather than the center, of the midrib is a shorter distance from the leaf surface and thus more easily reached with short mouthparts. As for adults, phloem tissue in mature leaves is surrounded by a thick fibrous ring of sclerenchyma. ACP feeding behavior on young and mature orange leaves, investigated using electrical penetration graph technology (George et al., 2017), showed that, while adults feed on both young and mature leaves the duration of phloem ingestion is shorter on mature leaves; this pattern is consistent with the role of the sclerenchymatous ring as a barrier to feeding in ACP and provides a potential explanation for the vulnerability of young leaves to ACP attack.

In support of the 2010 near-term ***Recommendation NI-7: Support research aimed at developing alternative ACP management strategies***, effects of abiotic factors such as temperature, humidity, wind speed, and barometric pressure on flight and dispersal behavior were documented, in some cases by novel marking methods; these findings have potential applicability for increasing the efficiency of management strategies. Relevant to ACP–host interactions was the finding that windbreaks are significant factors in ACP host finding; fewer psyllids infest edges of groves having windbreaks compared with groves lacking them. As well, replanting patterns influence host finding and infestation rates; ACP were more abundant in groves with solid set replanting compared with resets (trees planted within mature groves to replace dead or infected trees) (Martini et al., 2015).

Pitfalls

Unfortunately, virtually all new knowledge gained over the past decade about ACP interactions with citrus hosts has served to underscore the biological recalcitrance of the system to management. Psyllid treatments began in Florida in 2008. Early on the psyllid was not considered a major risk to citrus, but now, a decade later, essentially all citrus groves are infected, and most psyllids carry CLas (Coy and Stelinski, 2015; Stelinski, 2017). The variability of the behavior and ecology of ACP has slowed progress in developing effective and reliable management techniques. Unlike the case for many other pest insects, vibrational, volatile, and phagostimulatory cues influencing ACP courtship and mating are effective over only short distances and thus unlikely to be useful for trapping or monitoring. Moreover, although hundreds of compounds have been screened for ACP attractant or repellent activity, responses to chemical signals, in the context of both mate and host assessment, appear to be labile and subject to influence by prior experience and possibly learning. Similarly, with respect to host finding, although ACP responds to visual signals, including color, responses vary with the developmental state of the host. Any signal modality that elicits responses in laboratory conditions tends to be overwhelmed under conditions of leaf flushes of new growth and, at least in central Florida, flushing occurs continuously through the growing season. Moreover, the health status of the host tree affects attraction, with volatiles produced by infected trees altering relative ACP preferences and possibly accelerating pathogen spread (Mann et al., 2012). Finally, flight activity is influenced by temperature, barometric pressure, and light, even over the course of a single day, introducing variability into the reliability and reproducibility of monitoring by trapping and constraining the development of predictive models. The extreme variability of ACP behavior relative to its interactions with host trees is largely responsible for the fact that, to date, no reliable correlation has been found between trap capture and absolute densities of ACP.

In some ways, the interactions between ACP and CLas thwart conventional approaches to management. As described elsewhere in this report, defining host resistance to HLB is operationally difficult because a reduction in psyllid number is not necessarily reflected by a decrease in disease incidence. Nymphs can acquire the bacterium quickly and, due to their mobility and feeding behavior, they are particularly difficult to control. In Florida, disease spread has outstripped the pace of research aimed at managing psyllids; reducing psyllid numbers by any available means is best considered a stopgap, delaying the spread and reducing multiple infections until the disease can be cured or until disease-resistant citrus trees become commercially viable.

Another pitfall in efforts to combat HLB, not explicitly considered in the 2010 NRC report, is human behavior. Groves abandoned for economic reasons support psyllid populations that can reinfest managed groves and circumvent control efforts. Also not directly addressed in the 2010 NRC report was the willingness of consumers to embrace the release of insects altered by new gene editing or modification technologies into the environment, a concept that falls under ***Recommendation L-4: Explore possible control strategies based on release of modified psyllid males***. Even under conditions in which public benefits of such releases are more far reaching and immediate, as in the case of vectors of human pathogens (Ernst et al., 2015), public resistance has been encountered.

Future Directions

Long-term solutions for HLB are likely to be in the areas of citrus variety improvement resulting from new technology. Thus, ***Recommendation L-1: Support the development of transgenic HLB-resistant and ACP-resistant citrus*** from the 2010 report remains a goal. Although genetic traits for ACP resistance in *Citrus* and related genera have been identified, neither traditional plant breeding nor cutting-edge techniques for citrus germplasm improvement have advanced to the point of practicality.

Goals for future research should include significant improvements in genetic transformation, clustered regularly interspaced short palindromic repeats (CRISPR) technology, and transient expression breeding in citrus. The sequencing of the ACP genome has provided new targets for manipulation, including candidate genes associated with host finding (e.g., chemoreceptors for volatile and gustatory stimuli) or feeding efficiency (e.g., salivary sheath formation). The olfactory receptor co-receptor (Orco) gene in other insects has been a target for CRISPR-associated protein-9 nuclease (CRISPR-Cas9) editing (Sun et al., 2017). As well, the availability of the ACP genome will facilitate the exploitation of the CYPome (the genomic inventory of detoxification genes in the cytochrome P450 monooxygenase superfamily) to interfere with growth and development, targeting genes encoding enzymes that detoxify both phytochemical and pesticide toxins. Tiwari et al. (2010, 2011a,b,c,d) documented stage-specific differences in activity in both cytochrome P450 monooxygenases and glutathione-S-transferases; transcriptomic analysis across developmental stages could aid in identifying suites of genes associated with xenobiotic metabolism,[10] and characterizing the specific chemical substrates for these differentially expressed genes could provide new targets not only for counteracting insecticide resistance but also for interfering with host use efficiency by

[10] A set of metabolic pathways that modify the chemical structure of xenobiotics, which are compounds foreign to an organism's normal biochemistry, such as any drug or poison.

developing inhibitors to block metabolism of host plant phytochemicals. Other future research directions are in the realms of sociology and economics, with the goal of identifying obstacles to grower participation in area-wide management efforts and public acceptance of management tactics. These sociological and economic considerations are addressed in more depth in Chapter 3.

Pathogen–Host Interactions

Notable Research Outcomes

Identification of Pathogenesis Factors Involved in Pathogen–Host Interactions. The NRC (2010) recommended ***NI-6: Exploit the CLas genome sequence for new strategies of HLB mitigation.*** Relevant projects funded by CRDF and other funding agencies have significantly advanced the basic understanding of pathogen–host interactions in HLB. Systematic and comparative analyses among genomes of CLas and other Liberibacter spp. strains resulted in the identification of a number of pathogenicity-implicated Liberibacter effectors derived mainly from type I and general secretory systems (T1SS and Sec, respectively). Using bioinformatics tools and seven well-annotated Liberibacter genome sequences, varying numbers (133 to 214) of proteins in those genomes were predicted to contain Sec-dependent signal peptides (Prasad et al., 2016). Of a total of 166 such predicted proteins from one CLas strain (Las-psy62), 86 were experimentally confirmed, using the *Escherichia coli*-based PhoA assay to measure acid phosphatase activity, to be secreted into the extracytoplasmic milieu. Due to the conserved nature of the Sec system among microbes, it is likely that those effectors are also translocated into CLas extracytoplasmic spaces and interact with host components and biological processes (Prasad et al., 2016). Pitino et al. (2016) examined 16 putative CLas effector genes for their subcellular localization and host cellular responses in the nonhost plant *Nicotiana benthamiana* through ectopic expression. These effectors exhibited diverse subcellular location patterns when fused with green fluorescent protein reporter genes under the strong promoter CaMV 35S. One of them, Las5315mp (mature protein), induced hypersensitivity (rapid cell death at the infection site) and associated plant defense responses (Pitino et al., 2016). J. Li et al. (2017) have identified a CLas effector that functions as a salicylic acid (SA) hydroxylase. The encoded protein (SahA) enzymatically degrades SA and its derivatives. Ectopic expression of SahA in tobacco plants abolishes SA accumulation and consequently interferes with hypersensitive response caused by nonhost pathogens. CLas infection enhances citrus susceptibility to citrus canker and counteracts disease resistance induced by exogenous application of SA, a result attributable to SA degradation by SahA. When SA analogs BTH and INA were used to treat HLB, significant reduction of disease severity and pathogen growth were observed, suggesting the possibility of using SA analogs to control HLB (J. Li et al., 2017). An effector protein (SC2-gp095) encoded from a CLas prophage, shown to be a reactive oxygen species (ROS) scavenging peroxidase, may be implicated in scavenging host-generated ROS and suppressing ROS signaling events during CLas infection (Jain et al., 2015). Through computational analyses of the CLas proteome, Cong et al. (2012) curated all CLas proteins having predicted subcellular localization, structure, and function.[11] These effectors represent potential targets for disease mitigation.

Host Genes and Gene Activation Involved in Pathogen–Host Interactions. Significant breakthroughs have been made in identification and characterization of host genes and signaling pathways activated in response to CLas infection. Several groups funded by CRDF used transcriptomic, proteomic, and metabolomic approaches to characterize host responses to HLB and the molecular basis of citrus–CLas interactions. Transcriptomic research on infected versus healthy citrus fruits and leaves revealed significant differences in gene expression, mainly reflecting suppression of immunity (e.g., systemic acquired responses) and metabolic dysfunction (sugar biosynthesis, transportation, and metabolism) attributable to source-sink disruption (Kim et al., 2009; Martinelli et al., 2013). Similarly, mRNA profiling from healthy and CLas-infected fruit revealed significant changes in transcription, particularly in biological processes, such as the light reactions of photosynthesis and in adenesine triphosphate synthesis, protein degradation and misfolding, source-sink pathways, and phytohormone-mediated pathways (Martinelli et al., 2012). When citrus roots and stems of infected versus healthy citrus plants were analyzed for changes in gene expression, the numbers of genes affected were greater in stems than in roots. The affected genes were found to be involved in cellular functions, such as sugar metabolism, cell wall biogenesis, stress responses, signaling, and protein modification and degradation (Koh et al., 2012; Aritua et al., 2013). Transcription profile comparisons showed more genes expressed differentially in tolerant host plants than in susceptible ones during CLas infection, likely reflecting a molecular basis of host sensitivity and basal resistance to HLB (Albrecht and Bowman, 2012; Fan et al., 2012; Nwugo et al., 2013; Wang et al., 2016). Using meta-analysis and gene co-expression network modeling on 22 transcriptome datasets, Rawat et al. (2015) identified the 65 most common probe sets regulated by CLas infection; these represent transcriptional modulations by CLas in sugar metabolism, nutrient transportation, and stress responses. The identification of resistance-specific probe sets that

[11] See http://prodata.swmed.edu/citrusgreening/index.html. Accessed June 8, 2018.

represent leucine-rich repeat proteins, chitinase, miraculins, and constitutive disease resistance (CDR) elements are of significance (Rawat et al., 2015). The identification and molecular characterization of CDR genes from *Citrus* and its close relative, HLB-tolerant *Poncirus trifoliata*, may provide a useful resource for producing HLB-tolerant citrus (Rawat et al., 2017). Finally, proteomic analysis of CLas-infected leaves from susceptible and tolerant citrus revealed that four glutathione-S-transferases were up-regulated in the tolerant cultivar *Volkameriana* but not in the susceptible navel orange (Martinelli et al., 2016). These findings may yield prospects to boost host defense mechanisms or alter host susceptibility for mitigation of HLB.

Pitfalls

Despite considerable research effort, much remains to be learned about the mechanics of CLas–citrus interactions at the molecular level. Genetic and functional analyses of pathogen metabolic and effector genes, and their *in vivo* roles in the biology and pathogenesis of CLas and its close relatives, have been hampered by the inability to culture HLB-causing Liberibacters (***Recommendation NI-10: Develop* in vitro *culture techniques for CLas to facilitate experimental manipulation of the bacterium for insights into gene function***). Understanding of *in planta* interactions has also been limited by the need for effective tools for genetic manipulation of host genes relevant to HLB, another important aspect that requires citrus genetic engineering technologies, which remain elusive or very limited.

Future Directions

Ongoing research, including studies funded by CRDF, the NIFA SCRI Citrus Disease Research and Extension (CDRE) program, and the Citrus Research Board, characterizing the components of pathogen–host interactions continues to yield advances in the areas of functional characterization of bacterial effectors, identification and functional analysis of their host targets, and exploitation of molecular host–pathogen interaction for novel strategies to manage HLB. Identification and utilization of resistance (R) genes from within or beyond citrus that counteract bacterial effectors should be a priority of future research. Furthermore, identification of critical genes, proteins, or metabolites in hosts that are targets of Liberibacter spp. and more comprehensive characterization of their interactions will provide keys to interfering with the disease processes, resulting in both genetic and chemical means to control the disease.

Pathogen–Vector Interactions

Notable Research Outcomes

Considerable new information regarding ACP–CLas interactions has been generated since the release of the 2010 NRC report. Investigators have followed to some extent the 2010 NRC ***Recommendation NI-1c: Investigate the behavioral ecology and CLas transmission biology of ACP to improve timing and other aspects of insecticide application***, and ***Recommendation NI-7: Support research aimed at developing alternative ACP management strategies.***

CLas Transmission Parameters. While the general characteristics of CLas transmission by ACP, e.g., the duration of acquisition, latent, retention, and inoculation periods, have long been known (da Graça, 1991), the 2010 NRC report listed several deficiencies in an understanding of ACP–CLas interaction that would affect transmission and HLB epidemiology. These included the role of ACP nymphs in CLas transmission and HLB epidemiology, effects of CLas acquisition and infection on ACP biology and fitness, and the potential for vertical transmission of CLas through ACP. CRDF funded several projects to investigate several of these aspects of CLas transmission by ACP.

While early reports suggested that ACP nymphs were not important in the transmission of CLas (da Graça, 1991), more recent studies have shown that early instar nymphs acquire CLas more efficiently than do adults (Pelz-Stelinski et al., 2010; Grafton-Cardwell et al., 2013; Pelz-Stelinski, 2014; Wu et al., 2016; Canale et al., 2017). Furthermore, transmission efficiency from nymphs reared on CLas-infected plants is higher than that from adults, although long feeding times by adults can negate these transmission efficiency differences (Pelz-Stelinski et al., 2010). Initial studies suggested that CLas does not replicate in the ACP (Pelz-Stelinski et al., 2010), but more recent studies have shown that CLas titer increases over time (Ammar et al., 2016), suggesting that CLas replicates in the insect and is a pathogen of both plant and insect. Efficient CLas replication may occur in nymphs but not in adults (Inoue et al., 2009), possibly due to the fact that more ACP proteins involved in immunity to bacteria were differentially expressed in adults than in nymphs (Ramsey et al., 2017). Several CLas genes are differentially expressed depending on whether the bacteria are associated with plants or with ACP (Yan et al., 2013). While the bacteria may not replicate efficiently in all life stages of psyllids, it is clear they survive for long periods in the insect and can be vertically transmitted, albeit at a low frequency (Mann et al., 2011; Grafton-Cardwell et al., 2013). The efficiency of acquisition of the CLas pathogen by ACP is influenced by plant age and maturity (Luo et al., 2015; Hall et al., 2016), and pathogen acquisition can have profound effects on ACP behavior and life parameters, including dispersal, reproduction, and gene expression (Martini et al., 2015; Ramsey et al., 2015; Pelz-Stelinski and Killiny, 2016).

As documented in many other studies on pathogen transmission by insects, all of the measured parameters can vary widely depending on pathogen and insect populations (Coy and Stelinski, 2015), pathogen source materials, experimental design and methods, and environment. Nonetheless, the new information on CLas transmission by ACP is being used to develop and modify HLB management recommendations (Ukuda-Hosokawa et al., 2015; Udell et al., 2017).

Disrupting CLas Transmission. While not specifically detailed in any of the 2010 NRC report recommendations, several of the recommendations mentioned various aspects of replication and pathogenicity of the bacteria in the insect, as well as the molecular and cellular mechanisms that allow the bacteria to move and infect different tissues in the ACP. More information on these topics would provide the basis for interdiction strategies to prevent ACP from becoming a vector. There has been progress in characterizing CLas movement in the ACP (Ammar et al., 2011a,b, 2016) and in identifying molecules in the ACP that are involved in CLas infection and transmission and examining the potential methods to disrupt these interactions (Yan et al., 2013; Hoffmann et al., 2014; Killiny et al., 2014b; Pelz-Stelinski, 2014; Kruse et al., 2015, 2017; Ramsey et al., 2015, 2017; Ghanim et al., 2016; Arp et al., 2017; Gill et al., 2017). A key factor in this work has been research efforts toward the 2010 NRC ***Recommendation NI-11: Sequence, assemble and annotate the ACP genome to provide a basis for new approaches to ACP management.*** Initial seed money from CRDF, USDA Agricultural Research Service (ARS) and the Los Alamos National Laboratory provided preliminary ACP sequence data and led to a USDA NIFA-funded project to develop comprehensive bioinformatics resources for ACP (NIFA, 2018) as well as interdiction molecules targeting various ACP–CLas interactions and interfering with either CLas infection in ACP or CLas transmission by ACP. A key component of this work is the ability to test these molecules for biological activity either through the development of transgenic citrus expressing them, the use of CTV vectors to transiently express them (El-Mohtar and Dawson, 2014; Hajeri et al., 2014; Dawson et al., 2015), or the use of RNAi to directly target genes in the ACP (El-Shesheny et al., 2013; Hajeri et al., 2014; Andrade and Hunter, 2017; Galdeano et al., 2017). Several laboratories are identifying genes, proteins, and metabolites that are being tested for activities. Intellectual property concerns have kept many specific details out of this review. The expression and quantification of compounds, either in transgenic plants or transiently using the CTV vector, are in progress (El-Mohtar and Dawson, 2014; Hajeri et al., 2014; Dawson et al., 2015).

The 5-year $9 million NuPsyllid project formulated by CRDF and funded by USDA NIFA concluded in 2017. The very ambitious overall goal of the project, to develop a modified psyllid that would not transmit CLas and could be deployed in the field to displace the natural ACP population, was not achieved, but the project did contribute significant advances to the fundamental knowledge of ACP–CLas biology (Soderlund et al., 2014; Cicero et al., 2015; Ding et al., 2015, 2016, 2017; Yuan et al., 2015, 2016; Brown et al., 2016; Stover et al., 2016; Liu et al., 2017).

Pitfalls

A major concern in this research area is the apparent lack of communication among members of the research community investigating how to control ACP and those investigating how to reduce transmission of the pathogen. There is a disconnect between insect control and vector control with respect to management of HLB in the field. Insect control efforts should be integrated with studies on how those strategies will impact CLas transmission among hosts, within and between citrus groves. Reduction in ACP populations alone may not significantly slow the spread of the pathogen. Similarly, studies on host response to CLas infection as well as the progress and virulence of the infection are not always linked to studies on pathogen transmission. Furthermore, while there are active and productive collaborative groups working on CLas–ACP interactions, the groups themselves would benefit from frequent discussions and sharing of information to minimize redundancy and facilitate progress.

The ongoing studies focused on CLas–ACP interactions would be facilitated by an expansion of efforts to generate high-quality genomic data for the pathogen and insect vector, to characterize more extensively, and to inform research on host responses and better understand the genomic diversity of CLas (including bacteriophage genes) and ACP (including mitochondrial and endosymbiont genes). A NIFA-funded project has developed extensive ACP genomic data and analysis tools (NIFA, 2018), but it is unclear how these resources are being used by the greater HLB research community. It is also unclear how these resources will be supported beyond the life of the grant. Furthermore, intellectual property concerns hinder the sharing of some of the research results in this area.

Future Directions

That transmission is influenced by CLas titer and distribution in trees, as well as by differential host responses to infection that influence vector attraction, feeding, and dispersal, are all fruitful areas of research that can lead to predicting with greater accuracy how types and levels of host resistance or tolerance are likely to influence the epidemiology of HLB.

There is considerable effort to discover and develop molecules that interfere with some aspect of the CLas life cycle and lead to a reduction in CLas titer or distribution in the plant or insect host. Molecules are also being examined for their ability to reduce CLas transmission by ACP. Ultimately these molecules, along with durable host resistance, will be the bases for sustainable interdiction strategies that can slow or prevent the expansion of HLB. Promising preliminary results are still scientifically far from being translated to effective field management tools, a process that will necessitate regulatory and intellectual property considerations. With this in mind, the industry may want to consider how these hurdles specifically influence the selection of molecules for development. How each molecule would be classified by the regulatory agencies will affect what types of data will be needed to satisfy permitting decisions, which in turn may affect how the industry and funding agencies prioritize project proposals aimed at short- to mid-term field management tools. An overarching panel (see Recommendation 4.6 in the section Overarching Findings, Conclusions, and Recommendations for Future Research Efforts in HLB Management in this chapter) reviewing all research and extension proposals would help facilitate this decision making, especially if members had a presence on the advisory boards of all of the large multidisciplinary and multi-institutional projects. While intellectual property concerns must be acknowledged and respected, they are slowing progress and hampering data sharing. A respected review committee would be aware of the total research activities supported by all funding agencies and familiar with information and data that, if shared, would facilitate other research activities. With the appropriate confidentiality agreements in place, information could be shared, and management opportunities translated to the industry more quickly.

HLB MANAGEMENT

Cultural Control

Notable Research Outcomes

Research over the past decade on HLB management using cultural approaches has addressed the 2010 ***Recommendation NI-8: Support small-scale studies on the feasibility of alternative horticultural systems suited to endemic HLB***. Relevant CRDF-funded projects have investigated strategies that could reduce the immigration or inoculating efficiency of ACP, retard disease development in inoculated trees, and improve tree health and productivity.

ACP Immigration and Inoculation Efficiency. Several projects evaluated cultural strategies for reducing or eliminating ACP populations in citrus groves. Full-coverage screen houses prevented ACP access to grapefruit trees, while environmental parameters (such as air temperature, cumulative rainfall, and solar radiation) and tree development factors (including canopy surface area, water use efficiency, and leaf area index) remained suitable for grapefruit production (Ferrarezi et al., 2017a,b). The possibility that ACP could be diverted from citrus trees by interplanting them with alternate ACP hosts known to be highly attractive to the psyllid was investigated in field studies. Modest reductions in ACP numbers on citrus were noted after the first year of interplanting with orange jasmine (*Murraya exotica* L.) but did not continue into the second year (Hall et al., 2013a), and although intercropping citrus with pink guava (*Psidium guajava* L.) resulted in lower ACP numbers it did not prevent the introduction and spread of HLB (Gottwald et al., 2014). On the other hand, the use of metalized polyethylene mulch in citrus groves repelled ACP, resulting in significant reductions in both ACP populations and the incidence of HLB (Croxton and Stansly, 2014).

Data from microsimulation modeling led Lee et al. (2015) to conclude that entire citrus groves can become infested with up to 12,000 ACP per tree in under a year, prior to the appearance of HLB symptoms. However, the model showed that disease could be delayed significantly by applying control measures that reduce ACP numbers by 75% during leaf flush. Reduction of local inoculum by removal of HLB-infected trees has been studied primarily in Brazil, where experimental data and epidemiological modeling results confirm that immigration of inoculative ACP from source trees is a significant factor in epidemics and suggest that area-wide inoculum reduction strongly affects HLB control (Belasque et al., 2010; Bassanezi et al., 2013a,b; Bergamin Filho et al., 2016). However, these results have not led to any widely adopted management recommendations in Florida, due primarily to the high levels of CLas-infected trees throughout all production areas and the constant movement of CLas-infected ACP into and within citrus production areas (Gottwald, 2010; Gottwald et al., 2012, 2014; Hall et al., 2013b; Lee et al., 2015).

Slow Disease Development in Inoculated Trees. A number of cultural management approaches target CLas within plant phloem, either reducing existing titers or impacting bacterial reproductive rates.

Thermotherapy. The development of citrus thermotherapy to reduce or eliminate CLas in infected citrus trees has yielded beneficial results in greenhouse applications and some limited field tests in which trees have been enclosed for treatment (Ehsani et al., 2013; Hoffman et al., 2013; Fan et al., 2016; Pelz-Stelinski, 2016). In growth chamber experiments, Hoffman and coworkers (2013) measured lower CLas titers in 3-year-old citrus treated at 40–42°C for 48 hours than in control trees, while tree growth was unimpaired. Similar to the findings of Hoffman et al. (2013), but funded by other sources, Brazilian scientists Gasparoto and colleagues (2012) found that young citrus trees grown at 27/32°C did not become infected after graft inoculation in the greenhouse, in contrast to those maintained at lower temperature regimes. However, results are more variable and less beneficial when trees are more mature or are field grown or when solarization is used as the heat source, and the effect of thermotherapy on the acquisition efficiency of ACP feeding on treated trees is mixed (Yang et al., 2016; Doud et al., 2017; Pelz-Stelinski, 2017). Pelz-Stelinski (2017) reported mixed results depending on the experimental setup. Limited field experiments (Ehsani et al., 2013), in which single mature trees were enclosed in clear plastic tents, showed that trees heat treated in midsummer (but not in late summer) had yields and Brix content indistinguishable from those of untreated controls. The success of thermotherapy in the field may be hampered by natural weather conditions, such as cloudy or rainy days lowering the temperature or solar radiation resulting in much higher temperatures near the upper sections of the plastic enclosures (Ehsani et al., 2013).

Improve Tree Health and Productivity. There is increasing interest in supporting and extending tree health in the presence of HLB infection through advanced production systems that supplement water, macronutrients, and related materials both to foliage and to soil (Campos-Herrera et al., 2013, 2014; Kadyampakeni et al., 2014, 2016; Ferrarezi et al., 2017a,b). Potential benefits for growers include extending the productive lifespan of diseased trees and shortening the time required for new trees to become economically productive.

Advanced citrus production systems manage tree health in a “fertigation” approach by constraining root growth into small clumps that can be supplied directly and daily, through drip or microsprinkler irrigation, with predefined water and nutrient supplements (Kadyampakeni et al., 2014); such intensive fertigation resulted in higher nitrogen accumulation in plants as well as increases in growth rates (as much as 330%) and canopy area (Kadyampakeni et al., 2016). The same principle is being applied by some growers using intensified foliar-applied nutritional supplements (Ingram, 2017). High-density planting in new or replanted groves is also becoming more popular in Florida (Black, 2017; Ingram, 2017); growers anecdotally report that when this practice is performed in combination with intensified nutrient and water supplementation trees apparently do not suffer from competition, and the result can be greater per-acre productivity and greater economic gain.

While the successes of the projects are often difficult to measure in terms of contribution to slowing disease progress or improving fruit yield and quality, many of the outcomes from these studies are contributing to the apparent grassroots efforts in tree health management and intensive orchard management in Florida (Campos-Herrera et al., 2014).

Pitfalls

Although thermotherapy has shown promise in reducing or eliminating CLas populations within infected trees (Ehsani et al., 2013; Hoffman et al., 2013) and could be useful in greenhouse operations or for valuable individual trees, the requirement that plants be enclosed for treatment presents challenges for achieving positive cost–benefit ratios. Further economic analyses are needed to determine whether investment in additional research is likely to lead to effective and affordable disease management.

Intense efforts to nurture infected trees for extended productivity have led to mixed results and may have some unintended consequences. After 2 years of treatment, Gottwald et al. (2012) found no difference in bacterial titer between a number of different enhanced nutritional programs and control treatments. That result, combined with other findings from the same study, led to concern that such approaches, in the absence of other measures, may allow for inoculum buildup and spread.

In contrast, Shen et al. (2013) (with funding from the Emerging Pathogens Institute at the University of Florida and the Smallwood Foundation) found higher population levels of CLas after supplemental nutrient treatments compared to those without. The Ct (cycle threshold) value of CLas was positively associated with leaf contents of several elements, although no significant association was observed with leaf contents of nitrogen, phosphorous, and potassium (N, P, and K). Furthermore, the richness index[12] of endophytic α-proteobacteria was significantly greater for leaves that had received the nutrient treatment than the insecticide treatment. In particular, calcium and manganese (Ca and Mn) content and nutrient management were important environmental variables controlling the endophytic α-proteobacteria community structure (Shen et al., 2013). Some growers feel that the use of reflective mulches has an added benefit of stimulating tree development along with reducing numbers of alighting ACP (WBUR, 2017). Unfortunately, scant data have been generated on the utility of these nutritional approaches, and some reports are based on relatively short-term studies that would not reveal longer-term benefits. It will be important to continue to monitor, and in fact to design new and comprehensive field testing approaches to evaluate, the efficacy of these tree health practices so as to provide growers with robust research-based data with which to make sound management decisions.

Although most of the recent research on the citrus microbiome has focused on possible impacts on maintaining the health and productivity of infected trees, there is preliminary evidence that applications of beneficial

[12] An index based on the number of species per specified number of individuals, and the number of species per unit area (species density).

bacteria may also lead to lower CLas titers (Wang and Pelz-Stelinski, 2017). While this observation advances understanding of the system, the microbiome communities of citrus are so complex and variable that other CLas-directed management strategies are likely to be simpler and more effective.

It will likely be several years before the benefits of different therapies on trees can be assessed, and these will all need to be validated on a range of tree ages relevant to commercial production.

Future Directions

Elimination of abandoned groves currently serving as refugia seems to be absolutely essential to managing HLB. Research could explore simple and inexpensive ways to "neutralize" abandoned groves that can be accomplished with minimal landowner involvement. In some ways the HLB situation resembles the challenge of eradicating vector-borne human diseases, such as yellow fever from Havana and Panama—efforts to stop the disease were unsuccessful until breeding sites for the mosquitoes were eliminated.

While the intensive management of trees to reduce stress appears to improve overall tree health and productivity it is not clear if the pathogen populations are negatively affected or if the improved tree health will have any effects on reducing pathogen acquisition or transmission. If the infected trees remain as reservoirs of CLas for ACP, then the benefit of improved health and fruit production will need to be evaluated against the longer-term effects of the tree as an inoculum source for future dissemination of the pathogen. It is also not clear if fruit quality is correlated with improved tree health and yield. Commercial production groves that are practicing various components of enhanced citrus management are becoming potential laboratories for replicated trials that could provide the data needed to tease out the individual contributions of these new cultural management programs. While conducting research in commercial settings can be challenging, it may be possible to rent large blocks and adequately compensate growers to ensure their full cooperation in conducting stringently designed experiments.

The 2010 NRC report identified efforts in advanced production systems and recommended new cultural practices be evaluated for their effectiveness against HLB epidemics. While there are many examples mentioned above that have addressed alternative or enhanced management strategies, there is no evidence of a concerted and coordinated effort to consolidate information so the contributions of the individual and combined components can be experimentally evaluated and validated. This was a concern mentioned in the 2010 NRC report. Rather than the science providing impetus and supporting data to drive changes in cultural practices, the grower community through grassroots efforts has begun to experiment with and adopt a diversity of measures to enhance tree health and fruit production.

ACP Control

Notable Research Outcomes

The status of chemical and biological control in the Florida citrus industry was quite different in 2017 than it was at the time of the 2010 NRC report. From 2000 to 2009 scientists and growers worked to document integrated control strategies that could suppress the vector, reduce the numbers of infested trees or at least slow the spread of the pathogen, and support the long-term goal of managing the damaging effects of the pathogen (NRC, 2010). In 2017 the industry was in decline (Farnsworth et al., 2014), with packing sheds closing (Browning, 2017) and the 57,000+ hectares of abandoned orchards providing overwhelming amounts of inoculum and infected psyllids (Lewis-Rosenblum et al., 2015). Growers who spoke at the committee webinar held on November 20, 2017 (see Appendix B for agenda and speakers) expressed the belief that vector control is no longer useful since every tree in the state is considered infected with the *Ca.* Liberibacter pathogen and virtually every psyllid is assumed to carry the pathogen. However, continued psyllid control is still needed, and removal of abandoned trees is still of value since multiple or repeated inoculations result in faster spread within large trees and subsequently greater losses (Pelz-Stelinski et al., 2010). Thus, controlling the vector can provide benefits even for an infected tree. At the same webinar the committee heard that not all growers were interested in integrated control strategies because these programs did not always effectively manage HLB or provide a level of control that allowed citrus to continue as an economically viable industry.

Chemical Control. Recommendations in the 2010 NRC report relevant to chemical and biological control of ACP include ***Recommendation N1-1: Improve insecticide-based management of Asian citrus psyllid***, and ***Recommendation NI-7: Support research aimed at developing alternative ACP management strategies.*** Researchers have investigated which insecticides could provide control and if biological control agents could be used effectively to improve vector suppression. These goals have been largely met, at least with respect to performing and completing funded research.

Chemical control strategies for pests in most agricultural crops rely on sampling to determine if populations have reached an economic threshold[13] that justifies pesticide application. Considerable progress has been made to understand and optimize sampling and counting strategies that accu-

[13] The level of crop damage at which the lost value exceeds the cost of pest management.

rately reflect numbers of both nymphs and adults on trees so that pesticide applications are applied only when warranted by numbers, thereby reducing environmental impacts and likelihood for the development of insect resistance. Sampling plans for ACP and pesticide treatment thresholds have been developed for both young and mature trees, although these plans have not been widely adopted.

Nearly every pesticide available for insect control has been tested against psyllids in Florida (reviewed in Grafton-Cardwell et al., 2013; Qureshi et al., 2014; Boina and Blumquist, 2015), addressing ***Recommendation N1-1: Improve insecticide-based management of Asian citrus psyllid.*** Psyllid behavior, population size, and life stage at the time of acquisition influence pesticide effectiveness (Pelz-Stelinski et al., 2010). Psyllids infected with CLas are more susceptible to at least five commonly applied pesticides than those that are not infected (Tiwari et al., 2011d), possibly because esterase activity in infected psyllids is reduced (Tiwari et al., 2012). Not surprisingly, growers have focused on the most effective pesticides and applied them more frequently and at higher rates, actions that quickly led to resistance to organophosphates and carbamates (Tiwari et al., 2012), followed shortly thereafter by resistance to the most effective systemic pesticides, the neonicotinoids (Tiwari et al., 2011d). In response, researchers have designed and implemented a resistance assessment program to assist growers in documenting the effectiveness of key pesticides in their fields (Kanga et al., 2015). Most researchers have reported that timing of applications is critical for population reduction. One practice that has potential for organic farms in particular is to combine oils with insecticides, although this would not meet organic requirements if the pesticide is not organically certified. The only material reportedly being developed for commercial use is dimethyl disulfide, which has been incorporated into a slow-release matrix to repel adult psyllids (Mafra-Neto et al., 2013).

Researchers and growers alike are aware of the risk that psyllids can readily evolve resistance to pesticides that are applied repetitively. Pesticide resistance management plans have been developed and are being used in some area-wide management programs, such as Citrus Health Management Areas (CHMAs).

Biological Control. The benefits of indigenous predators and parasites of the psyllid have been evaluated and quantified, constituting progress on ***Recommendation NI-7: Support research aimed at developing alternative ACP management strategies.*** A wide variety of indigenous natural enemies attack ACP (Chong et al., 2010; Hall et al., 2012; Juan-Blasco et al., 2012). Their effectiveness has not been evaluated on the basis of individual species, but from cage experiments. Monzo et al. (2014) estimated that the collective suppression of immature psyllids in untreated orchards ranged from about 15% to 91%. These approaches may be most effective in abandoned orchards and in urban and suburban citrus because pesticides are applied intensively in active commercial groves. Considerable field research effort has been focused on the imported psyllid parasitoid *Tamarixia radiata* (Hymenoptera: Eulophidae) (Qureshi et al., 2009), which has been reared in large numbers and released throughout Florida. Again, the utility of this technique was limited by pesticide application in commercial groves, but it may have considerable value in reducing immigration from abandoned orchards and urban citrus. *T. radiata* is now established in at least 16 Florida counties, with levels of parasitism generally below 20% (Michaud, 2004; Chong et al., 2010), but as high as 56% in southwestern Florida in November (Qureshi et al., 2009). This parasitoid apparently spread through Florida, Texas, and Mexico (De Leon and Sétamou, 2010) and, in 2008, reached California (Gomes, 2008). A combination of host feeding and oviposition in the laboratory has been reported to kill about 50 nymphs per female (Skelley and Hoy, 2004), although Chien et al. (1993) stated that female *T. radiata* could kill between 16 and 245 nymphs, depending on the temperature. Entomopathogenic fungi have also been tested for psyllid management; most of the fungal isolates tested provided some suppression, but certain environmental conditions, such as low humidity and the application of fungicides for citrus pathogens, limit commercial control potential.

Pitfalls

Pesticides effective against the ACP have been a mainstay for HLB management programs in Florida. However, Hall et al. (2013a) suggest that the threshold for treatment is fewer than one per tree, so finding even a few psyllids results in a recommendation for treatment, and sampling strategies can vary in accuracy depending on environmental and host tree variables. Regardless of the sampling technique employed, psyllid reproduction in abandoned, untreated, or organic orchards can lead to their detection throughout the year in numbers that can trigger treatment (Tiwari et al., 2010; Lewis-Rosenblum et al., 2015). This has caused many growers to increase pesticide applications, which, in turn, has increased psyllid insecticide resistance levels, leading to further increases in pesticide use (Alverez et al., 2016). This "pesticide treadmill" has put growers in the economically difficult position of spending more money on pesticide application at a time when crop productivity (along with income) is declining due to HLB. Unfortunately, psyllid population suppression within active commercial groves requires nearly 100% control throughout the season even though the infection rate in psyllids averages about 70% (Coy and Stelinski, 2015). Missing even a few psyllids per tree can result in transmission of the pathogen. As a result, some growers either treat frequently, assume all plants are infected and do not spray, or spray on a schedule rather than as guided by insect surveillance.

Of the available insecticides, pyrethroids (synthetic compounds that are related to pyrethrins and have similar insecticidal properties), organophosphates, and neonicotinoids work reasonably well and can last several weeks (Qureshi et al., 2014; Boina and Blumquist, 2015). However, by the time psyllids are again detected feeding on the plants, transmission will have occurred and the action threshold will have been passed. So, although many of the pesticides initially tested offered excellent control, the short residual control provided by many of the insecticides, the occurrence of multiple flushes requiring additional pesticide treatments, the development of resistance by psyllids to the longer-lasting systemic insecticides, and the need for nearly 100% psyllid suppression in order to stop transmission of the HLB pathogen have led growers to apply pesticides more frequently and at maximum rates. Overapplication of pesticides not only increased costs but also led to additional resistance development. As a result, several CRDF projects were funded to examine how resistance could be managed within an IPM framework. Researchers implemented a resistance assessment program to assist growers in documenting the effectiveness of key pesticides. In a committee meeting on July 24, 2017 (see Appendix B for agenda), Stelinski (2017) stated that although some of the CHMA (UF IFAS, 2018) programs delayed resistance development in 2013–2014, resistance evolution began to accelerate again.

The implementation of additional IPM programs has resulted in partial success, particularly when used in large-scale area-wide programs (Grafton-Cardwell et al., 2013; Qureshi et al., 2014; Boina and Blumquist, 2015). However, these programs have not provided consistent results or reliable levels of HLB suppression across the Florida citrus industry. While the suppression of psyllid populations by both indigenous and imported biological control agents is undoubtedly useful in minimizing immigration into commercial orchards from urban trees (dooryard citrus) and abandoned groves, the level of control is not commercially adequate as a stand-alone practice. While useful, indigenous predators alone will not provide adequate suppression in commercial orchards. Given the extensive acreage of abandoned orchards in Florida and the large numbers of untreated trees in urban areas, this level of suppression will help to reduce the overall environmental load of inoculum and psyllids; but because the movement of psyllids from abandoned orchards to active commercial groves can be substantial (Lewis-Rosenblum et al., 2015), suppression will be inadequate unless additional control strategies are employed. Furthermore, use of biocontrol agents in active citrus orchards is severely limited by the frequent application of insecticides toxic to the beneficials. A persistent problem with biocontrol is that many pesticides effective against the ACP are also lethal to beneficial organisms. Much of the potential benefit of biocontrol agents in commercial citrus groves is likely lost because of application patterns that use multiple pesticides in rotation. This approach, while critical for preventing or slowing resistance development by the psyllid, essentially guarantees that insecticides toxic to the beneficials will be applied.

The parasitoid *T. radiata* attacks nymphs in the third to fifth instar (Skelley and Hoy, 2004), but trees can become infected by adult feeding well before any subsequent nymphs develop to an acceptable stage for parasitoid oviposition. As a result, the primary long-term benefit of psyllid suppression by *T. radiata* in commercial orchards would be to reduce the production of infected adults developing from nymphs within the orchard. While this outcome would clearly provide some benefit, a reduced adult population would not eliminate the loss of the infected trees or the eventual spread of the pathogen.

Future Directions

Despite the concern that most citrus trees may be infected by CLas, there is still a need for vector control using chemical, biological, and cultural control strategies. For example, while breeding for resistance or genetically engineering citrus may offer the best long-term strategy for control, even these programs require pathogen-free plants. The simplest strategy is to exclude the psyllid vectors (for example, in greenhouses) and chemically control any that escape the exclusion defenses. At this time, it is not known whether any resistant germplasm or genetic modification that may be found will result in 100% suppression of HLB or ACP. Therefore, as for many crops that have partial resistance, pesticide use may be required to prevent repeated inoculum transfer that can overwhelm the defenses (Prioul et al., 2008; Hariprasad and van Emden, 2010). Because the HLB pathogen spreads faster with repeated inoculations (Pelz-Stelinski et al., 2010), any strategy that reduces repeated inoculation will help slow the spread of the pathogen. Biological or other controls that can result in reduction in psyllid populations can help to reduce the overwhelming numbers of psyllids moving from abandoned orchards or urban environments to newly planted trees or to research facilities. In California, where HLB has not yet caused significant losses in commercial orchards, a reduction in inoculum (from infested psyllids and trees) could serve to slow the spread of the pathogen, allowing more time for scientists to explore other management strategies. The usefulness of most of the older pesticide chemistries has been well established. The focus should be on the newest chemistries—how to get them into or onto the trees and how they interact with changing cultural strategies, such as increased tree density.

Like nearly all members of the Hempitera, ACP possess a unique, highly developed microbial endosymbiotic system involving a highly specialized organ containing cells necessary for the endosymbiosis. The microorganisms have been hypothesized to compensate for the nutritional deficiency resulting from the imbalance of nutrients in plant phloem sap. Investigations on such systems can increase insights into the mechanisms allowing the psyllid to cope with infection of the CLas organism, the pathways mediated by endosymbiont associates resulting in vector competence, and ultimately the proteins and pathways that can be targeted for disruption of transmission.

There are many proposed treatment strategies, and determining which is best for a particular citrus grove is not a simple process. The distance from an abandoned orchard could easily change the effectiveness of a management approach. Most of the IPM research reported to date is accomplished by rotating chemical applications. Nonetheless, a number of strategies (such as reflective mulches, which work reasonably well with younger trees integrated with insecticide programs; Croxton and Stansly, 2014) have not been fully tested in conjunction with biocontrol agents or high-density plantings. Documenting which strategies can be combined and will provide cost-effective control (if any) will require cost–benefit analyses. Unfortunately, generating these analyses can be difficult (Farnsworth et al., 2014) and may be hampered by concerns about patent disclosure by researchers and by the need for chemical use strategies, yield data, and other information considered proprietary by the growers.

Bacterial Control

Notable Research Outcomes

There have been numerous research efforts over the past decade in response to the 2010 ***Recommendation L-2: Support development and testing of bactericides, therapeutics, or SAR [systemic acquired resistance] activators***. A number of research projects have identified useful enhancements for the use of, and application approaches for, chemical and antibiotic substances to control the pathogen.

Antibiotics and Chemotherapy. With increasing bacterial resistance to copper-containing formulations for bacterial diseases such as HLB, zinc (Zn) has been explored as an alternative. In a past CRDF-funded project a compound designated "TSOL," containing chelated Zn, was taken up into stems and roots of sour orange seedlings, and spraying grapefruit trees every 21 days with TSOL formulations reduced disease incidence by 34% (Santra et al., 2017). CRDF also funded the development of the Zn-based nanoparticle treatment, Zinkicide, which has undergone initial testing in the field. The product showed initial promise in field tests (Johnson, 2015), but the data have not been published. A project related to this formulation is now being supported through NIFA SCRI.

Sulfonamide antimicrobials (sulfadimethoxine and sulfathiazole) were highly effective in eliminating or suppressing CLas, with low infection rates and bacterial titers in scions and rootstocks (Zhang et al., 2014). Cell-wall inhibitors in the beta-lactam group (ampicillin, carbenicillin, penicillin, and cephalexin) were highly effective using a graft-based greenhouse evaluation assay on rootstocks, as was the transcriptional inhibitor rifampicin (Zhang et al., 2014). Effectiveness of sulfonamide antibiotics was increased when combined with thermotherapy (Yang et al., 2016).

Penicillin was more effective in controlling the CLas bacterium and decreasing disease severity than the antimicrobials EBI-602, silver nanoparticles, or carvacrol (Powell et al., 2016). Three of the beta-lactam antibiotics eliminated detectable CLas from infected scions, prevented HLB symptoms, and prevented transmission to the rootstocks (Zhang et al., 2014). Less effective were peptide antibiotics (vancomycin and colistinmethanesulfonate), the trehalase inhibitor validoxylamine, the cell wall inhibitor cycloserine, the quinolone ciprofloxacin, and the translation inhibitor chloramphenicol. Aminoglycoside antibiotics had either moderate effectiveness (zhongshengmycin, hygromycin B, spectinomycin, and streptomycin) or no effect (amikacin, gentamicin, kasugamycin, neomycin, and tobramycin). Lincomycin, the peptide polymixin B, and the quinolone cinoxacin had no effect on CLas (Zhang et al., 2014). Several tetracycline derivatives ineffective against human bacterial pathogens showed greater potency than oxytetracycline against strains of Liberibacter (Nelson, 2014). Formulated for delivery to the tree bark, two of the most potent compounds, EBI-601 and EBI-602, penetrated and translocated throughout the citrus tree. Both compounds showed the ability to suppress CLas growth in infected citrus, but as yet there have been no publications from this work. In some cases, combinations of antibiotics have been more effective than individual compounds (Zhang et al., 2010; M. Zhang et al., 2011). Antibiotic mixtures containing either penicillin and streptomycin or kasugamycin and oxytetracycline resulted in reduction of both the number of microbes on leaf midribs and the diversity of microbial taxa (Zhang et al., 2013a).

A number of research efforts have focused on improving bactericide treatment effectiveness by optimizing the method of plant delivery. Hu and Wang (2016) identified optimum parameters for trunk injection of oxytetracycline to achieve uniform antibiotic distribution *in planta,* thereby enhancing tree productivity (Hu and Wang, 2016). Others showed improved outcomes by adding adjuvants, creating nanoemulsion formulations (Yang et al., 2015; Powell et al., 2016), or using "soft" or nonclumping nanoparticles (SNPs) (Moudgil et al., 2015). Moudgil and coworkers (2014) are working to develop SNP nanoemulsions of two essential oils, EO A and EO B, that effectively inhibit bacterial growth. Project reports indicate that their characterization is progressing, but no data have yet been published. Even lasers can improve the uptake of foliar sprays (Etxeberria et al., 2016).

New approaches such as chemical genomics for identifying novel chemicals that are potentially antimicrobial (Patne et al., 2011), the use of Zn to replace Cu, which has been applied since the 1940s and to which bacterial resistance is a growing problem (Johnson, 2016; Santra et al., 2017), and the development of nanoparticle and nanoemulsion formulations that promote uptake into the plant (Moudgil et al., 2015; Yang et al., 2015; Powell et al., 2016) have produced promising preliminary results. Another area of significant progress has been the identification of factors, including chemical stimuli and pathogen effectors and signal molecules, of both pathogens and beneficial microbes that trigger pathogen-associated molecular pattern host defense induction in the citrus host resulting in suppression of disease progress, disease severity, or bacterial multiplication (Patne et al., 2011; Lu et al., 2013; Moore and Febres, 2014; Roose et al., 2014; Shi et al., 2015; Li et al., 2016; J. Li et al., 2017). Several new host defense molecules have also been identified and could be the target of research to develop new antimicrobial compounds. Hundreds of new chemical formulations have been tested in an effort to identify novel anti-CLas compounds (Triplett et al., 2017), and predictions were made about which of these would likely be phloem mobile, but these results have not yet been published. Through mutagenesis of *L. crescens* (Lai et al., 2016) 314 genes essential for growth in culture have been identified; 238 of these have homologs in *Ca*. Liberibacter asiaticus and could potentially be targeted through development of novel antibiotics. Furthermore, Pagliai et al. (2014, 2015) showed that a CLas transcriptional regulator (*ldt*R) and a transpeptidase (*ldtP*) affected CLas viability; such novel approaches may be useful as pathogen-targeted control elements.

Bacteriophage Therapy. Significant research efforts have led to the identification of bacteriophages present in CLas and its *Ca*. Liberibacter relatives, and their roles in the survival and fitness of their bacterial hosts (S. Zhang et al., 2011; Fleites et al., 2014; Zhang et al., 2014; Gabriel et al., 2017; Jain et al., 2017). Since CLas prophages were discovered through annotation of the CLas genome, these projects are relevant to both ***Recommendation L-2*** and ***Recommendation NI-6: Exploit the CLas genome sequence for new strategies of HLB management.*** CRDF funding has led to the characterization of regulatory factors mediating phage existence in and, in some cases, transition between lytic or lysogenic phases. Unexpectedly, phage status (prophage or lytic) differs depending on the host; some phage regulatory elements found in citrus are absent in the ACP. More about the biology of CLas phages can be found in section *Candidatus* Liberibacter asiaticus in this chapter. One of the CLas phage encodes a secreted peroxidase that suppresses plant defense and could support infection (Jain et al., 2015). An *in vitro* reporter assay has been developed in *L. crescens* to measure the suppressive activity of host extracts on phage lethal genes. That work has enabled screening for specific phage-inducing stresses and treatments (Fleites et al., 2014; Jain et al., 2017), resulting in the identification of a psyllid endosymbiont protein that strongly represses phage gene activation (Jain et al., 2017). Suppression of phage promoters is a heat-labile phenomenon, providing one potential explanation for the efficacy of thermotherapy treatments in suppressing CLas in citrus (Fleites et al., 2014). Ongoing work includes the attempt to engineer culturable *L. crescens* to take up phage in order to facilitate study in a living system, and to identify compounds or organisms that can trigger the phage lytic cycle or suppress entry into the lysogenic state (Gabriel, 2017).

Biological Control. Bacterial-based biocontrol is a desirable control method for many growers as it is generally compatible with organic production. A screen of 327 citrus-inhabiting bacteria uncovered 21 that have antimicrobial properties (Riera et al., 2017). The abundance of the beneficial bacteria *Bradyrhizobium* and *Burkholderia,* the two most dominant rhizoplane-enriched genera, was lower in HLB rhizoplane samples than healthy samples, suggesting that plant health might be enhanced by supplements containing these microbes. The production of antimicrobial compounds by beneficial bacteria isolated from HLB-escape citrus trees had antagonistic activity against several citrus root pathogenic fungi and oomycetes (Riera et al., 2017). Application of four antimicrobial species to infected citrus resulted in some slowing of disease progression, but only if applications were made during very early infection (Wang and Pelz-Stelinski, 2017). Beneficial microbes may suppress infection and slow symptom development by triggering host plant defense responses (Wang et al., 2017). Application of *Burkholderia* spp. also induced natural plant defenses (Zhang et al., 2017). Because biocontrol efforts can be hampered by low microbial survival, several candidate beneficial bacteria are being analyzed for antimicrobial compounds that could be extracted for concentrated application (Wang and Pelz-Stelinski, 2017). In as-yet unpublished work, these beneficial bacteria slowed HLB disease index increase and CLas titers for the asymptomatic or mildly symptomatic trees compared to the control, but they had no effect on severely symptomatic trees (Wang and Pelz-Stelinski, 2017).

Pitfalls

For HLB, every component of the disease triangle[14] (or pyramid, counting the insect vector) presents unusual challenges for research and applications. Intensive research and development efforts funded by CRDF and other agencies have yet to result in a robust set of strategies for managing HLB in Florida citrus groves and nurseries. The phloem habitat of the pathogen in citrus provides a safe haven for the bacteria from many bactericides and antibiotics, and the inability to culture CLas hampers screening of such compounds in the laboratory.

There are mixed opinions about the utility of bactericides and chemical sprays for CLas control, particularly in relation to recent legislation permitting the emergency use of tetracyclines in Florida groves (M. Zhang et al., 2011) or the applications of novel compounds generated through chemical genetic approaches. Concerns about chemical residues in fruit and juice, the likelihood of development of bacterial resistance, and even the ability of such applications to reduce or eliminate bacteria in the field influence whether such approaches will be widely adopted in the future. Other concerns include the high cost of application, the need for repeated applications since the compounds are bacteriostatic rather than bactericidal, the risk of phytotoxicity, and, as microbiome-related issues are revealed and characterized through ongoing research, deleterious effects on nontarget (and possibly beneficial) bacteria.

Much remains unknown about the ultimate impact of phage therapy, and researchers are far from translating current knowledge into an effective product. Even if it is possible to manipulate CLas phages to enter the lytic cycle and kill a bacterial host cell, it is not clear whether this action could be managed in such a way as to eliminate all of the bacteria in an individual host plant and, further, in all of the citrus trees in a grove or region.

Future Directions

Major approaches for managing an insect-transmitted plant pathogen are to enhance host resistance, control the vector, and reduce the inoculum. Thus, killing or significantly reducing pathogen populations is an important component of an overall disease management strategy. Research-based evidence has shown that some approaches for doing so are likely to be more effective than others. A recent review of challenges in the management of HLB (Blaustein et al., 2018) included thoughtful comments on pathogen-targeted approaches.

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[14] A conceptual model that shows the three factors required for disease development: a susceptible host, a disease-causing organism (the pathogen), and a favorable environment for disease.

It will be important to continue to explore the potential of novel chemicals such as those involved as regulators or intermediates in key host plant defense response pathways, which might be exploited in the development of resistant citrus cultivars, as well as substances identified through chemical genetics. Research to identify and develop strategies to enhance chemical uptake and translocation within the plant should continue, as they will help to reduce usage rates and increase effectiveness of formulations applied to plant surfaces. Combinations and seasonal rotations of materials should be explored further, for both their bactericidal and bacteriostatic impacts and their potential to reduce the development of bacterial resistance. Just as important will be verifying the overall effectiveness of any chemical or application strategy through controlled field testing over multiple years and in a variety of environmental conditions.

Promising current research is exploring possible applications of phages or their regulators, including some factors found in the ACP endosymbiont *Wolbachia*. Preliminary results from research on bacteriophage regulatory factors, and the potential applications of such regulators as antibacterials, should be continued.

CLas management strategies that target the pathogen operate under the premise that reduction of bacterial numbers will result in less disease, less pathogen transmission, and more productive trees. However, data to support these assumptions are scant. It will be important to investigate further whether trees currently receiving tetracycline applications are, in fact, more productive, and at what bacterial titers ACP acquisition or transmission rates are prevented or lowered to an acceptable level.

Host Resistance and Conventional Breeding

Notable Research Outcomes

The citrus breeding programs in Florida prior to HLB were innovative and advanced the knowledge of citrus genetics, breeding, and biotechnology. Over the years of breeding and selection these efforts produced a range of promising advanced selections and cultivars (Cooper et al., 1962; Gmitter et al., 2007; McCollum, 2007; Grosser et al., 2010).

After the arrival of HLB in Florida CRDF funding helped bring the conventional breeding programs through the first phases of work on identifying HLB-resistant or -tolerant genotypes. Some level of apparent tolerance in existing citrus fruiting varieties and rootstocks, such as Persian and Mexican limes, Eureka and Volkemer lemons, and Carrizo citrange and *Poncirus trifoliata* rootstocks was detected but most commercial citrus grown in Florida was found to be quite susceptible (Folimonova et al., 2009). Although high-level resistance was not detected in commercial-ready genotypes, useful levels of tolerance were found in both rootstock and fruiting selections that had been developed prior to the detection of HLB in Florida. These included rootstocks US-942 (USDA, 2010), US-802 (Bowman et al., 2016a,b; Albrecht et al., 2012), US-1516, 896, 1503, and 1524 (Bowman et al., 2016b), and US-1279, 1281, 1282, 1283, and 1284 (Bowman and McCollum, 2015) and fruiting varieties such as "Sugarbelle," "Tango," and "Bingo" (Grosser et al., 2015; McCollum and Stover, 2017; UCR, 2018). CRDF supported the testing of these materials, and they have been either released to growers or are undergoing advanced field trials (Albrecht, 2017; Grosser and Gmitter, 2017).

The committee noted that none of the recommendations in the 2010 NRC report addressed conventional breeding.

Pitfalls

Citrus breeding is a long-term activity marked by challenges such as long juvenility periods, high levels of heterozygosity, and large tree size, requiring large and expensive field test sites. Polyembryony in some citrus cultivars and germplasm makes it difficult or impossible to produce new genetic combinations through conventional hybridization, although it facilitates clonal propagation through seeds (Kepiro and Roose, 2007).

New cultivars of citrus that had been developed through breeding programs in place prior to HLB were only slowly, if at all, accepted by the industry because profitable production depends upon market acceptability, uniformity of product, and acceptable levels of fruit production. Acceptance levels changed dramatically after the destruction caused by HLB in Florida after 2005 (McClean and Schwarz, 1970; Miyakawa, 1980; Nariani, 1982; Miyakawa and Zhao, 1990; Lopes and Frare, 2008; Folimonova et al., 2009). Also on the rise is interest in the testing of advanced selections toward commercialization. Chaires (Giles, 2017) noted that, "Whereas there used to be one release every 20 years or so, we have seen approximately 22 fresh selections made available through the accelerated programs and a number of private or proprietary selections come into Florida for trial." Unfortunately, the most important commercial orange and grapefruit cultivars currently grown in Florida appear to be among the most susceptible to HLB (McClean and Schwarz, 1970; Miyakawa, 1980; Nariani, 1982; Miyakawa and Zhao, 1990; Lopes and Frare, 2008; Folimonova et al., 2009).

Future Directions

Apart from HLB, citrus trees face many challenges, biotic (including, but not limited to, *Phytophthora*, CTV, canker, and nematodes) and abiotic (including cold damage, flooding, and hurricanes), that must be considered along with productivity and fruit quality. That there is industry demand for new genotypes provides an opportunity for both breeders and growers. Although evaluations are being done, they need to be ramped up. The standard definitions of resistance, tolerance, and susceptibility for HLB may not follow those for other plant diseases. Instead, the community might be well served to regard host responses based on whether trees become unproductive or continue to thrive and produce marketable fruit in spite of HLB infection. A community-accepted standard for evaluation of host resistance may be accompaniedby or correlated with omics and molecular bases for host responses. Evaluations must take place in the field and must continue for multiple years before recommendations can be made with confidence. With that said, growers and industry supporters of research will need to be willing to take some risks with new citrus genotypes.

Citrus trees used in commercial production are composed of two interacting biological systems: the fruit-producing scion and the soil-inhabiting rootstock. Each must be evaluated for reaction to HLB and other biotic and abiotic factors. While fruit quality is not a factor for rootstock selection, ease of vegetative propagation and seed production, if polyembryonic, are important selection factors. Rootstock-scion combinations must be evaluated first for graft compatibility and then for overall tree health, abiotic stress resistance, effects on tree architecture, productivity, and fruit size and quality. Tolerance to HLB can be affected by environment and pathogen load, and perhaps by pathogen genetics. Therefore, considering the investment in developing a new grove and the time that young trees are nonproductive, growers need to have some assurance that they will profit, in the long run, from investment in a tolerant rootstock or scion.

Tolerant genotypes such as "Sugar Belle" mandarin, "Temple" tangor, and "Triumph" and "Jackson" grapefruit hybrids, released prior to the incursion of HLB into Florida, and a number of advanced selections (Stover et al., 2012; Gmitter et al., 2017; FFSP, 2017) will require focused investigations to ascertain the mechanisms of resistance or tolerance and guide the development of new resistant or tolerant material (Albrecht et al., 2016; Killiny and Hijaz, 2016; Killiny et al., 2017). Evaluation of the current collection of tolerant material should be shifted toward extension and industry, and field data and critical observations provided to breeders to assist in guiding next generation cultivar development. To some extent this ramped-up field testing is already under way; currently, through projects supported by CRDF and Multi-Agency Coordination (MAC) Group funding, more than 50,000 trees, including approximately 100 rootstocks, 50 scions, and 70 rootstock–scion combinations, are being field tested. These tests provide opportunities for the release of new HLB-tolerant rootstock and scion cultivars but also present challenges. The material being developed by the breeding programs can help provide industry with direction for the future, but it can do so only with strong industry collaboration in advanced selection and new cultivar testing programs. Mechanisms already in place for such collaboration, leading to rapid evaluation and distribution of promising new citrus selections, include the New Varieties Development & Management Corporation (NVDMC) and the Fast Track (NVDMC, 2015, 2017; Chaires, 2016) mechanism co-developed by NVDMC, University of Florida (UF) Institute of Food and Agricultural Sciences (IFAS), and Florida Foundation Seed Producers Inc. The latter program is strengthening connections between breeders and the grower community for their mutual benefit (UF IFAS, 2018). In the long term, these types of research and development efforts, managed collaboratively by the breeding community and the industry, will increase the likelihood of survival and perhaps healthy evolution of the Florida citrus industry.

A critical area of collaboration is that between breeding programs, especially between UF and USDA breeding programs in Florida (but extending also to California-based and other citrus breeding programs worldwide). The industry is depending on these programs for long-term solutions to HLB. Considering the challenges in citrus breeding described above, breeding programs must be fully integrated at the scientist and institutional levels. Funding, facility sharing, and intellectual property issues must be worked out by the respective institutions to facilitate multi-institutional breeding efforts. Certainly, the physical proximity of the Florida programs to each other and to the industry create a natural environment for close cooperation that must be fully exploited.

Host Resistance and Genetic Engineering and Editing

Notable Research Outcomes

Genetic resistance to HLB has been considered key to the survival of the Florida citrus industry and hence was a primary focus of research funding, but hybridization and selection, identification of naturally occurring sports (segments of the plant that are distinctly different from the parent plant in both phenotype and genotype), mutagenesis, and somatic hybridization[15]

[15] The fusion of enzymatically isolated somatic cell protoplasts (naked cells) from primary tissues, circumventing complex sexual incompatibility and enabling important traits to be transferred from donor to recipient species.

have not yet led to highly resistant or immune commercially acceptable citrus varieties. Unfortunately, no suitable sources of high-level resistance have been identified in citrus for use in breeding programs, but promising genes from other plants may be used in genetic engineering (GE) approaches (NRC, 2010).

In response to the 2010 ***Recommendation NI-4: Accelerate the sequencing, assembly, annotation and exploitation of a sweet orange genome to provide a powerful tool for all future citrus improvement research***, the genomes of several citrus cultivars and species have been sequenced[16] (Xu et al., 2013; Wu et al., 2014). Gene identification, now under way, must take into account the large and complex genomes of citrus species and cultivars.

The terms "resistance" and "tolerance" are rather loosely applied in relation to HLB. The term "tolerance," as discussed by Castle et al. (2015), is a "concept . . . of endurance . . . or thriving in the presence of the pathogen or pest." Horns and Hood (2012) state that "Resistance traits reduce the harm caused by disease by preventing infection or limiting subsequent pathogen growth and development within the host through avoidance or clearance of infection." In many cases for citrus it is not yet clear if particular genotypes express tolerance or some level of resistance.

Citrus Transformation. The 2010 NRC ***Recommendation L-1: Support development of transgenic HLB-resistant and ACP-resistant citrus***, has been very well addressed by CRDF funding. Many new transgenic lines have been developed in a number of projects; of these, most possess resistance to CLas, although a few are targeted for ACP control.

Transformation of citrus has been accomplished through *Agrobacterium*-mediated transformation of protoplasts (plant cell protoplasm whose cell wall has been removed), leaves, stem segments, seeds, epicotyls, and embryogenic cell suspensions (Dutt and Grosser, 2010). Particle bombardment has also been successful. Factors influencing transformation efficiency include the genotype, age and type of explant, *Agrobacterium* strain, inoculation procedure, preculture conditions, and selection regime (Febres et al., 2011). Transformation, which is specific to genus, species, and cultivar (Dutt and Grosser, 2009), has been successful in relatively few genotypes (Febres et al., 2011; Donmez et al., 2013). *P. trifoliata*, used for decades as a source of cold-hardiness genes for transfer to citrus germplasm, is at least tolerant of, if not moderately to highly resistant to, HLB. Unfortunately, while *P. trifoliata* is readily transformed, it generates offspring having poor quality or inedible fruit so it is of interest to the industry primarily for rootstock use. Transformation has generally been undertaken with explants from juvenile tissues

[16] Citrus genome database URL: https://www.citrusgenomedb.org/. Accessed December 18, 2017.

such as epicotyls and nucellar embryos produced from parent tree tissues. While these explants reproduce a tree having the original genotype, the time to fruiting is extended due to juvenility. Juvenile tissues have been used to produce transgenic rootstocks, sweet oranges, grapefruits, mandarins, limes, and lemons (Dutt and Grosser, 2010; Orbović and Grosser, 2015). Rates of transformation are still quite low, in the range of single digits, for many commercial genotypes (Hu et al., 2016).[17] The rootstock cultivar "Carrizo," a hybrid of *P. trifoliata*, and "Duncan" grapefruit, has among the highest transformation rates using juvenile tissues (~40%) (Dutt and Grosser, 2009). There are relatively few reports of mature citrus transformation (Cervera et al., 2008; Khan et al., 2012; Marutani-Hert et al., 2012; Orbović et al., 2015; Wu et al., 2015). When it does occur, transformation rates from mature tissue explants are low (1% or less), but the time to fruiting is shorter than when using explants from immature tissues. In general, major issues keeping transformation rates low are low rates of *Agrobacterium* infection and low integration efficiencies (Febres et al., 2011). The use of the *knotted1* (*kn1*) gene in transformation significantly increased transformation and transgenic plant recovery rates using juvenile tissue explants, but its effect on mature explants has not yet been fully evaluated.[18] While the *kn1* gene did not appear to affect the transgenic shoots, the effects on tree development, flowering, and fruiting remain to be investigated (Hu et al., 2016). Investigation of the *kn1* gene and other potential regeneration-inducing genes may be useful in increasing levels of regeneration, a critical process for most methods of stable genetic transformation (genetic engineering).

Low transformation rates make it difficult to reliably evaluate the effect of a particular gene since many factors influence transgene expression, including the site of insertion, copy number of the insert, the arrangement of copies, and base pair deletions in the gene of interest or promoter. Large numbers of plants from each transformation event are important for determining the effect of a particular gene and/or promoter. In the short term, testing of genes and constructs may also be accomplished through the use of virus-based vectors, which would not require transformation or regeneration of plants for evaluation.

Transgenic Plants. Hundreds of independent citrus lines have been produced despite challenges associated with citrus transformation; these transgenic plants express antimicrobial proteins (Stover et al., 2013b; Dutt et al., 2015b), disease resistance or R genes from citrus or other plant species (Lu et al., 2013; Dutt et al., 2015a,b), antibodies against CLas proteins,[19]

[17] M. Dutt, Citrus Research and Education Center (CREC), Lake Alfred, Florida, personal communication, December 12, 2017.

[18] J. Zale, CREC, Lake Alfred, Florida, personal communication, December 12, 2017.

[19] E. Stover, USDA ARS, personal communication, December 20, 2017.

genes to improve regeneration and transformation (Hu et al., 2016), and promoters targeting specific tissues (Dutt et al., 2012; Benyon et al., 2013; Belknap et al., 2015).

These plants, which represent the culmination of years of work, are a notable outcome that must be well cared for and critically evaluated in the long term for their impact on HLB resistance. They will provide a basis for future work and may provide the genetic material that the industry will need to prosper. Work with transgenic HLB-resistant citrus expressing a defensin gene from spinach is well advanced; field tests have been ongoing in Florida since 2009. The groups supporting this work (Texas A&M and Southern Gardens Citrus) have obtained permits from Animal and Plant Health Inspection Service (APHIS) Biotechnology Regulatory Services and the Environmental Protection Agency for more extensive field tests in both Florida and Texas. Methods of selection of transgenic shoots are improving (Acanda et al., 2017). Gene expression in particular tissues, such as phloem, can now be targeted using transgene promoters (Dutt et al., 2012; Benyon et al., 2013; Belknap et al., 2015). Development of transgenic plants using anthocyanin markers rather than antibiotic resistance (Stover et al., 2013a; Dutt et al., 2016) could facilitate selection of plants already containing another marker for use in retransforming, or stacking, transformation events.

CRDF funding has supported research efforts to identify genes that are expressed differentially in susceptible and tolerant citrus cultivars, and to produce transgenic HLB-resistant or -tolerant citrus[20] (Dutt et al., 2015b; Wang et al., 2016). These studies have moved forward the fundamental knowledge of the *Citrus* and *Poncirus* genomes with respect to how plants react to HLB infection and how they might be manipulated to provide resistance (Rawat et al., 2015). These, as well as mapping studies, have demonstrated that naturally occurring tolerance and resistance in citrus relatives is complex and is likely the result of multiple genes and multiple pathways (Wang et al., 2016). This apparent complexity makes it critical to have open communication between genomic researchers, breeders, and researchers studying the disease process and reactions to inoculations in order to identify genes that are responsible for resistance or tolerance.

Resources Required for Genetic Engineering. Resistance screening of research-derived HLB-resistant genetically engineered citrus is a bottleneck in delivery of new varieties to the industry. CRDF supported research on high-throughput screening using ACP, an approach that is more similar to the natural infection pathway than the use of graft inoculation, but that may require additional greenhouse capabilities in which to select a smaller advanced set for field testing. Using new flushes of leaves significantly in-

[20] Z. Mou, University of Florida, Gainesville, Florida, personal communication, December 4, 2017.

creased rates of disease transmission (Hall et al., 2016), and ACP nymphs were more efficient transmitters of CLas than adults (Ammar et al., 2016). It is critically important that resistance screening be supported and that vetted data be meticulously collected in order that decisions can be made on what material is to be moved to the next stages of replicated testing. Data from these tests will also be useful for regulatory submissions.

Acceptance of Genetic Engineering Traits. CRDF funding has supported investigation of many potentially useful R genes from a variety of sources that may be candidates for gene editing. Many in the field assume that gene-edited products will be readily accepted by consumers. Ishii and Araki (2016) reported finding no basis at this time to support such a claim; however, House (2017) found that a majority of surveyed Florida consumers reported willingness to purchase genetically engineered citrus if cost incentives were provided (see also Future Directions in this chapter, and Chapter 3). The citrus industry would do well to keep informed and be proactive in interacting with the public on all of the technologies that are being developed (genetically modified or non-genetically modified) to save U.S. citrus production. Additional discussion of sociological and economic factors influencing public and market acceptance of genetically engineered citrus is provided in Chapter 3.

Pitfalls

In addition to the scientific and technical challenges of citrus transformation, progress is hampered by a reduction, in severe cases, in transformation rates due to low seed quality from HLB-infected trees,[21] and this issue is holding back the development of transgenic lines. While the use of a more tractable model system, such as *Arabidopsis,* or the use of viral-based vectors can provide preliminary data to guide further research it will be important to move the work into more relevant systems as quickly as possible. Sourcing disease-free citrus trees will be essential for transformation work.

Despite optimism that improvements to HLB resistance produced through genetic engineering technologies will ultimately provide solutions for HLB management, there has, as yet, been only very limited interaction between citrus developers and representatives of the regulatory agencies whose approvals will be required for dissemination of new genetically engineered and gene-edited citrus cultivars. The regulatory process can be both challenging and time consuming, and Florida growers have little time to spare in implementing industry-saving measures.

[21] V. Orbović, CREC, Lake Alfred, Florida, personal communication, December 1, 2017.

Future Directions

Many potentially useful transgenic lines developed over the past decade must be fully tested so that only the most promising material, selected based on reliable, replicated data, moves to intense field testing. Field, and in some cases greenhouse, testing will require sufficient and long-term funding in order to achieve practical results for the industry. Preparation for release of enhanced citrus varieties must include consideration of intellectual property and regulatory issues.

There is a critical need for a coordinated effort to develop highly efficient transformation systems for citrus genotypes of commercial interest. In the meantime, it may be useful for the community to select a susceptible citrus host—not necessarily of commercial interest—that provides consistently high levels of transformation as a model that could be used as a community-wide standard for evaluating genes of interest. Such a cogeneric model may be more useful than an *Arabidopsis* system, or it may be used to follow up on *Arabidopsis* rapid screening. For example, if a transgene is effective in "Duncan" grapefruit (now being used in work at UF Citrus Research and Education Center), a genotype having a transformation rate of 40% or greater (Dutt et al., 2009), that gene would be a good candidate to move into a transformation-recalcitrant but commercially important genotype. A constitutive promoter such as 35S should be considered for the initial tests of a candidate gene because constitutive expression may provide a more robust assay; tissue-specific expression could miss important tissue targets for providing resistance.

Long-term solutions to the citrus greening crisis will involve a combination of tactics, and disease management will likely depend on reducing inoculations by ACP coupled with durable host resistance. The citrus industry (juice and fresh) can work to eliminate barriers to the implementation of genetic solutions, including the incorporation of disease resistance genes into desirable citrus varieties and modification of ACP to render them incompetent vectors.

The Picos Farm and other citrus field test sites are critical for the pipeline from gene discovery, transformation, and greenhouse testing to field testing and cultivar release. Although these screening goals are different from those mentioned in the 2010 NRC report (***Recommendation NI-3: Establish citrus orchard test plots for evaluation of new scouting and therapeutic methods***), such field plots are critical for real-world assessment of genetic improvements as well as therapeutics and scouting approaches. These sites will need long-term CRDF, institutional, and industry support.

More rapid advancement in citrus transformation should be a major goal of all teams working on the genetic engineering projects, and more robust communication on methodologies and strategies—both successful and unsuccessful—would be beneficial. Once efficient productive transformation systems are developed, all research groups will benefit, as will the growers who are waiting for a breakthrough in transgenic citrus highly resistant or immune to HLB. Information on genes of interest, promoters, and selection strategies should be shared as soon as possible throughout the CRDF-supported community.

In summary, the following are important steps for this stage of the research and development (R&D) pipeline:

Screening plants that have already been developed for resistance and moving the promising clones into replicated field test for critical evaluation by researchers and industry. This work will ultimately provide valuable information and guidance for the ongoing search for useful genes, native to citrus and related species, or from unrelated organisms. It could also lead to a major breakthrough in HLB resistance. Support is needed for long-term evaluation of field tests for all promising plants (whether GE or conventionally bred) developed through CRDF funding. MAC grants are recognized as a great benefit in this area.

Improving transformation. Transformation efficiency is a major bottleneck. There seems to be a rather wide range in transformation efficiencies within the community, particularly with the more recalcitrant genotypes. It is not clear if the more successful approaches are being effectively shared; information on regeneration protocols, the effects of promoters, and genes of interest could help to prevent duplication of efforts and increase research efficiency. Communication could be supported by having frequent principal investigator–sanctioned communications among technical personnel, graduate students, and postdocs within and between research groups.

In addition to generally improving citrus transformation efficiency, a model citrus system should be developed that can be readily transformed, with a consistently high transformation rate, for use in citrus genetic engineering and/or gene-editing research. It would also be wise to come to a consensus on a model vector and transgene cassette.

Understanding the regulatory and intellectual property landscape of the genes, promoters, and vectors being used in CRDF-funded projects. There should be a clear understanding of the regulatory potential of any gene to be used for resistance studies. If a gene is unacceptable for regulators it should be avoided and its use not funded by CRDF. Communicating with genetic engineering plant regulators is critical. The citrus industry can learn from the experience of others who have already received regulatory approvals for crops including apples, plums, and papayas, or are currently going through the process (chestnuts). Ideas on public outreach, website development, and the use of consumer focus groups can also be obtained from developers of other GE fruit and nut crops. The interactions of Texas A&M and Southern

Gardens Citrus with regulatory agencies and with consumers can serve as additional examples. By sharing their experiences these groups can learn from one another the best approaches to constructing a successful regulatory dossier and perhaps, when appropriate, could present a unified voice on issues pertaining to regulatory oversight of transgenic tree crops.

While there will always be consumers opposed to the use of biotechnology, there are some who will benefit from factual information presented in lay terms in order to make an informed decision on the use of the technologies.

Diagnostics

Notable Research Outcomes

Significant advances have been made in HLB detection technologies, notably those that detect biomarkers produced by the infected host, a biomarker secreted by the pathogen in the host, or volatile organic compounds (VOCs) released by an infected host (Cevallos-Cevallos et al., 2011, 2012; Aksenov et al., 2014; Ding et al., 2017; Pagliaccia et al., 2017; Slupsky, 2017). These results have addressed the 2010 ***Recommendation NI-2: Support searches for biomarkers that may be exploited to detect CLas-infected citrus.***

It is unlikely that further development of conventional diagnostics will result in increased pathogen sensitivity, although gains are possible in adapting some of the technologies to field-based assays that may help growers conduct diagnostic tests on the farm rather than sending samples to accredited laboratories. The primary continuing need for CLas diagnostics is for methods that detect infection shortly after inoculation, well before any symptoms of disease can be observed. The diagnostic breakthroughs are likely to come from alternative technologies that are geared toward detection of disease rather than the pathogen: to identify unseen symptoms rather than signs or to look for markers of disease at the tree level rather than the leaf, branch, or root level (or by extension the vector population level rather than the individual insect). These approaches, described collectively as early detection technologies (EDTs), will detect the pathogen indirectly by detecting subtle changes in the plant triggered by pathogen invasion, prior to the onset of obvious visual symptoms of the disease.

One promising detection method is based on the chemical analysis of VOCs that are released by HLB-infected trees. Biomarkers specific to CLas have been found and could be analyzed using gas chromatography/mass spectrometry and gas chromatography/differential mobility spectrometry (Aksenov et al., 2014). Greenhouse tests showed that a mobile differential mobility spectrometry system was able to distinguish volatile differences between closely related citrus cultivars and to show volatile-profile differences between healthy and infected citrus (McCartney et al., 2016). The use of canines to sniff out these volatiles specific to infected trees has been an effective strategy for citrus canker detection, and preliminary observations show promise for dogs to be able to detect HLB infections before symptom expression (Berger, 2014).

Detection of HLB through optical sensing has also been explored, but this technology is still being optimized and tested. Field experiments have demonstrated that optical sensors, such as a rugged, low-cost multiband active sensor that measures reflectance of tree canopy in two visible bands at 570 and 670 nm, and two near-infrared bands at 870 and 970 nm (Mishra et al., 2011), and a field portable spectroradiometer that collects the spectral reflectance data in the range of 350 to 2,500 nm (Sankaran and Ehsani, 2011) can be used to identify HLB-infected trees from healthy trees. Differentiation of infected and healthy trees based on tree canopy reflectance was also achieved using airborne multispectral and hyperspectral images (Kumar et al., 2012). These types of field imaging also yielded useful classifications of disease status if coupled with high-quality ground truth records (Li et al., 2012). Unmanned aerial vehicle imaging in combination with remote sensing and support vector machine technology produced higher classification accuracies than images obtained from aircraft (Garcia-Ruiz et al., 2013).

The data presented by invited speakers at the committee webinar[22] on omics biomarkers are impressive and appeared to minimize the effects of cultivar, environment, time after infection, and CLas strain, but it is not clear if there are enough data to draw broad conclusions. Furthermore, whether the uneven distribution of the pathogen in trees, especially large field trees, affects biomarker quantity and quality is as yet undetermined.

Pitfalls

CRDF has not been at the forefront of funding research on CLas diagnostics, presumably due to the high incidence of HLB in Florida and the emphasis on disease management rather than disease detection. A basic tenet of plant disease management is to remove or abate sources of inoculum. This principle was discussed in the 2010 NRC report (***Recommendation NI-2: Support searches for biomarkers that may be exploited to detect CLas-infected citrus.***) along with the need to improve CLas diagnostics so the early infections could be detected and trees removed. The 2010 report did elaborate on the need to look beyond diagnostics that detect the

[22] Webinar on Citrus Greening (HLB) Diagnostics and Detection, August 24, 2017. Agenda and speaker information are located in Appendix B of this report.

pathogen directly and to consider biomarkers that would allow detection of disease well ahead of detecting the pathogen.

Research results on remote optical sensing clearly identified differences in reflectance spectra between healthy and infected trees, but the differences were not detectable much before the onset of symptoms or detection by conventional pathogen diagnostics. An apparent shortcoming of efforts to employ canines for detection is that much of the canine training is done using potted, young trees rather than mature trees in a commercial grove setting. Nevertheless, canines may be useful for a quick preliminary screening of large acreages, and initial detections can be supported using laboratory instrument–based HLB diagnostics. Presentations at a committee webinar revealed data supporting the hypothesis that VOCs are cultivar independent (Davis, 2017), but it is not clear if there are enough data to indicate that VOC profiles of cultivars do not differ significantly.

Identifying ACP carrying CLas is more a problem of sampling than of detection since qPCR and other conventional assays can easily detect small copy numbers of bacterial DNA if it can be recovered from the insects. Because only a relatively small proportion of the ACP individuals are actually competent vectors (Coy and Stelinski, 2015), the insect sample size must be large enough to determine infection pressure accurately. Furthermore, the trapping method used to collect ACP must allow quality DNA to be recovered from the insects. Sticky cards are effective for trapping insects but not for the recovery of quality DNA. Alternative traps are being studied, but it is important to consider trap designs that will not only accurately estimate ACP seasonal phenologies and numbers but will also allow for accurate determination of CLas infection in the ACP population.

Future Directions

The choice of diagnostics must reflect the ultimate goal of the diagnostician. Identifying infected trees in commercial settings (orchards and nurseries) may demand different diagnostics than identifying CLas-infected ACP. Furthermore, as already mentioned, the major issue is not the sensitivity of pathogen detection but finding the pathogen within large trees occupying extensive acreages, or in large ACP populations. Continued efforts to improve the sensitivity of CLas diagnostics should be minimized with the possible exception of optimizing reliable, user-friendly, cost-effective, field-based diagnostics that can be used on the farm to test ACP immigrating into the orchards or trees in later stages of infection. These are unlikely to be effective early detection methods. The most critical and time-sensitive diagnostic need is to find and validate methods of detecting infection during the latency period when CLas titers are, for the most part, undetectable.

These will be methods that indirectly detect disease (i.e., HLB) rather than the CLas pathogen.

In the area of volatile sensing, a comparison of canines and laboratory instruments to detect the disease in the same trees would be informative, as would parallel studies to see if the same trees can be identified on subsequent days, weeks, or months. It is important to know whether there are diurnal or seasonal patterns to VOCs and, if so, how they affect diagnosis using VOCs. Also important is whether infection by other pathogens or other stresses or farm inputs affect VOCs and HLB detection. For all of these parameters, cost–benefit ratios are needed to support decision making by growers.

Exploration of the use of biomarkers should continue, with comparisons of omics biomarkers and VOCs. Adding other diseases to the analyses seemed to lessen the separation between data clusters in the principal component analyses, which could be a major issue for the validation of omics biomarkers. Furthermore, additional biotic and abiotic stresses or farm inputs may have effects on the ability of biomarkers to separate HLB-infected and non-HLB-infected trees. All of these issues should be addressed in continuing research.

Although remote optical sensing has the potential for monitoring large acreages for symptomatic disease, it may not be an effective early detection diagnostic. Additional research is needed to address whether the reflectance spectrum in a large tree has a uniform distribution or whether it reflects the nonuniform distribution of the pathogen.

While Florida citrus is effectively 100% infected and early diagnostics might seem irrelevant to some, this view is short sighted because a future citrus industry in Florida will need to minimize CLas inoculum sources before starting over with either noninfected or HLB-cured trees that must be monitored for new or resurgent CLas infection. Such monitoring will require early detection technologies so this avenue of research should be a high priority for the entire U.S. citrus industry. Furthermore, the 2010 NRC report recommended the establishment of field test plots of trees whose health status is controlled so that any detection technologies could be validated on trees of different ages representative of trees in commercial orchards. This critical need has not been addressed anywhere in the United States and represents a significant impediment to the testing and validation of any EDT.

It is unlikely that "any one diagnostic" approach for HLB, targeting either the disease or the pathogen, will be sufficient. More likely, grower decisions on treatment will be based on results from a series of diagnostic assays. As well, diagnostics differ with respect to economics and throughput, increasing the likelihood that a variety of assays will be required in the future.

Pertinent to the recommendations to pursue HLB rather than CLas diagnostics is the adoption of these technologies by the state and federal regulatory agencies responsible for delineating quarantine and regulated areas, certifying testing laboratories, and approving eradication and compensation programs. Currently, qPCR followed by conventional PCR and DNA sequencing are the approved methods that must be used by laboratories accredited under the National Plant Protection Laboratory Accreditation Program.[23] A diagnostic result that will result in federal regulatory action must be confirmed by an appropriate federal laboratory, and currently federal confirmation requires direct detection of the pathogen. This means that any of the EDT diagnostics could not be used by regulatory agencies to make decisions, but research laboratories and, more importantly, growers and grower organizations are free to use information from the EDTs to make disease management recommendations and decisions. With this distinction in mind, one approach moving forward could be to work with the industry and growers to adopt EDTs as the primary tools for making management decisions, downplaying the regulatory aspects of diagnostics. The qPCR test is unsatisfactory from a plant pathology and disease management standpoint since trees are a source of inoculum long before the regulatory-approved diagnostics can provide information on the infection status. Data available from Brazil clearly support the basic tenets of vector-borne plant disease management—minimize inoculum and reduce vector pressure (Belasque et al., 2010; Bassanezi et al., 2013a,b; Ayres et al., 2015; Bergamin et al., 2016). While current strategies are attempting to reduce vector pressure, little is being done to identify and remove inoculum sources early on.

OVERARCHING FINDINGS, CONCLUSIONS, AND RECOMMENDATIONS FOR FUTURE RESEARCH EFFORTS IN HLB MANAGEMENT

Since 2010 the impact of HLB on the U.S. citrus industry has continued to worsen, not only in Florida but, as the disease moved westward through the southern citrus-producing states into California, in an increasing diversity of environments. Disease management approaches and research priorities for rejuvenating the heavily damaged citrus industry in Florida will differ in significant ways from those aimed at preventing similar devastation of the industries in other U.S. states which have yet to feel the full brunt of HLB. However, some of the research projects funded by CRDF (and other agencies) have led to a greater understanding of the interactions between

[23] Philip Berger, USDA Animal and Plant Health Inspection Service (APHIS) PPQ, Raleigh, North Carolina, personal communication, September 19, 2017.

citrus and CLas and citrus and ACP, and the biology of CLas and ACP at the organismal and molecular levels, while others have yielded results that have led or may eventually lead to more effective HLB management strategies in any location. Some, despite investment and effort, have been unsuccessful, but even negative findings can be helpful in guiding future research directions.

Finding 4.1: Although research supported by CRDF and other agencies has advanced our knowledge of HLB since 2010, the disease remains an intractable threat to the Florida citrus industry and has progressed from an acute to a chronic disease present throughout the state.

The establishment of the Citrus Research and Development Foundation, with its formalized structure and framework for identifying, prioritizing, and allocating funding for research on HLB in Florida, was a response to a major organizational recommendation in the 2010 NRC report (***O-2: Identify one organization and empower it to have oversight responsibility over huanglongbing [HLB] research and development efforts***). The organizational framework of the CRDF has supported significant improvements to the effectiveness of HLB research prioritization, fund distribution, and communication within the Florida citrus production community.

Recognizing the threat of HLB to the U.S. industry, other research funding agencies (California's Citrus Research Board, the Texas Citrus Producers' Board, USDA NIFA and MAC competitive grants programs, as well as USDA ARS and APHIS, and others) are also supporting efforts to combat the disease. The disease in California and the southern United States is still considered acute, rather than chronic, and therefore requires site- and situation-appropriate management approaches that differ from those in Florida. Stronger communication and research linkages among those in the citrus-producing states will benefit all involved. Both the administrators and the researchers involved in these programs acknowledge the importance of communication, cooperation, and information and resource sharing at the interagency, interinstitutional, and interlaboratory levels, and a number of interactive planning meetings have taken place to address overarching needs.

Finding 4.2: A number of technical obstacles have been addressed by funded projects but continue to represent significant barriers to research progress and the generation of HLB solutions. Technical hurdles that remain unresolved include the following: transformation of citrus, *in vitro* cultivation of CLas, the need for optimizing nutritional approaches for maintaining tree health and productivity in the presence of HLB, the development of a transformable citrus model system, the development of diagnostic advancements addressing early detection (whole tree diagnostics) and improved tree sampling, and the development of standardized methods for research protocols, controls, assay validation, and assessment, for enhanced reliability and comparison across studies. Resolution of any one of these issues would constitute a significant step, supporting, in turn, the advancement of a number of other research fronts.

Conclusion 4.1: Citrus growers, particularly in Florida, still need short-term solutions for the industry to remain viable while researchers continue to generate longer-term approaches for managing HLB.
Recommendation 4.1: Continue support for both basic and applied research and both short- and long-term research efforts.

Conclusion 4.2: Longer-term solutions to HLB are likely to involve citrus variety improvement, much of it derived from new molecular techniques.
Recommendation 4.2: Continue to support the development and application of gene modification, including gene editing, focusing on targets mediating molecular interactions among plant, bacteria, and vector.

Conclusion 4.3: HLB research is hampered by the lack of standardized methods and parameters for measuring, evaluating, and analyzing factors including vector transmission rates, fruit yield, plant tolerance and resistance, citrus variety performance, antibacterial compound effectiveness, and diagnostic assay evaluation. Inconsistency in experimental designs, sampling methods, and field investigations limit the ability to compare findings and use previous research as a springboard for further exploration.
Recommendation 4.3: Support the development of community-accepted standards for the conduct, evaluation, and assessment of research to facilitate comparisons of research results across teams and institutions.

Finding 4.3: Novel approaches to foster communication, collaboration, and innovation among HLB researchers and representatives of funding agencies and the citrus industry may advance research progress and facilitate solutions to HLB.

The committee discerned that, at a practical level, both the planning and the execution of HLB research still operate primarily within individual states, programs, projects, institutions, and laboratories. Although opportunities to share information have increased through periodic conferences such as the International Research Conference on Huanglongbing (most recently held in Florida in March 2017, sponsored by CRDF, California Citrus Mutual, California Citrus Research Board, Cutrale Citrus Juices USA, Fundecitrus, KeyPlex, SunKist, and Texas Citrus Mutual), these events have focused primarily on brief, formal presentations of research results rather than futuring or brainstorming discussions to garner diverse community expertise and foster visionary thinking about HLB solutions. Furthermore, the confidentiality required by the development of patent applications has, to some extent, interfered with the sharing of information.

A number of researchers and funding agency representatives were invited to share their HLB expertise, experiences, research directions, and accomplishments in National Academies of Sciences, Engineering, and Medicine forums and webinars on HLB by addressing a set of focused, objective-based issues (see Appendix B). The committee noted that, in this setting and framework, most addressed the problem from broad and overarching perspectives and engaged in innovative and synergistic discussion of challenges, priorities, and solutions. Providing future opportunities in which members of the research community and the industry move beyond presentations of individual research outcomes and encouraging direct community effort to generate fresh, broad-based thinking could be a good investment.

Finding 4.4: There are inconsistencies in the format, content, and frequency of CRDF-funded research progress reporting by researchers, as well as in the inclusion of specific outcomes, impacts, and products.

With respect to the CRDF funding program in particular, this committee found a lack of consistency in regular reporting, by principal investigators, of research progress, including outputs, outcomes, and impacts, presentations at conferences, and publications. A number of projects have resulted in no, or relatively few, publications in peer-reviewed journals, and midterm and final research progress reporting to CRDF was inconsistent in both compliance and the amount of detail provided. For example, for 75 completed CRDF-funded research projects related to the Asian citrus psyllid, comprehensive final reports were available from only 11, of which only 8 listed publications. Of the 38 publications mentioned in those reports, 18 were published, peer-reviewed journal articles; 4 were articles in preparation or in press in peer-reviewed journals; 2 were articles to be published after a patent was received; 10 were abstracts or papers presented at conferences; and 1 was a dissertation. The requirement for annual reporting, using a standard, thoughtfully developed template that ensures the reporting of both research accomplishments and their current or expected impact on HLB solutions, could be a condition for continuation of funds in multiyear projects. Report information, like the scientific literature mentioned above, could then be used to populate a database for use by the research community.

Conclusion 4.4: Improved reporting consistency is needed to reduce constraints in reviewing research progress and delays in applying new information to HLB solutions.
Recommendation 4.4: CRDF should develop a standardized format, procedure, and timeline for mandatory reporting of midterm project research progress and final reports, to include outputs (publications and presentations), outcomes, practical applications, and impacts. CRDF should consider hiring a staff person to review and analyze HLB research findings annually.

Finding 4.5: More timely publication of research results in refereed scientific journals and trade journals would facilitate communication among the research community and between researchers and growers and support research assessment efforts.

Finding 4.6.1: Engaging with a disease that threatens the survival of an industry and requires a short-term and sustainable solution could benefit from a nonacademic research model or approach.

The committee observed that a majority of current HLB research efforts are taking place in academic settings in which the tenure and promotion system emphasizes publication and funding, training students and postdocs, and strengthening the careers of individual investigators, while discouraging out-of-the-box thinking and high-risk research. In this model, individuals or small groups typically compete for the same funding with relatively little coordination. It is a model that works in some cases but does not appear to be the best approach for dealing with HLB. With this in mind, CRDF might consider looking into adopting a product-focused industry-model of R&D and best practices. In the case of HLB research, this model could facilitate cross-institutional collaboration/teaming of multidisciplinary investigators; creating research teams (consortia) for each of the HLB research areas, with clear responsibilities set out for team leaders to review research and to communicate with other team leaders; and the setting aside of funds for innovative and/or high-risk projects.

Finding 4.6.2: Despite the commendable efforts of multiple funding agencies to coordinate funding and encourage appropriate interstate, interagency, and interdisciplinary collaborations, decisions about research funding priorities and allocations occur largely within the domain of each agency.
Conclusion 4.6: The committee concludes that the current system of research prioritization and funding, accomplished primarily within each relevant funding agency, is not optimally efficient and has not led to the

development of an overarching master plan for HLB research and its translation to management solutions.

Recommendation 4.6: CRDF should consider working, together with representatives of other agencies at the national and state levels, to create an overarching HLB research advisory panel to develop a fresh, systems approach to HLB research prioritization and the strategic distribution of resources for research leading to effective HLB management.

This new approach could involve national-level and multiagency research prioritization and emphasize a strategic distribution of resources for research leading to effective HLB management. It could also encourage and facilitate timely and effective information sharing as well as translation of research outcomes to field-deployable actions. Finally, a multiagency approach could facilitate the achievement of coordination between regulatory, research, and production priorities.

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Appendix A

Committee Biographies

Jacqueline Fletcher (*Chair*), is currently Regents Professor Emerita at the National Institute for Microbial Forensics and Food and Agricultural Biosecurity (NIMFFAB), Department of Entomology and Plant Pathology, Oklahoma State University (OSU), Stillwater, Oklahoma. Previously, she was the director of NIMFFAB (2007–2015), Sarkeys Distinguished Professor of Agricultural Science (2001–2008), professor (1992–2001), associate professor (1988–1992), and assistant professor (1983–1988) at OSU. Dr. Fletcher established a strong research program on the molecular biology, genetics, and host–pathogen interactions of phytopathogenic spiroplasmas and phytoplasmas at OSU. Her research team is recognized internationally for its contributions to the field of plant mycoplasmology, particularly on molecular determinants mediating transmission by insect vectors. Dr. Fletcher was also part of a team of U.S. Department of Agriculture Agricultural Research Service (USDA ARS), Texas A&M University, and OSU scientists that worked on cucurbit yellow vine disease, a damaging cucurbit disorder in the Midwest and East Coast states that is caused by a phloem-inhabiting bacterium. She has also participated in several international research initiatives, such as the International Soybean Program in Mexico and Costa Rica, and has worked on sugarcane whiteleaf disease in Thailand, and on several phytoplasma diseases in Italy. Dr. Fletcher has been invited to speak at numerous national and international venues. She has also served on numerous panels and committees, including the USDA National Institute of Food and Agriculture (NIFA) Departmental Review Panel: Department of Plant Pathology, University of Nebraska (2016); National Academies of Sciences, Engineering, and Medicine, Forum on Microbial Threats (2010–2016);

American Association for the Advancement of Science Consortium of Affiliates for Security Policy (2010–2015); Biosurveillance Subject Matter Expert Panel, Defense Threat Reduction Agency/Los Alamos National Laboratory (2011–2012); USDA ARS National Programs 303 Research Plan Review Panel, Emerging Plant Diseases (chair, 2011–2012); the National Research Council Committee on Global Science and Technology Strategies and Their Effect on U.S. National Security (2009–2010); Inter-Agency Working Group on Citrus Variegated Chlorosis, Animal and Plant Health Inspection Service (APHIS) Select Agent (2007–2008); and Inter-Agency Working Group on Citrus Greening, APHIS Select Agent (2005–2006). Dr. Fletcher is on the editorial board of *Frontiers in Microbial Forensics* and was senior editor for the American Phytopathological Society (APS) Press and associate editor for *Plant Disease*. She has served as an ad hoc reviewer for agencies such as the National Environmental Research Council (Great Britain), the National Science Foundation, the U.S. National Park Service, and USDA National Resources Inventory (NRI)/NIFA and for numerous scientific journals including *Applied and Environmental Microbiology*, *Journal of Bacteriology*, *Molecular Plant–Microbe Interactions*, *Phytopathology*, and *Proceedings of the National Academy of Sciences of the United States of America*. Her honors and awards include the Plant Pathologist of Distinction award from APS (2016); OSU President's Cup–Promoting Creative Interdisciplinary Competition Award (2012); Phoenix Award, Outstanding Graduate Faculty Educator from OSU (2011); and Sigma Xi Lectureship Award (2006). Dr. Fletcher is a member and a fellow of the American Association for the Advancement of Science and the APS. She served as the president of the APS from 2003 to 2004. She is also a member of the American Society for Microbiology, the International Organization for Mycoplasmology, Gamma Sigma Delta (National Agricultural Honor Society), American Women in Science, Oklahoma Academy of Science, and Sigma Xi. She received a B.S. in biology from Emory University in 1970, an M.S. in botany from the University of Montana in 1972, and a Ph.D. in plant pathology from Texas A&M University in 1979.

May R. Berenbaum (NAS) has been on the faculty of the Department of Entomology at the University of Illinois at Urbana-Champaign since 1980, serving as head since 1992 and as Swanlund Chair of Entomology since 1996. She is known for elucidating chemical mechanisms underlying interactions between insects and their host plants, including detoxification of natural and synthetic chemicals, and for applying ecological principles in developing sustainable management practices for natural and agricultural communities. Her research, supported primarily by the National Science Foundation (NSF) and U.S. Department of Agriculture (USDA), has produced over 230 refereed scientific publications and 35 book chapters. She has authored numerous magazine articles and six books about insects for the general public. Dr. Berenbaum is a member of the National Academy of Sciences and has chaired two National Research Council committees, the Committee on the Future of Pesticides in U.S. Agriculture (2000) and the Committee on the Status of Pollinators in North America (2007). Her most recent awards and honors include fellow, Entomological Society of America (2002); Entomological Society of America Distinguished Teaching Award (2006); Tyler Prize for Environmental Achievement (2011); honorary fellow, Royal Entomological Society (2012); fellow, Ecological Society of America (2012); Entomological Foundation Medal of Honor (2014); 2012 National Medal of Science (2104); and John P. McGovern Science and Society Award, Sigma Xi (2015). She has been invited to speak at various symposia and conferences and has served as associate editor for *Entomology*, *American Midland Naturalist*, *CRC Reviews in Plant–Insect Interactions*, *Evolution*, *Journal of Chemical Ecology*, and *Chemoecology* and as a reviewer for numerous scientific journals. She has also served as a program reviewer for the NSF, USDA, National Geographic Society, and National Institutes of Health, among others. She is a member of the American Association for the Advancement of Science, Sigma Xi, Entomological Society of America, Ecological Society of America, International Society for Chemical Ecology, Society for the Study of Evolution, Phytochemical Society of North America (invited member 1985–1995), Lepidopterists Society, and the American Institute of Biological Sciences. Dr. Berenbaum graduated summa cum laude, with a B.S. degree and honors in biology, from Yale University in 1975 and received a Ph.D. in ecology and evolutionary biology from Cornell University in 1980.

Stewart M. Gray is a senior research plant pathologist with the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) and a professor of plant pathology at Cornell University in Ithaca, New York. He received his M.S. in entomology and Ph.D. in plant pathology, both from North Carolina State University. Since moving to Ithaca in 1987, his research has focused on understanding insect vector–virus–plant interactions, the genetics of vector competence in aphid populations, and biological and cultural factors that influence virus epidemics in cereal and potato crops. His long-term goal is to develop sustainable virus disease control practices based on cultural practices, various types of host plant resistance, and the interference of efficient transmission of viruses by their insect vectors. Dr. Gray is internationally recognized as an authority in the field of vector biology and plant virus epidemiology and has authored comprehensive and highly cited review papers on plant virus–aphid–plant interactions and virus disease management. Since 2002, he has worked closely with the U.S. potato industry and USDA Animal and Plant Health Inspection Service

(APHIS) to develop and implement National Harmonization Standards for the management of tuber necrotic viruses. He serves on the National Potato Council subcommittee for Seed Certification and Plant Disease Management and the North American Plant Protection Organization committee on potato diseases. He is the project leader for a national research program (2009–2019) on tuber necrotic viruses funded by USDA ARS, USDA National Institute of Food and Agriculture, USDA APHIS, and the potato industry involving more than 30 investigators at 12 institutions. For his contributions to the potato industry, Dr. Gray was awarded the 2009 Meritorious Service Award by the National Potato Council and U.S. Seed Potato Growers. His other awards include being named a fellow of the American Association for the Advancement of Science (2013) and the American Phytopathological Society (2014) and being recognized as the Senior Scientist of the Year (2013), USDA, ARS, North Atlantic Area. Dr. Gray has received more than $15 million in competitive funding, has published more than 110 refereed publications and book chapters, and has been invited to speak at more than 60 regional, national, and international meetings. He served as senior editor of *Plant Disease* (2007–2010), senior editor of *Phytopathology* (2015–2016), associate editor of *Virology* (1994–2000), and associate editor of the *Journal of General Virology* (2010–2017).

Russell L. Groves currently serves as a vegetable extension specialist and professor in the Department of Entomology at the University of Wisconsin–Madison, where he also served as associate professor (2012–2016) and assistant professor (2006–2012). Prior to working at UW–Madison, he was a research entomologist at U.S. Department of Agriculture Agricultural Research Service (USDA ARS), San Joaquin Valley Agricultural Sciences Center in Parlier, California, where he worked on the bacterium *Xylella fastidiosa* and its insect vector, the glassy-winged sharpshooter. His extension and research program at UW–Madison is centered on the ecology and management of insects of commercial and fresh market vegetable crops. Among his recent awards are the UW ARS Researcher Award, University of Wisconsin, Agricultural Research Stations, College of Agricultural and Life Sciences (2015); Second Mile Award from the Wisconsin Association of County Agricultural Agents (2014); Glen Pound Extension Award from the College of Agricultural and Life Sciences (2013); American Society of Agronomy, Certificate of Excellence, 2013 Educational Materials Award; Outstanding National Extension Project Award (2011) from the Potato Association of America; and Researcher of the Year award from the Wisconsin Potato and Vegetable Growers Association (2009). Dr. Groves has served on the research board of several grower associations in Wisconsin. He has also served as grants reviewer for the Ecological Genomics Institute, Kansas State University (2008); the Citrus Research and Development Foundation

(2010–2015); and the National Science Foundation (2009, 2012, 2013) and for several USDA National Institute of Food and Agriculture grants programs, including the Specialty Crop Research Initiative, Citrus Disease Research and Extension (2015), and the Special Grants Program, Citrus Tristeza Virus (2007). Dr. Groves served on the Frito-Lay North America Zebra Chip Technical Advisory Board (2006–2014), was the chair for the Frito-Lay Research Grant Panel (2006–2008), and was a reviewer for the grants program of the California Department of Food and Agriculture Pierce's Disease Research and Control Program (2006–2015). He has been invited to give seminars at various universities and to speak at numerous conferences. He has published more than 50 peer-reviewed articles and more than 150 nonrefereed/technical papers. Dr. Groves is a member of the Gamma Sigma Delta National Honor Society, the Phi Kappa Phi National Honor Society, the Entomological Society of America, and the American Phytopathological Society. He holds an M.S. degree in entomology from the University of Arkansas and a Ph.D. in entomology from North Carolina State University.

Ralph Scorza was employed by the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) as a research horticulturist and lead scientist from 1980 until his retirement in September 2016. He received his B.S. and M.S. degrees in agronomy and plant physiology, respectively, from the University of Florida, and received his Ph.D. in plant genetics and breeding from Purdue University. His peach, nectarine, and plum breeding programs combined classical and molecular approaches to improve tree form, disease resistance, and fruit quality. Dr. Scorza led the development of the genetically engineered (GE) plum pox virus-resistant plum cultivar "HoneySweet," which was the first GE temperate tree fruit approved for cultivation in the United States and the first crop of any kind to be approved for commercialization in the United States exhibiting RNA interference–based genetic modification. Dr. Scorza currently leads an international team that is working to submit HoneySweet for cultivation approval in the European Union. Dr. Scorza worked closely with the USDA Foreign Agricultural Service to provide information and guidance on plant biotechnology regulation to government agencies in a number of countries, including Bosnia-Herzegovina, Bulgaria, Chile, France, and Serbia. He is a co-developer of the "FasTrack" breeding technology, a novel breeding approach that incorporates genetic engineering to reduce the time required for developing new fruit tree varieties by at least half. Using the products of FasTrack technology he worked with NASA to adapt miniature and ever-fruiting plum trees for long-distance space flight and for Mars colonization. Dr. Scorza is the recipient of the Flemming Award for "exceptionally creative and useful research and leadership in the area of stone fruit breeding and genetics," a recipient of a USDA ARS Senior Research Scientist of the Year Award, and has been co-recipient of three U.S. Secretary of Agriculture Honor Awards. He is a recipient of the USDA Foreign Agricultural Service Distinguished International Service Award and the U.S. National Peach Council Carroll R. Miller Award. He has developed and released 12 stone fruit cultivars that are currently marketed in the United States. Dr. Scorza is the co-inventor of four biotech patents, and has authored over 200 research publications. He is a fellow of the American Society for Horticultural Science, and in 2015 he was honored by induction into the USDA Science Hall of Fame. Dr. Scorza is currently the principal of Ralph Scorza LLC, providing consultation services in plant biotechnology science, the regulation of the products of plant biotech, and in plant breeding and plant patenting.

Lindsay R. Triplett is the primary investigator and assistant agricultural scientist II at the Jenkins-Waggoner Laboratory in the Department of Plant Pathology and Ecology at the Connecticut Agricultural Experiment Station. She studies the DNA sequences of plant-infecting bacteria to answer questions about their virulence mechanisms and phylogenetic diversity, and to develop diagnostic tools for disease detection. In the past, her research has characterized the molecular interactions between *Xanthomonas oryzae* and rice, focusing on resistance to a few secreted proteins that act as toxins or transcriptional activators. She is now continuing that line of research on other plant pathogenic *Xanthomonas* species that infect a variety of crops. Dr. Triplett is a member of the American Phytopathological Society and the American Society for Microbiology. She has also received many grants and awards, such as the American Phytopathological Society award for the "Face of the Future in Phytobacteriology." Dr. Triplett received her B.A. in biology from Earlham College and her Ph.D. in plant pathology from Michigan State University.

John Trumble is currently a distinguished professor at the Department of Entomology, University of California (UC), Riverside, where he also served as full professor (1990–2011), associate professor (1985–1990), and assistant professor (1980–1985). His current research covers both basic and applied problems in agricultural and natural ecosystems and includes the effects of air and water contaminants on plant–insect interactions, particularly how increasing concentrations of contaminants impact plant physiology and how any resulting chemical or growth changes may impact insect development and behavior. Dr. Trumble was previously engaged in zebra chip research and was co-director of the Zebra Chip Research Team, which was formed in 2005 to address the devastating effect of the zebra chip pathogen, *Candidatus* Liberibacter psyllaurous/solanacearum, on the potato industry. This research team received the Entomological Society of America (ESA) Integrated Pest Management Team Award in 2012 for developing techniques and strategies that helped control the disease. His recent honors and awards include the National Institute of Food and Agriculture Partnership Award for Mission Integration of Research, Extension, and Education (2014); the Oscar Lorenz Award (2013) from the UC Davis Plant Sciences Department, in recognition of his meritorious service to the California vegetable industry; the Texas A&M Award in Excellence (2013); plenary apeaker, International Congress on Climate Change (2009); the Outstanding Entomology Alumnus Award, Virginia Tech (2007); team leader, International team to evaluate Biosecurity and Applied Entomology in New Zealand (2007); and the ESA Research Recognition Award (2003). Dr. Trumble is a fellow of the American Association for the Advancement of Science and ESA. He is the editor in chief of the *Journal of Economic Entomology* (2001 to present) and is a member of the editorial board of the *Annual Review of Entomology* (2000 to present) and *Insect Science* (2002 to present). He was an editor for the *Annals of Applied Biology* (2009–2015). Dr. Trumble has served on numerous grant panels, including those of the National Institutes of Health, the Environmental Protection Agency, and U.S. Department of Agriculture National Resources Inventory and has been invited to speak at more than 30 symposia and seminars since 2005. He has published more than 200 peer-reviewed journal articles and more than 100 technical publications. Dr. Trumble received an M.S. and a Ph.D. in entomology from Virginia Tech in 1977 and 1980, respectively.

Bing Yang is currently an associate professor of development and cell biology at Iowa State University. His research focuses on the molecular mechanism of plant–microbe interactions and crop disease resistance engineering, and the development and application of TALEN (transcription activator-like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeats) technologies for targeted genome editing in plant species. For the past 19 years, Dr. Yang has identified and characterized several important naturally occurring transcription activator-like (TAL) effectors in the rice pathogen, *Xanthomonas oryzae*, for their disease-promoting ability and, most recently, he has helped harness the disease-causing TAL effectors for targeted gene editing. His group has generated the first disease-resistant crop plant by using the TALEN technology. Previously, he was research assistant professor of plant pathology at Kansas State University. Dr. Yang received his B.S. in 1986 and his M.S. in 1989 from Southwest Forestry College in China. In 2000, he received his Ph.D. from Kansas State University, where he continued his studies as a postdoctoral research fellow from 2001 to 2004.

Appendix B

Open Session Meeting Agendas

FIRST MEETING AGENDA
March 15, 2017

In conjunction with the
5th International Research Conference on Huanglongbing
Caribe Royale Hotel
8101 World Center Drive, Orlando, FL 32821

3:00 pm **Welcome, Introductions, Process for Open Session**
Robin Schoen, Director, Board on Agriculture and Natural Resources
Jacqueline Fletcher, Committee Chair

3:15 **HLB Multiagency Initiative-Funded Projects**
Mary Palm, Director, Pest Management PHP, PPQ, APHIS, USDA

3:35 **APHIS/PPQ HLB-Related Activities**
Don Seaver, National Science Manager USDA APHIS PPQ S&T

3:55 **Overview of HLB in Florida and the Citrus Research and Development Foundation**
Harold Browning, CRDF
Q&A with Harold Browning (clarification on the Statement of Task, expectations from this study/review, materials they will provide us, what types of info we want from them, etc.)

4:55 **Review of HLB Research Supported by the California Citrus Research Board**
Gail Wisler, USDA ARS (Retired)

5:15 **Public Comments**

5:25 **Closing Remarks and Adjournment of Closed Session**
Jacqueline Fletcher, Committee Chair

SECOND MEETING AGENDA
May 22–23, 2017

Forum on Citrus Breeding and Transformation for HLB Resistance
Arnold and Mabel Beckman Center of the
National Academies of Sciences, Engineering, and Medicine
100 Academy Way, Irvine, CA 92617

Monday, May 22, Board Room

8:15 am **Welcome, Introductions, and Overview of Forum Agenda**
Jacqueline Fletcher, Committee, Forum Attendees

PART I – PIs of projects on citrus breeding/transformation will be given 15 minutes maximum to address the following questions:

1. What approaches/techniques are you employing to come up with a citrus plant that is resistant to HLB?
2. What are your major accomplishments to date?
3. What are the hurdles you have encountered/what do you need to overcome in order to achieve your goals?
4. If relevant to your work, how close are you to identifying any target(s) to modify by genome editing?

8:30 **Fred Gmitter,** University of Florida (UF) Citrus Research and Education Center (CREC)

8:45	**Jude Grosser**, UF CREC
9:00	**Nian Wang**, UF CREC
9:15	**Ed Stover**, USDA ARS U.S. Horticultural Research Laboratory (USHRL)
9:30	**Zhonglin Mou**, UF Institute of Food and Agricultural Sciences
9:45	**Yi Li**, University of Connecticut, College of Agriculture, Health, and Natural Resources
10:00	**COFFEE BREAK**
10:15	**Janice Zale**, UF CREC Mature Citrus Facility
10:30	**Vladimir Orbović**, UF CREC Transformation Lab
10:45	**Tim McNellis**, Pennsylvania State University, College of Agricultural Sciences
11:00	**James Thomson**, USDA ARS Western Regional Research Center
11:30	**Kim Bowman**, USDA ARS USHRL
11:45	**Mike Irey**, Southern Gardens (to discuss GE CTV)
12:00 pm	**LUNCH**

PART II – PIs, committee members, and invited guests to discuss experiences and insights from work on citrus/other crops and possible ways to overcome the current challenges in citrus breeding and transformation for HLB resistance and approaches/techniques that are worth exploring.

Discussants: All Part I participants, committee members, and invited discussants:

- **Chris Dardick**, USDA ARS, Appalachian Fruit Research Laboratory (via WebEx)
- **Erik Mirkov**, TAMU Agrilife Research and Extension Center (via WebEx)
- **Gloria Moore**, UF (via WebEx)
- **Mikeal Roose**, UC Riverside
- **Dan Voytas**, University of Minnesota (via WebEx)
- **Yinong Yang**, Pennsylvania State University

1:00 **Discussion Objectives and Guidelines**
Jacqueline Fletcher, Committee Chair

1:05 **Development of a GE Citrus and Field Screening**
Erik Mirkov, TAMU
Mike Irey, Southern Gardens

1:20 **Discussion**

3:00 **COFFEE BREAK**

3:10 **Continuation of Discussion**

4:30 **End of Forum**

Tuesday, May 23, Board Room

9:00 am **Welcome, Introductions**
Jacqueline Fletcher, Committee Chair

9:05 **CA Citrus Industry and CRB Perspectives on HLB**
Gary Schulz, President, Citrus Research Board
Jim Gorden, Chairman, Citrus Research Board

9:45 **COFFEE BREAK**

10:00 **Introduction of Keynote Speaker: Anthony James**
Jacqueline Fletcher

10:05 **Synthetic Biology and the Malaria Eradication Agenda**
Anthony James, NAS
Donald Bren Professor, Microbiology & Molecular Genetics School of Medicine
Molecular Biology and Biochemistry School of Biological Sciences, UC Irvine

11:00 **Adjourn Open Session**

THIRD MEETING AGENDA

Forum on Asian Citrus Psyllid (ACP) Control/Management
July 24, 2017

Keck Center of the National Academies of Sciences, Engineering, and Medicine
500 Fifth Street, NW, Washington, DC 20001

Monday, July 24, Room 105

8:15 am **Welcome, Introductions, and Overview of Forum Agenda**
Jacqueline Fletcher, *Committee, Forum Attendees*

PART I – Invited speakers will be given 30 or 45 minutes to address the questions below. Each speaker is requested to represent and present the overall state of ACP-related research on his/her topic, to include not only his/her own work but also that of others in the research community, so that the committee will gain an understanding of each of these critical areas of research.

1. In general, what have been the major research accomplishments, over the past 10 years, by your own research group as well as other groups, in the area about which you are speaking?
2. What research results/outcomes generated by the research community have been useful to ACP or HLB control, and how?
3. What are the important current research efforts in these areas?
4. To what extent are research collaborations and shared resources contributing to the progress and success of these projects?
5. (If applicable) What is slowing down the translational aspects of this area of research?
6. What other information/research is needed in the future to come up with better strategies for controlling ACP, and therefore to control HLB?

8:30 **ACP Chemical Control, Biocontrol, and Integrated Pest Management**
Lukasz Stelinski, UF Citrus Research and Education Center
Jawwad Qureshi, UF Indian River Research and Education Center (IRREC)
Beth Grafton-Cardwell, UC Riverside

10:15 **Comments from other PIs of projects on ACP control (chemical, biological, IPM) and other forum participants**

10:30 **BREAK**

10:45 **RNAi and Other Genomic Approaches for ACP Control, and Use of CTV Vectors to Deliver Transmission-Disruptive Molecules into ACP**
William Dawson, UF

11:30 **Comments from other PIs of projects on RNAi and other genomic approaches for ACP control use of CTV vectors to deliver transmission-disruptive molecules into ACP and other forum participants**

12:00 pm **LUNCH**

1:00 **Vector–Pathogen Interactions and Interrupting CLas Transmission by ACP**
Kirsten Pelz-Stelinski, UF CREC

1:45 **Ecology and Behavior of ACP and Vector–Host Interactions**
Lukasz Stelinski, UF CREC

2:30 **Comments from other PIs working on vector ecology, interactions of vectors with the pathogen and the host, and transmission of CLas and other forum participants**

PART II – Discussion of experiences and insights from work on ACP or other insects that are vectors of plant pathogens and possible ways to overcome the current challenges in ACP research and approaches or techniques that are worth exploring.

Discussants: All Part I participants, committee members, and invited experts in ACP/psyllids/other vectors, insect-vectored plant pathogens, insect genomics, RNAi:

- **Susan Halbert**, Florida Department of Agriculture and Consumer Services (via WebEx)
- **David Hall**, USDA ARS, U.S. Horticultural Research Laboratory (USHRL)
- **Michelle Heck**, USDA ARS/Boyce Thompson Institute/Cornell University (via WebEx)
- **Wayne Hunter**, USDA ARS, USHRL

- **Jawwad Qureshi,** UF Indian River Research and Education Center (IRREC) (via WebEx)
- **Mamoudou Sétamou,** Texas A&M University, Kingsville (via WebEx)
- **Robert Shatters, Jr.,** USDA ARS, USHRL

2:45 **Discussion Objectives and Guidelines**
Jacqueline Fletcher, Committee Chair

2:50 **Discussion of Challenges/Ways to Overcome Challenges and Other Approaches to Explore**

3:30 **BREAK**

3:45 **Continuation of Discussion**

5:00 **End of Forum/Adjourn Open Session**

FIRST WEBINAR AGENDA

Citrus Greening (HLB) Diagnostics and Detection
August 24, 2017

10:00 am **Welcome, Introductions, and Overview of Webinar Agenda**
Jacqueline Fletcher, Committee Chair; Webinar Participants

PART I – SPEAKER PRESENTATIONS

10:10 **Greg McCollum,** U.S. Horticultural Research Laboratory, USDA ARS

10:40 **Q&A**

10:50 **HLB Detection Using Canines**
Tim Gottwald, U.S. Horticultural Research Laboratory, USDA ARS

11:00 **HLB Detection Using Optical Sensors**
Sindhuja Sankaran, Washington State University/Reza Ehsani, UC Merced

11:10 **HLB Detection Using VOCs**
Cristina Davis, UC Davis

11:20 **HLB Detection Using Plant Metabolites**
Carolyn Slupsky, UC Davis

Invited speakers are requested to use the questions below as a guide for their presentations; each speaker is requested to represent and present the overall state of HLB diagnostics- and detection-related research on his/her assigned topic, to include not only his/her own work but also that of others working in that research area(s) so that the committee will gain an understanding of each of these critical areas of research.

1. In general, what have been the major research accomplishments, over the past 10 years, by your own research group as well as other groups, in the area about which you are speaking?
2. What research results/outcomes generated by the research community have been useful to ACP or HLB control, and how?
3. What are the important current research efforts in these areas?
4. To what extent are research collaborations and shared resources contributing to the progress and success of these projects?
5. (If applicable) What is slowing down the translational aspects of this area of research?
6. What other information/research is needed in the future to come up with better strategies for diagnosing/detecting HLB, and therefore controlling HLB?

PART II – DISCUSSION

Discussants: All invited speakers, committee members, registered participants, and invited experts in HLB diagnostics and detection:

- **Manjunath Keremane**, USDA ARS, National Clonal Germplasm Repository for Citrus (via WebEx)
- **John Rascoe**, USDA ARS, APHIS (via WebEx)

11:30 **Discussion Guidelines**
Jacqueline Fletcher, Committee Chair

11:45 **Discussion**

- Experiences and insights from work on HLB diagnostics and detection
- Possible ways to overcome the current challenges in this area of research
- Approaches or techniques that are worth exploring

12:45 pm **Closing Remarks/Adjourn Webinar**

SECOND WEBINAR AGENDA

CLas and Bacterial Control
September 28, 2017

2:30 pm **Welcome, Introductions, and Overview of Webinar Agenda**
Jacqueline Fletcher, Committee Chair; Webinar Participants

PART I – SPEAKER PRESENTATIONS

2:40 **CLas Culturing Needs, Phage, and Quorum Sensing**
Dean Gabriel, University of Florida

3:10 **Q&A**

3:15 **Efforts to Exploit CLas Requirements for Interaction with the Insect and Plant**
Yong-Ping Duan, U.S. Horticultural Research Laboratory, USDA ARS

3:45 **Q&A**

3:50 **Efforts to Control CLas/HLB Using Bactericides or Other Chemical Compounds**
Robert Shatters, Jr., U.S. Horticultural Research Laboratory, USDA ARS

4:20 **Q&A**

Invited speakers are requested to use the questions below as guide for their presentations; each speaker is requested to represent and present the overall state of CLas/bacterial control-related research on his/her assigned topic, to include not only his/her own work but also that of others working in that research area(s) so that the committee will gain an understanding of each of these critical areas of research.

1. In general, what have been the major research accomplishments, over the past 10 years, by your own research group as well as other groups, in the area about which you are speaking?
2. What research results/outcomes generated by the research community have been useful to CLas or HLB control, and how?
3. What are the important current research efforts in these areas?
4. To what extent are research collaborations and shared resources contributing to the progress and success of these projects?
5. (If applicable) What is slowing down the translational aspects of this area of research?
6. What other information/research is needed in the future to come up with better strategies for controlling CLas/HLB?

PART II – DISCUSSION

Discussants: All invited speakers, committee members, registered participants and invited discussants:

- **Gitta Coaker,** UC Davis
- **David Gang,** WSU
- **Claudio Gonzalez,** UF
- **Michelle Heck,** USDA-ARS/Cornell
- **Graciela Lorca,** UF
- **Swadesh Santra,** UCF
- **Nian Wang,** CREC

4:25 **Discussion Guidelines**
Jacqueline Fletcher, Committee Chair

4:30 **Discussion**

- Experiences and insights from work on CLas/bacterial control
- Possible ways to overcome the current challenges in this area of research
- Approaches or techniques that are worth exploring

5:00 **Closing Remarks/Adjourn Webinar**

THIRD WEBINAR AGENDA

Economic/Sociological Impacts of HLB/HLB Management Strategies
October 18, 2017

10:00 am **Welcome, Introductions, and Overview of Webinar Agenda**
Jacqueline Fletcher, Committee Chair

PART I – SPEAKER PRESENTATIONS

10:10 **Economic Impact of Florida Citrus Industry and Importance of Investment in HLB Mitigation and Management Strategies in Preserving the Industry for the Long Term**
Marisa Zansler, Florida Department of Citrus

10:40 **Q&A**

10:45 **Economic Evaluation of ACP/HLB Control/Management Strategies**
Fritz Roka, UF Southwest Florida Research and Education Center

11:05 **Q&A**

11:10 **Economic Barriers to Participation in Citrus Health Management Areas (CHMAs)**
Ariel Singerman, UF CREC

11:30 **Consumer Attitudes Toward Genetic Modification to Manage HLB**
Lisa House, UF

11:50 **Q&A**

Invited speakers may address these questions in their presentations or during the discussion (Part II of webinar).

1. In what ways do sociological and economic factors influence the successful implementation of scientifically derived HLB management strategies?
2. How can the social science and agricultural economics research communities help the citrus industry with HLB management?
3. Is there a need for more economic/sociological studies, and if so, on what topics related to HLB/HLB management?
4. Are the current assessment methods suitable and adequate or is there a need for different models and resource bases?
5. How can we increase research utilization and ensure that the best available knowledge is used to inform policy and grower practice?

PART II – DISCUSSION

Discussants: All invited speakers, committee members, and registered participants.

11:55 am **Discussion Guidelines**
Jacqueline Fletcher, Committee Chair

12:00 pm **Discussion**

- Experiences and insights from work on the economic aspects of HLB/HLB management
- Possible ways to overcome the current challenges in this area of research
- Approaches or techniques that are worth exploring

1:00 **Closing Remarks/Adjourn Webinar**

FOURTH WEBINAR AGENDA

Forum on Cultural Practices to Keep HLB-Infected Trees Productive
November 20, 2017

9:30 am **Welcome, Introductions, and Overview of Discussion Agenda**
Jacqueline Fletcher, Committee, Forum Attendees

At this forum, Florida citrus growers will discuss the cultural practices they currently employ in their groves to keep HLB-infected trees productive. The growers were asked to address the following questions:

1. Can you describe how you are taking care of HLB-infected trees?
2. About how much yield increase (if any) have you seen since employing your current practices?
3. Are you also spraying for ACP/participating in a CHMA?
4. How much of your current grove practices are based on research results generated by the HLB research community?
5. What current HLB management strategies that resulted from HLB research community are you employing in your groves and why did you choose to employ them?

9:40 **Larry Black, Jr.**, Peace River Packing Company

9:50 **Bruce Ingram**, Southern Gardens Groves, Inc.

10:00 **Ned Hancock**, Hancock Citrus

10:10 **Marty McKenna**, McKenna Brothers Citrus

10:20 **David Howard**, Graves Brothers Co.

10:30 **Lee Jones**, Gardinier Florida Citrus

10:40 **Q&A**

11:00 **Adjourn Open Session**

Appendix C

Glossary

α-proteobacteria: A class of proteobacteria that are gram-negative; included in this class are most genera of phototrophic bacteria and several genera of bacteria that metabolize chemical compounds containing only one carbon atom, bacteria that are symbionts of plants and animals, and a group of pathogens (the Rickettsiaceae).

Abaxial (leaf surface): The upper side of the leaf.

Acquisition: For circulative pathogens like *Candidatus* Liberibacter spp., passage of ingested pathogens through the gut epithelial cells into the insect vector hemocoel (the blood-filled cavity of arthropods).

Acyl-homoserine lactone (AHL): A class of signaling molecules that are involved in bacterial quorum sensing (see quorum sensing).

Adaxial (leaf surface): The lower side of the leaf.

Advanced citrus production systems: See advanced production systems.

Advanced production systems: Production systems aimed at bringing new citrus groves into commercially viable production quicker than in traditional plantings by employing open hydroponics or intensive fertigation, high planting density, and a suitable rootstock capable of developing a compact tree and an efficient root system in the fertigated soil zone.

***Agrobacterium*:** A bacterium that causes crown gall disease in a variety of plant hosts by horizontal transfer of pathogen genes for expression in the host; also an important tool in molecular biology for genetic engineering.

Aminoglycoside antibiotics: Traditional gram-negative antibacterial therapeutic agents that inhibit protein synthesis.

Annotation (genome sequence): The process of determining the location of genes and coding regions in a genome and their functions.

Anthocyanin marker: A blue, violet, or red flavonoid pigment found in plants that has been found to be a suitable visible selectable marker for plant transformation.

Antimicrobial proteins (AMPs): Low-molecular-weight proteins having broad spectrum antimicrobial activity against bacteria, viruses, and fungi. Those larger than 100 amino acids are often lytic enzymes or nutrient-binding proteins, or contain sites that target specific microbial macromolecules.

***Arabidopsis*:** A genus of small, flowering plants in the Brassicaceae family that is popular as a model organism in plant biology and genetics research because of its small genome and rapid life cycle.

Assembly (genome): Aligning and merging sequenced fragments of DNA so as to reconstruct the original complete sequence in proper order.

Attract-and-kill: A pest control approach or strategy that involves luring the insect (for example, with pheromones) and then killing it with an insecticide.

Aurantioideae: A taxonomic subfamily within the family Rutaceae, which contains the citrus.

Bacteriome: A specialized organ, found mainly in some insects, that hosts endosymbiotic bacteria.

Bacteriophage: A virus that parasitizes a bacterium by infecting it and reproducing inside it (see also phage).

β-lactam antibiotics: A class of broad-spectrum antibiotics, consisting of all antibiotic agents that contain a beta-lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems.

Biofilm: A slimy film of microorganisms and extracellular polysaccharides that adheres to a surface; cells within a biofilm undergo phenotypic shifts in which large suites of genes are differentially regulated.

Bioinformatic: Related to the analysis of biological information using computers and statistical techniques.

Biomarker: A substance, chemical, or gene used as an indicator of disease or a biological state.

Biosensor: A device that uses a living organism or biological molecules, such as enzymes or antibodies, to detect the presence of chemicals.

Brassinosteroids: A class of polyhydroxylated steroidal phytohormones with structures similar to steroid hormones produced by animals. Brassinosteroids regulate a wide range of physiological processes, including plant growth, development, and immunity.

Brix: The sugar content of an aqueous solution.

Callose: A plant polysaccharide composed of glucose residues linked together through β-1,3-linkages secreted by an enzyme complex (callose synthase), resulting in the hardening or thickening of plant cell walls.

***Candidatus*:** A modifier appended to the binomial taxonomic name of an organism that cannot be fully characterized because it cannot be maintained in artificial culture medium.

cDNA library: A combination of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which together constitute some portion of the transcriptome of the organism and are stored as a "library."

Chelate: To form a compound from an organic ligand and a central metal ion at two or more points; chelation often facilitates the uptake of nutrients or removal of toxic substances.

Chemical genetics: The technique of screening for small-molecule modulators.

Chemical genomics approach: The systematic screening of targeted chemical libraries of small molecules against individual drug target families with the ultimate goal of identification of novel drugs and drug targets.

Circulative transmission: Movement of a pathogen from the insect foregut to the mid- and hindgut, from which it is transported to the hemolymph and further to the salivary gland, from which it is released into the plant tissue during insect feeding.

Citrus canker: An economically damaging disease caused by the bacterium *Xanthomonas axonopodis* pv. *citri*; canker outbreaks have occurred periodically in Florida despite a 10-year effort to eradicate the disease from Florida. A series of legal challenges and an unprecedented rash of storms in 2004 and 2005 led to disease spread to a point in 2006 at which eradication was no longer considered possible.

Citrus Health Management Areas (CHMAs): Groupings of commercial citrus groves in close proximity where growers work cooperatively to manage the spread of HLB. The goal of CHMAs is to coordinate the timing and ensure the proper rotation of pesticide mode of action to obtain the best psyllid control possible while minimizing the potential for pesticide resistance development.

Citrus tristeza virus (CTV): An aphid-transmitted, phloem-residing *Closterovirus* that causes the most destructive disease of citrus in the Western Hemisphere and has a worldwide distribution; viral vectors based on modifications of the *Citrus tristeza virus* RNA are useful for transfecting citrus trees for beneficial purposes.

Clustered regularly interspersed short palindromic repeats (CRISPR): Segments of prokaryotic DNA containing short, repetitive base sequences occurring naturally as an acquired immune response system in bacteria and archaea; these have been adopted recently as a component of a gene-editing system (see gene editing).

Cofactor: A nonprotein chemical compound or metallic ion that is required for a protein's biological activity to happen.

Cost–benefit ratio: An indicator, used in cost–benefit analysis, that attempts to summarize the overall value for money of a project or a proposed approach.

Defense marker gene(s): Markers that are associated with a plant response to pathogen infection.

Diapause: A period of suspended or arrested development during an insect's life cycle.

Downregulation: The process by which a cell decreases the quantity of a cellular component, such as RNA or protein, in response to an external variable.

Ectopic: In an abnormal place or position.

Effector (bacterial): A protein such as an inducer, a corepressor, or an enzyme, secreted by pathogenic bacteria into host cells, that activates, controls, or inactivates a process or action; effectors usually help the pathogen to invade host tissue, suppress its immune system, or otherwise help the pathogen to survive, and are usually required for virulence.

Electrical penetration graph (EPG): A technology used to study the interactions of insects with plants through the creation of an electrical circuit by attaching a conductive wire to the insect and to the plant, with resulting signal patterns displayed as a waveform graph; often used to study the basis of plant pathogen transmission, host plant selection by insects, and the way insects can find and feed from the plant phloem.

Endosymbiont: An organism living symbiotically inside the cells or body of another organism.

Entomopathogen: Organisms capable of causing disease in insects.

Explant: Small piece of plant tissue that is aseptically cut and used to initiate a culture in a nutrient medium.

Fecundity: The ability to produce an abundance of offspring.

Fertigation: The injection of fertilizers, soil amendments, and other water-soluble products into an irrigation system.

Fitness: The ability of organisms to survive and reproduce in the environment in which they find themselves.

Flight mill: A device for measuring the speed, distance, and periodicity of insect flight.

Flush (citrus flush): New leaves produced simultaneously on all branches of a bare plant or tree; results in an abundance of young, tender leaf tissue to which citrus-feeding insects are attracted.

Gene editing: Unlike genetic engineering, this technology can be used to very precisely edit or change the genetic code of an organism's own native genome at precise locations; nucleases are used to cut DNA at a specific location in the genome, and the cell's DNA repair mechanisms can be directed to introduce, delete, or replace specific sections of the genetic code.

Genetic engineering: A technology that is employed to alter the genetic material of living cells by introducing foreign DNA (from the same or a different species, or even artificially synthesized DNA) so that they produce new substances or perform new functions; typically the location where the new DNA sequence inserts into the genome is random.

Glutathione-S-transferase: An enzyme catalyzing the conjugation of reduced glutathione to xenobiotic substrates, increasing their solubility, for the purpose of detoxification.

Graft: To unite a shoot or bud (i.e., scion) to an established plant (i.e., stock) by insertion or attachment, or the plant construct resulting from that union.

Graft inoculation: Method of inoculation often used for the initial establishment of infection of a nonmechanically transmissible pathogen, involving the grafting (see graft) of scions excised from symptomatic parts of the infected plant.

Gram-negative (bacteria): Characterized by cell envelopes composed of a thin peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane. Named for a characteristic staining reaction.

Gustatory: Of or relating to taste or tasting.

Hemipteran: Any of various insects of the order Hemiptera, having biting or sucking mouthparts and two pairs of wings.

High-throughput: An approach or method that involves automation such that large-scale repetition becomes feasible.

Hybridization (citrus): The crossing of two individuals or plants or lines with dissimilar genotype, resulting in a hybrid.

Immunity: A condition of being able to resist a particular disease, especially through preventing development of a pathogenic microorganism or by counteracting the effects of its products.

Inoculation: For *Ca.* Liberibacter spp. it is the passage of pathogens in saliva from the salivary glands (of the insect vector) into phloem sieve elements via salivation.

Inoculative (insects): Infective insects that will transmit during a given test access period.

Inoculum: Biological material, cell, or part of a pathogen that induces disease.

Instar: A developmental stage of insects, between each molt, until sexual maturity is reached.

Integrated pest management (IPM): A strategy aimed at long-term prevention of pests or their damage by using a combination of techniques such as biological control, habitat manipulation, modification of cultural practices, and use of resistant varieties.

Intellectual property: Work or invention to which one has rights and for which one may apply for a patent, copyright, trademark, etc.

Juvenility: Plant growth stage during which a plant cannot be induced to flower.

Latent period (incubation period): In plants, the interval in the course of a disease between plant infection and the first appearance of the symptoms; in insects, the interval between pathogen acquisition and inoculativity.

Leucine-rich repeat (LRR): A protein composed of repeating 20– to 30–amino acid stretches that are unusually rich in the hydrophobic amino acid leucine; frequently involved in protein–protein interactions.

Loop-mediated isothermal amplification (LAMP): A technique for the amplification of DNA, which involves a reaction that takes place in a single tube containing buffer, target DNA, DNA polymerase, and primers.

LuxR: A protein (the transcriptional activator of luminescence) involved in quorum sensing (see quorum sensing) and intercellular communication in bacterial species.

Lysogenic (cycle): One of two cycles in bacterial virus reproduction, characterized by the integration of the bacteriophage nucleic acid into the host bacterium's genome or formations of a circular replicon in the bacterial cytoplasm. See also lytic.

Lytic (cycle): One of two cycles in bacterial virus reproduction that results in the destruction of the infected cell and its membrane. See also lysogenic.

Master regulator: A gene at the top of a gene regulation hierarchy, particularly in regulatory pathways related to cell fate and differentiation.

Meta-analysis: An approach that assumes that there is a common truth behind all conceptually similar scientific studies, but which has been measured with a certain error within individual studies and uses statistical approaches to derive a pooled estimate closest to the unknown common truth.

Metabolomics: The study of the unique chemical fingerprints left by specific cellular processes, and of their small-molecule metabolite profiles.

Microbiome: The ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share an environmental niche such as a living organism.

Microfluidics: A multidisciplinary field producing systems in which low fluid volumes are processed to achieve multiplexing, automation, and high-throughput screening.

Miraculin: A glycoprotein extracted from *Synsepalum dulcificum* berries that can serve as a natural sugar substitute.

Mitogenome: The DNA located within mitochondria.

Morphotype: Any of a group of different individuals of the same species in a population, a morph.

Mutagenesis: A process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation.

Nanoemulsion: An emulsion in which the disperse phase consists of nano-sized particles.

Nanoparticle: A particle between 1 and 100 nanometers in size with a surrounding interfacial layer.

Neonicotinoid: A class of neuroactive insecticides chemically similar to nicotine.

Nucellar embryony: A form of seed reproduction in which embryos genetically identical to the parent plant develop from the nucellar tissue.

Nymph: An immature form of an insect that does not change greatly as it grows.

Olfactory receptor: A protein that binds odor molecules.

Olfactory receptor co-receptor (Orco): Odorant co-receptor that complexes with conventional odorant (olfactory) receptors to form odorant-sensing units.

Omics: A field of study in biology ending in -omics, such as genomics, proteomics, or metabolomics.

Ortholog: Any gene found in two or more species that can be traced to a common ancestor.

Oviposition: The process of laying eggs.

Parasitoid: An insect whose larvae live as parasites that eventually kill their hosts, typically other insects.

Pathogen-associated molecular patterns (PAMPs): Small molecular motifs associated with groups of pathogens, conserved within a class of microbes, that are recognized by cells of the innate immune system.

Phage: Shortened form of bacteriophage (see bacteriophage).

Phage therapy: Therapeutic use of bacteriophages to treat pathogenic bacterial infections.

Phagostimulant: A chemical that stimulates feeding.

Phenology: The study of cyclic and seasonal natural phenomena.

Phototaxis: The movement of an organism either toward or away from a source of light (adj: phototactic).

Phylogenetic: Relating to the evolutionary development and diversification of a species or group of organisms, or of a particular feature of an organism.

Phytobiome: Plants, their environment, and their associated communities of organisms.

Phytoplasmas: Cell wall–less prokaryotic parasites of plant phloem and of insect vectors involved in their transmission.

Plant growth regulator (PGR): A natural or synthetic chemical that alters plant growth or development, also referred to as plant hormones.

Polyembryonic: Forming multiple embryos from a single fertilized ovum or in a single seed.

Polymerase chain reaction (PCR): A molecular technique to amplify a single or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Probe: A fragment of DNA or RNA that can be labeled and used to detect the presence of nucleotide sequences that are complementary to the probe sequence.

Promoter: A region of a DNA molecule that forms the site at which transcription of a gene begins.

Propagative transmission: Pathogen transmission characterized by a long period of acquisition of the pathogen by a vector, a latent period before the vector is able to transmit the pathogen, and retention of the pathogen by the vector for a long period because the pathogen reproduces or replicates in the vector.

Prophage: The genetic material of a bacteriophage, incorporated into the genome of a bacterium and able to produce phages if specifically activated.

Protease: An enzyme that digests proteins and peptides.

Protein marking (of insects): The application of a distinctive and detectable marker protein to populations of insects such that they can later be detected by a method, such as enzyme-linked immunosorbent assay (ELISA), specific to the marker.

Proteome: The entire set of proteins expressed by a genome, cell, tissue, or organism at a given time, under defined conditions.

Quantitative PCR (qPCR): Also called real-time PCR, a polymerase chain reaction that monitors the amplification of a targeted DNA molecule during the PCR, i.e., in real time, and not at its end, as in conventional PCR.

Quorum sensing: A system of stimuli and response correlated to population density that enables bacteria to restrict the expression of specific genes to the high cell densities at which the resulting phenotypes will be most beneficial.

Reactive oxygen species (ROS): Chemically reactive compounds containing oxygen, formed as byproducts of the normal oxygen metabolism and having important roles in cell signaling and homeostasis.

Reflective mulch: Also called metallic mulch; mulching material containing metal that reflects sunlight, providing weed control, soil moisture retention, and soil warming, and repelling some insects.

Replanting (reset): Replacing individual diseased citrus trees one-by-one within mature groves.

Replanting (solid set): Planting new solid sets of seedlings across large areas following large acreage removals.

Reservoir (for pathogen or psyllid): A population of organisms or the specific environment (usually a susceptible host) in which an infectious pathogen naturally lives and reproduces, or upon which the pathogen primarily depends for its survival.

Resistance gene (R gene): A plant gene that conveys plant disease resistance against pathogens by producing resistance (R) proteins.

Resistant (to plant disease): Having a genetic makeup allowing prevention or reduction of pathogen growth on or in the plant, leading to the absence or reduction of disease.

Retention (vector transmission phase): After acquisition, the period of time during which the pathogen remains present and viable in the insect. For *Ca.* Liberibacter spp., it is the act of pathogen moving through the hemocoel infecting various organs, including the salivary glands.

Rhizoplane: The root surface.

RNA interference (RNAi): One type of small RNA, RNAi is a defense mechanism in plants, fungi, and animals against foreign double-stranded RNA, such as viruses; characterized by the prevention of messenger RNA (mRNA) translation by specialized protein complexes in the host.

Rootstock: In grafting of citrus, a plant stump having an established, healthy root system, onto which a cutting or a bud from another plant is grafted.

Rutaceae: A family of plants ("rutaceous") including citrus and related species.

Salicylate hydroxylase: An enzyme that catalyzes the decarboxylative hydroxylation of salicylate to form catechol, a product that does not induce resistance. Salicylate hydroxylase (SahA) produced by *Candidatus* Liberibacter asiaticus (CLas) has been found to degrade salicylic acid and suppress plant defenses.

Salicylic acid: An important signal molecule in plant defense and signaling in plants.

Salivary sheath: An envelope surrounding the stylets of some phytophagous insects formed during stylet propagation inside the plant.

Scion: A young shoot or twig of a plant, especially one cut for grafting onto a rootstock during plant propagation.

Sclerenchyma: Strengthening tissue in a plant, formed from cells with thickened, typically lignified, walls.

Sec: A bacterial secretion system, conserved among a number of bacterial species, that translocates proteins, primarily in their unfolded state, across bacterial membranes.

Semiochemical: A chemical substance or mixture that carries a message for intraspecific or interspecific communication.

Sequencing (genome): A process that determines the complete DNA sequence of an organism's genome at a single time.

Sieve tube: A collection of specialized phloem cells, connected end-to-end, forming a continuous tube through which organic solutes (and phloem-restricted pathogens, including some bacteria and viruses) are translocated.

Small RNA: RNA molecules <200 nt and usually noncoding; one example is RNAi, which is often involved in RNA silencing.

Source-sink: With respect to in-plant translocation, the movement of materials from a source (such as carbohydrates from areas of photosynthesis such as leaves) to other, nonphotosynthetic, tissues throughout the plant.

Sticky card (yellow sticky card): A trap consisting of a sticky glue layer mounted on a piece of cardboard, used to monitor and catch insects and other pests.

Stylet sheath: See salivary sheath.

Sulfonamide(s): A class of synthetic drugs, derived from sulfanilamide, that are able to prevent the multiplication of some pathogenic bacteria.

Susceptible (to plant disease): Having a genetic makeup that permits the development of a particular disease.

Systemic acquired resistance (SAR): A "whole-plant" resistance response that occurs following an earlier localized exposure to a pathogen, when plants use pattern-recognition receptors to recognize conserved microbial signatures.

Thermotherapy: The use of heat to eliminate or reduce numbers of a pathogen in a host plant without significant detriment to the plant.

Tolerant (to plant disease): Able to grow and produce a good crop or maintain an acceptable appearance even when infected with a plant pathogen.

Transcription: The first step of gene expression, in which a particular segment of DNA is copied into RNA (mRNA) by the enzyme RNA polymerase.

Transcriptional regulator: A chemical that stimulates or represses the process of transcription.

Transcriptome: The set of all messenger RNA molecules in one cell or a population of cells.

Transformation: The natural or laboratory genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous DNA from its surroundings through the cell membrane.

Transgene: A gene or genetic material that has been transferred naturally, or by any of a number of genetic engineering techniques, from one organism to another.

Transmission (pathogen): A process involving pathogen escape from the host, and travel to and infection of a new host.

Transpeptidase: An enzyme that catalyzes the transfer of an amino group from one peptide chain to another.

Upregulation: The process by which a cell increases the quantity of a cellular component, such as RNA or protein, in response to an external variable.

Vector: An agent, such as a plasmid or virus, used to carry DNA into a cell; an insect or other living entity that transmits a pathogen.

Vertical transmission: Transfer of an infectious agent from parent to offspring via transovarial transmission.

Virulence: Expression of the degree of damage caused to a host plant by infection with a particular pathogen, generally negatively correlated with host fitness.

Virulence factor: A molecule produced by a pathogen that enables it to colonize the host, evade or suppress the host's immune response, obtain nutrition from the host, and/or enter or exit from host cells (if an intracellular pathogen).

Volatile organic compound (VOC): An organic substance easily evaporated at normal temperatures.

Windbreak: A row of trees or a fence, wall, or screen that provides shelter or protection from the wind.

Wing aspect ratio: The ratio of the span of a wing to its mean chord (length in the direction of wind travel over the wing); a high ratio describes a long, narrow wing, while a low ratio describes a short, wide wing.

***Wolbachia*:** A genus of gram-negative bacteria that infects or lives endophytically within arthropods, often in complex relationships that may be parasitic, mutualistic, or beneficial.

Appendix D

Selected Citrus Research Development Foundation Projects

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
002	Characterize the roles of callose and phloem proteins in citrus huanglongbing (HLB) symptom development	Albrigo, Gene University of Florida	4/1/2010 - 9/30/2012	$250,000.00
045	Examine the response of different genotypes of citrus to citrus greening (huanglongbing) under different conditions	Dawson, Bill University of Florida	7/1/2010 - 6/30/2011	$453,322.00
046	Identify and deliver antibacterial peptides and/or proteins for control of citrus greening (huanglongbing or HLB)	Dawson, Bill University of Florida	8/1/2010 - 7/31/2012	$618,388.00
048	Attempts to *in vitro* culture *Candidatus* Liberibacter asiaticus isolates in order to fulfil Koch's postulates	Dollet, Michel	4/1/2010 - 4/30/2012	$177,120.00
065	Genomic sequencing to closure of a curated Florida citrus greening strain of *Candidatus* Liberibacter asiaticus	Gabriel, Dean University of Florida	4/1/2010 - 3/31/2012	$186,285.00

Objectives

1. Characterize the blockage materials with immunoassay techniques with TEM and test for induction signals and virulence factors.
2. Transgenic approaches to disrupt HLB-associated callose and phloem protein plugging by reducing their production.
3. Reduce sieve element blockage in field and greenhouse HLB-infected plants through treatment with chemicals that are expected to alter callose and p-protein production. The information learned from the proposed research may lead to alternative control methods and will contribute to the understanding of the virulence mechanisms of Las and will be distributed through an extension program.

1. Continue to examine the susceptibility of citrus genotypes to Las.
   a. Compare the response of commercial varieties and more citrus relatives to Las.
   b. Examine the sensitivity of elite lines and select hybrids for the Plant Improvement Group.
2. Examine the reduction in Las titer in *Poncirus trifoliate*.
3. Characterize the pathogen that induces symptoms in *Citrus latipes*.

1. Continue to screen peptides for activity against Las.
   1a. Screening against Las in citrus.
   1b. Screening against Liberibacter in tobacco.
2. Examine ability to export peptides from the rootstock.
3. Improve *Citrus tristeza virus* (CTV)-based vector.
   3a. Develop a vector to express 2–5 peptides.
   3b. Develop a vector to overcome cross protection.
   3c. Remove aphid transmissibility from the vector.
   3d. Develop a vector that is restricted to transgenic tree parts.
4. Examine survival of peptides in fruit and juice.
5. Develop transgenic citrus trees with effective peptides—mature transformation.

To obtain a pure axenic culture of several strains of *Ca*. Liberibacter asiaticus maintained at this time in periwinkle and in infectious *D. citri* populations by the use of feeder cells: in this case insect cells cultures. Objectives of the project are numerous but the first of all is to obtain pure axenic cultures of Liberibacters in order to be able to fulfil Koch's postulates. Furthermore, some indirect objectives are to enable and facilitate development of technologies for improved detection of *Ca*. Liberibacter spp. and epidemiological studies, to facilitate host/pathogen/insect vector interactions studies, and to facilitate studies on genomics and molecular biology of Liberibacters.

The principal objective of this funding request is to completely close the Las UF506 genome.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
090	An effective trap for Asian citrus psyllid that can be used to monitor groves and plants for sale	Mizell, Russell University of Florida	11/1/2010 - 10/31/2012	$98,282.00
091	Efficacy of seasonal insecticide programs for suppressing HLB in new citrus plantings	Hall, David USDA	7/1/2010 - 9/30/2012	$199,402.00
093	Pathogen–vector relations between asian citrus psyllid and liberibacter asiaticus	Hall, David USDA	7/1/2010 - 8/31/2012	$296,414.00
123	Bioinformatic characterization and development of a central genome resources website for *Ca.* Liberibacter asiaticus	Lindeberg, Magdalen Cornell University	11/1/2010 - 4/30/2013	$286,337.00
125	Development of SSR markers for detection, genotyping, phenotyping, and genetic diversity assessment of *Candidatus* Liberibacter strains in Florida	Lin, Hong USDA	11/1/2010 - 1/31/2013	$215,166.00

Objectives

1. Develop a better method for monitoring both psyllids and HLB pathogens in the environment.
2. Identify attractants for the psyllids. Project will address the needs of regulatory agencies as well as citrus growers.

In our proposed project, we would evaluate ACP and HLB control under intensive and moderate insecticide programs.
1. Development of HLB in young citrus subjected to intensive or moderate insecticide control programs or an oil program for psyllid control.
2. Development of HLB in young citrus subjected to an intensive insecticide program for ACP control compared to development of HLB in young citrus subjected to a moderate insecticide program in combination with growing *Murraya paniculata* as a potential trap plant or source of ACP natural enemies.
3. The effect of planting density on development of HLB in young citrus subjected to an intensive insecticide program for ACP control.
4. Protecting citrus resets from HLB.

1. To study pathogen–vector cellular interactions, transmission barriers, and other factors affecting vector specificity and vector competence.
2. To clarify various acquisition and transmission parameters between the psyllid vector and the HLB pathogen.

To characterize the molecular interactions between the citrus-FLS2 and CLas/*X. citri* flagellins and increase disease resistance by regulating the expression of the functional FLS2
To understand the roles of FLS2 and a leucine-rich repeat receptor kinase (LRR-RK) gene by mutating them using the newly developed CRISPR/Cas9 system.

Identify molecular mechanisms associated with host response during heat treatment of Las-infected citrus plants. We hypothesized that novel mechanisms will be identified in heat-treated Las-infected citrus tissues that are absent in tissues exposed to only heat or Las infection.
Induced citrus plants to mimic the molecular conditions associated with heat-induced Las resistance. We hypothesized that citrus plants can be genetically engineered to express the molecular mechanisms associated with heat-induced Las resistance.
Develop genetically modified citrus plants that will enhance defense response to HLB infection. We hypothesized that there will be a net gain in fitness and resistance to Las in genetically modified plants compared to non-genetically modified plants.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
15-003	Metabolomic profiling to accelerate development of HLB-tolerant rootstocks	Bowman, Kimberly USDA	7/1/2015 - 6/30/2018	$539,104.00
15-009	Exploiting the Las phage for potential control of HLB	Gabriel, Dean University of Florida	8/1/2015 - 7/31/2017	$419,500.00

Objectives

Susceptibility of existing citrus rootstock cultivars to HLB and other diseases poses a great risk for tree and crop loss. The availability of resistant rootstocks with other qualities essential for superior production of citrus would effectively eliminate disease as a threat and permit continuous higher production at much lower production cost. The proposed research will be complementing the USDA rootstock development program and is expected to identify metabolite profiles in citrus rootstock cultivars, and conferred to commercial citrus scions, which are associated with tolerance to HLB, tolerance to other stresses, and the excellent production of citrus fruit. Metabolite profiles of field-grown plants established during the first year of research will be correlated with data from previous and ongoing rootstock trials regarding HLB tolerance, growth performance, and citrus fruit production. Continuous experiments involving greenhouse and field studies during the following years will allow us to refine metabolic profiles and to integrate findings into our breeding efforts. The ultimate goal of this project is the early selection of the most promising candidate rootstocks prior to long-term field testing, therefore reducing the time and expense of testing, and accelerating the release of trees for commercial use. In addition, we expect to identify metabolites that are associated with resistance to HLB and that may be employed for development and improvement of early detection methods of HLB and therapeutic strategies to reduce Las titer levels in infected citrus trees.

The overall goal of the project is to enable the practical exploitation of the presence of lytic prophage in all known Florida Las strains by identification of chemicals or a biological control method that could be used to artificially trigger the phage lytic cycle in psyllids and thereby cure the psyllids by provoking an innate immune response and cure citrus by provoking a stronger innate immune response; suppress the expression of phage-encoded lysogenic conversion genes, particularly peroxidase in citrus; disable phage peroxidase enzymatic function in citrus; or any combination of the above in existing Las infected trees in the field and thereby cure the trees.
Objectives
Control of HLB using the putative Las LexA target
Control of HLB using the psyllid repressor as target
Control of HLB using Las peroxidase and Las lytic cycle activator(s) as targets
Field and greenhouse testing of lead compounds

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
15-016C	High-throughput inoculation of transgenic citrus for HLB resistance	Hall, David USDA	8/1/2015 - 7/31/2018	$375,000.00
15-017	Disrupt LuxR solo quorum sensing that mediates plant virulence and insect transmission of *Candidatus* Liberibacter asiaticus to control the disease	Killiny, Nabil University of Florida	8/1/2015 - 7/31/2017	$157,144.00
15-020	Create citrus varieties resistant to huanglongbing (HLB) through transgenic and nontransgenic approaches	Mou, Zhonglin University of Florida	7/1/2015 - 6/30/2018	$358,922.00

Objectives

Citrus plants transformed to express AMPs must be inoculated in order to evaluate HLB resistance. A startup inoculation program was established in 2011 using funds from the Citrus Research and Development Foundation (CRDF) (Hall-502, ends July 2015). Colonies of ACP are maintained in cages on potted citrus plants infected by CLas and showing HLB symptoms. A two-step inoculation program is used. First, individual plants are subjected to a 2-week no-choice infestation by ACP from these colonies; and second, the plants are held for 6 months in a greenhouse with an open infestation of ACP coming from CLas-infected source plants. Over 7,000 transformed scion or rootstock plants have passed through this program and are currently being evaluated for resistance. Meanwhile, the citrus breeders continue developing new transformed germplasm. This proposal asks CRDF for renewed/expanded support for inoculations. To maintain the inoculation program, funding is needed for labor (we request funds for two technicians 100% dedicated to the project), insect cages, materials, and supplies, notably for qPCR assays, which have proved critical in selecting which ACP colonies to use in the first inoculation step. Our funding request is based on an inoculation pace of at least 300 plants monthly. This pace will require 10 to 20 individual colonies of hot ACP.

To enhance citrus resistance to CLas by quenching the quorum-sensing signals; CLas quorum-sensing signals will be quenched by expressing acyl-homoserine lactonase (AHL-lactonase) in citrus plants.
To test the effect of AHL-producing citrus plants on the pathogencity of CLas; an AHL-producing citrus plant will be produced to study the effect of AHL on the pathogencity of CLas.
To prevent CLas from infecting citrus plants by jamming bacterial communication of CLas; quorum-sensing (QS) antagonists will be used to block the cell-to-cell signaling of CLas.
Identification of AHLs in Asian citrus psyllids hemolymph using GC-MS. Because different types of symbionts are confined in a small environment “arena” within their insect host, cooperative interactions and communication among theses.
Test commonly used bioassay strains to find the best reporter strain for the AHLs present in ACP or AHL mimic compounds present in the phloem sap of citrus. In addition, we aim to develop a more sensitive reporter (double sensors).

The overall goal of this proposal is to create citrus varieties resistant or tolerant to HLB using two different approaches. One approach is to generate HLB-resistant/tolerant transgenic citrus plants, and the other approach is to screen for HLB-resistant/tolerant citrus mutants. These two approaches complement each other.
Confirm HLB resistance or tolerance in citrus transgenic lines and putative mutants.
Stack the ELP3 and NPR1 genes in citrus.
Generate and test transgenic citrus plants overexpressing newly cloned disease resistance genes.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
15-023	Citrus nutrition studies for improved survival of HLB-affected trees	Schumann, Arnold University of Florida	7/1/2015 - 6/30/2018	$281,804.00
15-024	Predicting when, why, and where asian citrus psyllids move to increase effectiveness of insecticide sprays	Stelinski, Lukasz University of Florida	8/1/2015 - 7/31/2017	$161,116.00
15-027	Developing a culture medium for Liberibacter asiaticus through comparative multi-omics analysis with its closest cultured relative, *L. crescens*	Triplett, Eric University of Florida	7/1/2015 - 6/30/2017	$325,912.00

Objectives

Our main goal is to find the reasons for inconsistent responses of HLB-affected citrus to EN programs and to develop feasible and economical remedies that can consistently replicate successful HLB mitigation with ENs in all Florida groves. Specific objectives are
1. Establish nutrient sufficiency guidelines for leaf tissues of HLB-affected trees that have successfully responded to nutritional programs in order to help growers replicate the successes and achieve higher nutrient efficiencies.
2. Determine soil conditions that favor root hair and VAM proliferation of citrus roots, thus maximizing root uptake of calcium and other deficient nutrients in HLB-affected citrus trees.
3. Establish testing protocols and remedial soil amendments that can be deployed to maximize root uptake of calcium and other deficient nutrients in nonresponsive HLB-affected groves.
4. Deliver results of the research project to the Florida citrus industry through extension/outreach to all stakeholders (growers, contractors, supporting industries).

Overall Goal: Determine the effects of abiotic factors on Asian citrus psyllid dispersal and flight capability.
Determine the fight initiation thresholds of ACP depending on temperature and humidity.
Determine the effect of wind speed on flight and the direction of psyllid flight with respect to wind.
Determine the effects of barometric pressure changes on psyllid dispersal.
Measure how psyllid dispersal is affected by abiotic factors in the field.
Establish a model to predict the risk of ACP dispersal/invasion based on prevailing abiotic conditions. Deliver this model as an online tool for growers.

Overall goal: The development of a robust, defined medium that provides sustained and reproducible growth of an L. asiaticus culture. Use comparative genomics approaches to identify means to culture L. asiaticus, including metabolic reconstruction, based on our new defined media and the identification of the essential gene set needed to culture *L. crescens*.
Continue to integrate multi-omics approaches to improve the medium for *L. crescens*, which, by extension, will get us closer to a robust medium for L. asiaticus.
Overarching—embedded with all other objectives. Test each new media design for its ability to sustain growth by L. asiaticus.
Characterization of the physiology and genome of the L. asiaticus culture.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
15-028	Control citrus huanglongbing (HLB) by counteracting the SA hydroxylase of *Candidatus* Liberibacter asiaticus	Wang, Nian University of Florida	7/1/2015 - 6/30/2018	$430,697.00
15-033C	Support role of the Citrus Core Transformation Facility remains crucial for research leading to production of citrus plants that may be tolerant or resistant to diseases	Orbović, Vladimir University of Florida	7/1/2015 - 6/30/2018	$270,000.00
15-036C	Correlating pesticide residue analysis with psyllid feeding to improve protection of young trees	Rogers, Michael University of Florida	7/1/2015 - 6/30/2018	$451,603.00
15-038C	Insecticide resistance monitoring and management in Florida citrus to maintain sustainable control of Asian citrus psyllid within Citrus Health Management Areas	Stelinski, Lukasz University of Florida	7/1/2015 - 6/30/2017	$129,491.00

Objectives

The goal of this project is to develop management strategies that boost natural defense mechanisms to control Huanglongbing (HLB) disease by counteracting salicylic acid (SA) hydroxylase of *Ca*. Liberibacter asiaticus (Las).
Control HLB by optimization of application of SA and its analogs. Application of SA and its analogs have potential to neutralize the SA hydroxylase. Based on our previous study, foliar spray of SA and its analogs slowed down the increase of Las population in citrus and HLB disease severity, whereas trunk injection of SA and its analogs significantly reduced Las population. The previous study suggest that we can improve the HLB management by optimizing the application methods of SA and its analogs.
Control HLB using a combination of SA, SA analogs, or SA hydroxylase inhibitors. By combining SA or SA analogs with SA hydroxylase inhibitors, we could improve the efficacy of plant defense inducing to control HLB. SA hydroxylase inhibitors can directly counteract Las SA hydroxylase. We have identified six SA hydroxylase inhibitors in our previous studies. We will continue to optimize those SA hydroxylase inhibitors.

The most important result of this project will be availability of an efficient service for production of transgenic plants according to a variety of ideas representing different approaches to fight citrus diseases. Considering the magnitude of crisis the citrus industry is in, CCTF as a place that routinely produces transgenic citrus plants will encourage researchers not to abandon any scientifically sound ideas but to test them through production of transgenic plants. Practical applications of this project are
Cost-effective completion of important phase of research contained in many projects.
Human resources as well as equipment in different research groups can be used for other activities while CCTF produces transgenic material for them.
Timely realization of ideas into life through production of transgenic plants that can be challenged by pathogens/vectors to test their susceptibility to diseases within a short period of time.

Residue analysis

We have already documented resistance in regional FL ACP populations, where prescribed MOAs are applied up to 12 times/yr to suppress new HLB infections, which began in earnest in 2007. A recent investigation from Mexico has shown that ACP had become 100-fold and 4,000-fold resistant to organophosphates and neonicotinoids. This is alarming and illustrates how lack of resistance management can allow ACP populations to become grossly resistant to our best tools leading to product failures. Our goal is to prevent this from happening proactively by monitoring resistance within CHMAs and prescribing appropriate management protocols.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
15-039C	Secure site for testing transgenic and conventional citrus for HLB and psyllid resistance	Stover, Ed USDA	1/1/2016 - 12/31/2018	$260,980.00
15-042	Control citrus huanglongbing using endophytic microbes from survivor trees	Wang, Nian University of Florida	7/1/2015 - 6/30/2018	$467,000.00
16-001	Enhancing genetic transformation efficiency of mature citrus	Li, Yi University of Connecticut	7/1/2016 - 6/30/2019	$316,168.00
16-005	GFP labeling of *Candidatus* Liberibacter asiaticus *in vivo* and its applications	Wang, Nian University of Florida	10/1/2016 - 9/30/2019	$472,753.00
16-020C	Dyed kaolin to repel Asian citrus psyllid in field conditions	Vincent, Christopher University of Florida	12/1/2016 - 11/30/2019	$273,908.00

Objectives

Transgenic strategies for controlling HLB and its psyllid vector will be tested in a secure environment in which ideal care is provided for the invaluable research material in full compliance with regulatory requirements. Nontransgenic citrus with reasonable probability of resistance will be tested and may provide resistant planting materials with fewer regulatory constraints. This care will be provided with no compromise to the research teams' ownership of tested intellectual property and no USDA/ARS expectation of inclusion in the related patents secured when USDA/ARS was not involved in development of the plant materials, expression vectors, or other material tested.

The goal of the proposed study is to characterize the effect of using endophytic microbes in controlling HLB. Our hypothesis is the outcome of the interaction among Las, psyllid, and citrus is affected by the citrus phytobiome. In order to achieve the goal of this study, the following objectives will be conducted:
To characterize the phytobiomes and endophytic microbes from HLB survivor trees and HLB diseased trees
To illustrate whether the endophytic microbes from survivor trees could efficiently manage citrus HLB

Using P19 co-expression to improve *Agrobacterium* infection/transient expression efficiency of mature citrus tissues
Using transient down regulation of AGO2 and NRPD1a expression to improve *Agrobacterium* stable transformation efficiency of mature citrus tissues
Using H2A co-expression to improve *Agrobacterium* stable transformation efficiency of mature citrus tissues
Using a combination of genes to develop a super-transformation-vector that is genotype independent, highly effective for transformation and regeneration of mature citrus
Using root specific auxin biosynthetic gene to engineer rootstock of citrus to improve success rates of micrografting

1. GFP labeling of *Candidatus* Liberibacter asiaticus *in vivo*
2. Elucidation of plant–Las interaction through real-time monitoring of Las movement and multiplication in planta using GFP labeled Las
3. Investigate the effect of different control approaches on the dynamic population of Las in planta using GFP labeled Las

1. Determine the optimum adjuvant-kaolin rate combinations to improve rainfastness of dyed kaolin particle film on citrus foliage.
2. Determine the optimum concentration of red-dyed kaolin residue to deter ACP.
3. Determine the effect of dyed kaolin particle films on ACP populations and CLas transmission under field conditions.
4. Assess horticulturally relevant physiological impacts of optical properties of red-dyed kaolin particle films as compared to undyed films.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
162	Dissecting the disease complex of citrus huanglongbing in Florida	Duan, Yongping USDA	7/1/2010 - 6/30/2013	$670,183.00
164	Sampling plans to guide decision making for control asian citrus psyllid	Qureshi, J. A. University of Florida	8/2/2010 - 7/31/2012	$124,786.00
176	Effects of nutrition and host plant on biology and behavior of the Asian citrus psyllid and implications for managing psyllid populations	Rogers, Michael University of Florida	4/1/2010 - 3/31/2012	$87,607.04
212	Enhanced biological control of asian citrus psyllid in Florida through introduction and mass rearing of natural enemies	Stansly, Phil University of Florida	5/1/2010 - 4/30/2011	$148,775.00
214	Quantitative measurement of the movement patterns and dispersal behavior of Asian citrus psyllid in Florida for improved management	Stelinski, Lukasz University of Florida	8/1/2010 - 7/31/2011	$71,614.00

Objectives

Genome sequencing and gap closure using computational approaches, construction of a large-insert library, combinatorial PCR, and chromosomal walking.
Culturing Las bacterium *in vitro* running metabolic pathway analyses.
Role of the microbial community and genetic diversity of Las bacteria in HLB development.
Seed transmission of Las bacterium.
Monitor Las population dynamics in citrus and periwinkle plants after chemical and heat treatment.

1. Evaluate and refine the use of the tap sample to monitor adult psyllids and methods of assessing flush density, and infestation rates in blocks of growing and dormant citrus trees of different ages and varieties planted at experimental and commercial groves.
2. Test the influence of these parameters on precision of estimated means and the distribution of the population within blocks using the appropriate aggregation and regression models.
3. Evaluate and integrate methods for assessing these parameters into a user friendly system accessible to consultants and managers.

Determine the effects of varying rates of N, P, and K fertilization rates on the duration and survivorship of nymphal stages; adult fecundity and longevity and body mass.
Examine psyllid fitness on different commercial citrus varieties grown in Florida and the effects of rootstock selection on suitability of scion material for psyllid development.
Evaluate psyllid fitness when reared on non-commercial citrus species and other rutaceous noncitrus host plants.
Examine the effects of host plant quality on feeding behaviors of adult psyllids.

1. Import, release, and evaluate new strains and species of parasitoid specific to *D. citri*.
2. Identify genetic markers that can be used to track *T. radiata* in the environment.
3. Develop efficient methods for mass rearing and release of *T. radiata* and possibly other species to increase biological control through augmentation of natural populations.
4. Transfer technology to industry clientele.

Determine the seasonality and frequency of psyllid dispersal behavior.
Determine how flush availability impacts ACP movement and whether HLB-infected ACP disperse more than noninfected counterparts.
Quantify ACP dispersal distance within managed groves and distance of dispersal between managed groves.
Confirm the impact of abandoned groves on psyllid populations in neighboring managed groves.
Determine height at which psyllids disperse.
Develop management recommendations based on understanding of ACP dispersal behavior patterns.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
232	Characterization of the virulence mechanism of the citrus huanglongbing pathogen *Candidatus* Liberibacter asiaticus	Wang, Nian University of Florida	4/1/2010 - 3/31/2012	$218,134.00
306	Culturing Liberibacter asiaticus	Davis, Michael J. University of Florida	5/1/2010 - 4/30/2011	$65,000.00
307	Infection traits and growth of "*Candidatus* Liberibacter asiaticus" inside microfluidic chambers	De La Fluente, Leonardo Auburn University	9/1/2010 - 5/30/2013	$100,000.00
312	Functional study of the putative effectors of "*Candidatus* Liberibacter asiaticus" using *Citrus tristeza virus* vector	Gowda, Siddrame University of Florida	5/1/2010 - 4/30/2013	$238,400.00
314	Insight into the causative agent of citrus greening disease (HLB) using computational structure/function analysis of genome-encoded proteins	Grishin, Nick Southwestern Medical	3/1/2010 - 8/30/2011	$99,902.00

Objectives

1. Transcriptional and microscopic analyses of different citrus varieties that are either susceptible or tolerant to *Ca*. L. asiaticus infection at different infection stages in greenhouse and citrus grove.
1.1. Identify citrus genes with altered regulation caused by inoculation with *Ca*. Liberibacter asiaticus in susceptible and tolerant citrus cultivars.
1.2. Characterize the relationship between the population of *Ca*. Liberibacter asiaticus in the phloem, anatomical changes of phloem, nutrient transport, and symptom development in susceptible and tolerant citrus cultivars.
2. Transcriptional and microscopic analyses of model system tobacco to Las infection.

We are proposing to attempt the establishment of co-cultures of Las with these cell lines. Such cultures could then be used as a source of inoculum in attempts to grow Las axenically.

Optimize culture conditions of Las inside microfluidic chambers.
Collect inoculum and phloem fluid from HLB-affected and healthy plants.
Observe movement and aggregation of Las inside microfluidic chambers.

1. Identify the putative effectors based on the genome sequence of Las and cloning into CTV vector.
2. Expression of selected putative effectors of Las in citrus using CTV vector and transcriptional analysis of the host in response to expression of Las effectors.

To classify and predict spatial structures and functions of all *Candidatus* Liberibacter asiaticus proteins and suggest their relevance in the citrus disease.
To compile a database from all the data obtained in the course of the project and make it available at our group website and at the central depository www.citrusgreening.org.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
315	Speedy evaluation of citrus germplasm for psyllid resistance	Hall, David USDA	5/1/2010 - 7/31/2013	$497,503.00
325	Development and evaluation of psyllid management programs for protection of resets and young tree plantings from HLB	Rogers, Michael University of Florida	5/1/2010 - 4/30/2012	$127,288.40
330	Targeting the asian citrus psyllid feeding mechanism as a means of blocking psyllid feeding on citrus	Shatters, Bob USDA	5/1/2010 - 6/30/2014	$483,000.00
334	How does Liberibacter infection of psyllids affect the behavioral response of this vector to healthy versus HLB-infected citrus trees?	Stelinski, Lukasz University of Florida	4/1/2010 - 3/31/2011	$30,867.00

Objectives

Screen citrus germplasm for resistance to the Asian citrus psyllid (ACP, *Diaphorina citri*); explore host plant resistance as a management tactic for the Asian citrus psyllid. Two different types of plant resistance would be good candidates for psyllid control in commercial citrus: antixenosis and antibiosis (Painter 1951). A plant that adult psyllids avoid for either food or oviposition would constitute the antixenosis type of resistance. Plant species avoided by the adult psyllid for food and oviposition could lack specific plant traits (e.g., kairomones, color, or plant morphology) that attract the psyllid, or they could contain traits that repel the psyllid (e.g., kairomones).

1. Determine the most effective rates and application methods for soil-applied systemic insecticides to control psyllids on nonbearing trees of different sizes.
2. Evaluate the use of Kaolin clay particle films in conjuction with various adjuvants/stickers to increase the rainfastness of these applications.
3. Determine the ability of soil-applied systemic insecticide applications in combination with Kaolin clay particle film or dimethyl disulfide (DMDS) to protect nonbearing trees from HLB in a commercial grove setting.

Conduct chemical and molecular analyses of the isolated ACP salivary sheaths to determine structure and mode of synthesis. (year 1)
Identify specific salivary proteins secreted in both the gelling and the watery saliva. (year 1 and 2)
Use information from 1 and 2 above to construct a model describing the ACP feeding process. (end of year 2)
Use artificial diet system to screen for salivary sheath/feeding inhibitory compounds. (initiate in year 1 and continue through year 2)
Conduct plant spray tests to determine if inhibitory compounds currently identified (i.e., EDTA and related chelators) or identified in the future, will work to prevent ACP from feeding on plants. (initiate in year 1 and continue to field trials in years 2 and 3)
Use information to identify a transgenic plant approach to create citrus that block the ACP feeding process and thus are not at risk of becoming HLB infected. (year 3)

1. To determine whether healthy ACP distinguish between healthy versus HLB-infected citrus plants
2. To determine whether behavior of *Ca*. Las-infected ACP to healthy versus infected citrus plants differs from the behavior of ACP that are not carrying the pathogen
3. To determine whether the settling preference of healthy and *Ca*. Las-infected ACP on healthy versus HLB-infected citrus plants differs from the behavior of ACP that are not carrying the pathogen
4. To identify the active synthetic plant compounds that mediate ACP response to infected plants by GC-MS
5. To determine whether behavior of ACP to healthy versus HLB-infected trees differs in the field
6. To determine whether synthetic chemicals identified from HLB-infected plants attract ACP

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
335	Evaluation of methyl salicylate as a simultaneous repellent of Asian citrus psyllid and attractant for psyllid natural enemies	Stelinski, Lukasz University of Florida	4/1/2010 - 9/30/2011	$42,054.00
336	Genome-enabled metabolic reconstruction of *Ca*. Liberibacter asiaticus and its use in culturing and controlling the pathogen	Triplett, Eric University of Florida	7/1/2010 - 3/31/2014	$834,163.00
407	Culturing Liberibacter asiaticus	Davis, Michael J. University of Florida	5/1/2011 - 4/30/2013	$130,000.00
418	Analysis of the colonization of citrus seed coats by "*Candidatus* Liberibacter asiaticus" the causal agent of citrus huanglongbing and their use as a concentrated, pure source of bacteria for research	Hilf, Mark USDA	6/1/2011 - 5/31/2013	$175,000.00

Objectives

1. Determine if field release of methyl salicylate (MeSA) will enhance biological control organisms.
2. Determine if MeSA is repellent to ACP.

Build a genome-scale metabolic model of *Ca*. Liberibacter asiaticus and use this model to identify the likely growth conditions necessary to culture *Ca*. L. asiaticus.
Determine which fully sequenced, culturable bacterium is most closely related metabolically to *Ca*. L. asiaticus and then apply those growth conditions to culture *Ca*. L. asiaticus.
Perform the experiments necessary on citrus with the cultured pathogen to satisfy Koch's postulates.
Predict the antibiotic sensitivity of *Ca*. L. asiaticus based on its sequenced genome and verify this sensitivity in the cultured organism as well.
Identify genes in *Ca*. L. asiaticus that can be targeted for inhibition based on comparative genomic analyses with all other sequenced bacteria.

We propose to continue modifying our media formulations until continuous culture of Las is obtained. This study is to provide culturing tools for Las that will support efforts to manage HLB. The capacity to grow Las in axenic culture is an advantage to many areas of study of HLB and its control.

Develop an effective protocol for isolation of viable "*Ca*. L. asiaticus" cells from citrus seed coats.
Evaluate the viability of extracted "*Ca*. L. asiaticus" cells.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
424	Functional disruption of the NodT outer membrane protein of *Candidatus* Liberibacter asiaticus for rootstock-mediated resistance to citrus greening using a phloem-directed, single-chain antibody	McNellis, Timothy Pennsylvania State University	7/1/2011 - 6/30/2017	$55,000.00
425	Effect of application rate, tree size, and irrigation scheduling on leaf imidacloprid concentration, psyllid populations, and soil leaching	Morgan, Kelly University of Florida	8/1/2011 - 10/31/2013	$199,225.00
434	Mass rearing and release of parasitic wasps to augment biological control of the Asian citrus psyllid (ACP)	Stansly, Phil University of Florida	6/1/2011 - 5/31/2014	$370,975.36
447	Role of nutritional and insecticidal treatments in mitigation of HLB in new citrus plantings	Stansly, Phil University of Florida	2/15/2012 - 2/14/2016	$324,430.00

Objectives

The ultimate objective of this proposal is to develop citrus rootstocks that can be used to control HLB in fruiting variety scions. A transgenic approach will be used. Transgenic citrus plants producing a phloem-targeted, single-chain antibody recognizing an outer membrane protein of *Ca*. L. asiaticus will be developed and tested for resistance to HLB. The antibody will be translationally fused with the FLT protein, which is a phloem-mobile protein that gets efficiently translocated from lower parts of the plant to upper parts of the plant, even through graft unions. The FLT-antibody fusion protein is expected to be functional and to move systemically through the phloem, including from a transgenic rootstock into a nontransgenic scion. However, the current proposal is limited to producing and testing the transgenic plants for resistance to HLB and does not include testing of graft transmissibility of any HLB resistance that may be observed.

1. Documentation of citrus greening management by application of the imidacloprid over time after selected irrigation applications.
2. Determine sorption and transformation of imidacloprid in soils.
3. Assess the soil transport and plant uptake of the applied imidacloprid and its metabolites over time after selected irrigation applications.
4. Characterize root zone water movement using bromide tracer.
5. Monitor psyllid populations and incidence of HLB over time.

1. Augment biological control of *D. citri* through mass production and release of previously established and imported colonies of *T. radiata* and *D. aligarhensis*.
2. Assess parasitism rates at release and control sites using feral populations of *D. citri* and infested sentinel plants.
3. Develop a system of genetic identification of the parasitoids recovered from the field to prove the establishment and effectiveness of released biotypes.
4. Transfer technology to industry clientele.

The overall objective of this research is to evaluate the individual and combined contributions of vector control and foliar nutrients in order to bring a new solid-block planting of juice oranges into profitable production.
1. Evaluate psyllid populations, HLB incidence and intensity, gene expression, tree growth, and eventually yield in newly planted citrus blocks.
2. Assess separate contributions of vector control and foliar nutritional applications to the above parameters.
3. Evaluate the effectiveness of reflective mulch to control ACP.
4. Provide economic analysis of costs and projected benefits.
5. Extend results to clientele.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
502	High-throughput screening of transgenic citrus for HLB resistance	Hall, David USDA	6/1/2012 - 7/31/2015	$190,200.00
516	Develop citrus resistant or tolerant to HLB using the CTV vector and transgenic approaches	Dawson, Bill University of Florida	8/1/2012 - 1/31/2016	$1,239,174.00
523	Screening and cloning of resistance related genes by RNA-seq in huanglongbing (HLB) resistant and susceptible citrus breeding lines	Duan, Yongping USDA	5/1/2012 - 4/30/2015	$458,000.00
535	Exploiting the Las and Lam phage for potential control of HLB	Gabriel, Dean University of Florida	5/1/2012 - 4/30/2013	$117,028.00
536	Identification and mapping of the genes controlling resistance to huanglongbing (HLB)	Gmitter, Fred University of Florida	12/1/2012 - 11/30/2014	$190,000.00

Objectives

The purpose of this proposal is to support a high-throughput facility to evaluate transgenic citrus for HLB resistance. Briefly, individual plants are caged with infected psyllids for 1 week, and housed for 6 months in a greenhouse with an open infestation of infected psyllids. Plants are then moved into a psyllid-free greenhouse and evaluated for growth, HLB symptoms, and Las titer. To expedite this process, additional labor, space, and supplies are needed.

The first goal is to find anti-HLB or antipsyllid genes that are effective in controlling the disease and that will also be acceptable for consumers and regulatory processes. I see this as two different stages. We now are in the first stage—finding genes that are efficacious in controlling the disease. Screening for effective genes is the most difficult and time-consuming challenge of my career. It would be ideal to quickly screen the activity of the gene products against the bacterium in culture.
The second phase will be to choose from the effective genes ones that can be effectively marketed or to modify selected ones such that they are suitable.

1. Generate transcriptome profiles of both susceptible and resistant citrus responding to HLB infection using RNA-Seq technology.
2. Identify key resistant genes from differentially expressed genes and gene clusters between the HLB-susceptible and HLB-resistant plants via intensive bioinformatics and other experimental verifications, such as RT-PCR.
3. Create transgenic citrus cultivars with new constructs containing the resistant gene(s).

A principal objective of this funding request is to establish a model in *E. coli* that can be used to assay if chemicals can be used to activate phage lytic (late) genes.
Cloning of previously identified early/late gene promoter regions fused with lacZ as a reporter.
Cloning and expression of both Las and the Lam repressors and determining responsiveness of the lacZ reporter.
Cloning and expression of all four Las and the one possible Lam antirepressors, and determining responsiveness of the reporter and clones from Milestone 2.
Development of a chemical assay for Las-responsive SOS.

The overall goal of this project is to accelerate the utilization of the HLB resistance in *Poncirus* for production of HLB-resistant citrus cultivars. This project aims to develop the knowledge and tools needed for this goal. Specifically, this project is intended to
1. Phenotype 92 replicated progeny and determine the genetic basis and mode of inheritance of the HLB resistance in *Poncirus*.
2. Construct a linkage map of the *Poncirus* genome and locate and map the Poncirus genes conferring resistance to HLB.
3. Increase the marker density and mapping resolution to refine the map location of identified resistance genes.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
538	Host genetic control of interference in Asian citrus psyllid life cycles	Gmitter, Fred University of Florida	12/1/2012 - 11/30/2014	$100,000.00
567	Acoustic trap for Asian citrus psyllids	Mankin, Richard USDA	7/1/2012 - 12/31/2013	$112,200.00
579	Citrus Core Transformation Facility (CCTF) as a platform for testing of different genes and/or sequences that have potential to render citrus plants tolerant or resistant to diseases	Orbović, Vladimir University of Florida	5/1/2012 - 6/30/2015	$255,000.00
581	Key unknowns about Asian citrus psyllid biology in Florida: Overwintering sites and alternative hosts	Pelz-Stelinski, Kirsten University of Florida	5/1/2012 - 9/30/2014	$200,029.00
581-1	Enhancement—Key unknowns about Asian citrus psyllid biology in Florida: Overwintering sites and alternative hosts	Pelz-Stelinski, Kirsten University of Florida	7/1/2013 - 6/30/2014	$11,868.00

Objectives

This project will address another approach that can contribute to the management of HLB disease spread, by targeting the vector, specifically to study the potential for host interference in ACP life cycles. If life cycles are significantly disrupted or impeded, population levels of ACP in the field may be reduced, which in turn should likewise reduce the potential spread of Clas and HLB disease. We will test whether the previously observed characteristic, of decreased fitness of ACP when reared on Cleopatra mandarin (*Citrus reshni*) seedlings, can be transmitted to hybrid offspring of Cleopatra.

In the proposed study:
Characteristics of the female reply that most effectively induce attraction of calling males will be identified in a laboratory setting.
Optimal replies will be tested in a field or greenhouse environment.
The best reply will be incorporated into a sticky (or alternative) trap with a piezoelectric buzzer that plays back the reply whenever a male call is detected.
The trap will be tested and modified as needed in laboratory and field tests with male ACP to determine whether infestations can be detected and targeted more precisely than with current methods.
If the trap is demonstrated to have enhanced efficacy, additional devices will be constructed for general field use.

The objective of this project is for CCTF to continue to produce transgenic plants, maintain its capacity from the aspect of output and turnaround time, and provide a service to scientists involved in the *Citrus* field of research, especially those who have a goal to create *Citrus* lines that will be tolerant/resistant to bacterial and other emerging diseases. By doing so, CCTF will reconfirm its prominence as the central place for production of transgenic *Citrus* plants and present itself as platform for testing of genes and sequences that are candidates to render *Citrus* resistant to diseases.

Identify overwintering habitats and alternative hosts of ACP.
Determine the capacity of alternative hosts as food sources and oviposition sites.
Directly sample ACP in citrus tree canopies and surrounding habitats all season long.
Determine effects of cold and heat acclimation on susceptibility of ACP to insecticides.

Enhancement

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
590	Enhancing psyllid control through a better understanding of the effects of pesticide applications on psyllid feeding and mortality	Rogers, Michael University of Florida	5/1/2012 - 4/30/2015	$889,701.00
600	Management tactics based on psyllid movement and distribution in Florida citrus	Stansly, Phil University of Florida	6/1/2012 - 5/31/2015	$296,000.00
603	Non-neurotoxic chemicals as alternatives to conventional insecticides for Asian citrus psyllid management and prevention of insecticide resistance	Stelinski, Lukasz University of Florida	4/1/2012 - 12/31/2014	$191,322.00

Objectives

The goal of this proposed work is to improve psyllid control and minimize the spread of the HLB pathogen through more effective use of pesticide applications.
Objective 1, an electrical penetration graph (EPG) monitor will be used to determine whether insecticides can disrupt the psyllid feeding behaviors responsible for pathogen transmission. For insecticides that disrupt psyllid feeding behavior, the longevity of feeding disruption provided will be determined.
Objective 2 will examine the duration of psyllid control provided by foliar-applied insecticides in a typical grove setting. This objective will address whether certain insecticides perform better at certain times (seasons) of the year and examine the use of adjuvants to increase the longevity of psyllid control provided.
Objective 3 will examine the use of different application methods of neonicotinoid insecticides for young and intermediate-sized trees to maximize the level of psyllid control provided by soil-applied systemic products.
Objective 4 is a multiyear evaluation of 4 different season-long approaches to young tree care to determine which approach is most likely to prevent HLB in young trees in order to bring them into production.
Objective 5 will examine whether, once a young tree has become infected with the HLB pathogen, there is a benefit to continuing to control psyllids on those plants already infected.

1. Assess effects of abiotic factors (light quality, photoperiod, air flow, and temperature fluctuations) on psyllid movement.
2. Evaluate physiological limits and biotic factors effecting of movement including feeding, egg load, infection status, and population density.
3. Evaluate techniques for tracking psyllid movement in the field for mark recapture studies.
4. Characterize seasonal patterns of ACP distribution and movement at different scales in the field.
5. Develop strategies to protect young trees from colonization by ACP utilizing UV reflection for repellency and insecticide-treated trap crops (such as *Bergera koenigii*) to attract and kill.

The goal of this project is to evaluate the efficacy of non-neurotoxic chemicals, known for their insecticidal properties, against ACP. The mortality, feeding behavior, host selection, development, and fecundity will be determined in controlled laboratory experiments. Subsequently, thorough field investigations will be conducted with the most promising chemicals under Florida conditions.
Objective 1. Determine the effect of non-neurotoxic compounds on mortality of ACP.
Objective 2. Investigate sublethal effects of non-neurotoxic compounds on development and fecundity of ACP.
Objective 3. Quantify the effect of non-neurotoxic compounds on ACP feeding behavior.
Objective 4. Determine the effectiveness of proven non-neurotoxic chemicals against ACP under field conditions in Florida citrus.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
609	Control of citrus huanglongbing by exploiting the virulence mechanisms of *Candidatus* Liberibacter asiaticus and inducing plant defense	Wang, Nian University of Florida	5/1/2012 - 4/30/2015	$413,783.00
701	Exploitation of visual stimuli for better monitoring and management of ACP in young citrus plantings	Allan, Sandra USDA	5/1/2013 - 4/30/2015	$119,096.00
701-1	Enhancement—exploitation of visual stimuli for better monitoring and management of ACP in young citrus plantings	Allan, Sandra USDA	7/1/2013 - 6/30/2014	$115,000.00

Objectives

This project is a combination of projects No. 609 and 613 as requested by CRDF. The goal is to develop management strategies that boost plant defense to protect citrus from HLB by exploiting the virulence mechanisms of *Candidatus* Liberibacter asiaticus (Las), how Las manipulates plant defense, and control HLB by blocking the translocation of Las inside the phloem from psyllid feeding sites. The virulence mechanisms of Las are largely unknown. Understanding the virulence mechanisms is important for HLB management. Importantly, we identified an enzyme salicylate hydroxylase encoded by sahA of Las, which breaks down salicylic acid (SA) and its derivatives. SA plays a central role in plant defenses. Degradation of SA is likely one important strategy of Las to suppress plant defense. In addition, phloem blockage caused by Las due to phloem proteins and callose has been suggested as a plant defense response. However, the deposition of phloem proteins and callose caused by Las seems to be very slow and could not be observed in the asymptomatic leaves, even though Las is present. Phloem-feeding insects cause rapid and significant host gene expression to insect feeding. It is probable that psyllid feeding could significantly affect citrus gene expression. Thus, modifying the promoters of phloem proteins and callose synthase genes with psyllid-induced promoters has the potential to block Las from moving by accelerating local phloem blockage around psyllid feeding sites. In order to achieve the goal of this study, the following objectives will be conducted: to characterize how Las causes HLB disease symptoms and how Las manipulates plant defense response by investigating the roles of putative virulence factors, to test different compounds in controlling HLB and characterize their mechanisms in controlling HLB, and to control HLB by blocking the translocation of Las inside the phloem from psyllid feeding sites.

The long-term goal of this project is to develop a push-pull system for protection of young citrus plantings from infestation of Asian citrus psyllids to complement insecticidal control and to protect young trees from citrus greening. This push-pull system will be developed through developing a better understanding of the visual cues that influence ACP attraction and repellency.
Objective 1. Evaluate factors that enhance visually induced flight behavior, including take-off tendency, flight duration, and light choice of psyllids.
Objective 2. Optimize attraction of psyllids to visual targets to provide a pull system.
Objective 3. Develop and evaluate push-pull systems in small orchards and commercial groves.

Enhancement

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
711	Identification of *Bacillus thuringiensis* endotoxins active against adult Asian citrus psyllid	Bonning, Bryony Iowa State University	5/1/2013 - 4/30/2017	$500,000.00
726L	A bacterial virus based method for biocontrol of Liberibacter	Gonzalez, Carlos Texas AgriLife Research	5/1/2013 - 10/31/2016	$386,902.00

Objectives

The overall goal of this application is to identify a Bt crystal toxin with toxicity to ACP and to further enhance toxicity by genetic modification. The long-term goal of the proposed research is an effective management strategy for the psyllid and associated huanglongbing disease that is more sustainable, less costly, and more environmentally benign than the repeated application of broad spectrum insecticides. This work will provide the foundation for sustainable management of the ACP through the use of transgenic citrus, or through delivery of the ACP-active Bt toxin using a nonpathogenic phloem-limited virus, such as the *Citrus tristeza virus* vector.

Screen Bt strains for activity against the ACP and identify ACP-active Cry toxins. We will screen toxins from up to 200 Bt strains with diverse insect toxicities and toxin profiles for ACP toxicity.

Modify a Bt toxin for psyllid toxicity. We will isolate peptides that bind to ACP gut membrane by screening a phage display library, and confirm gut binding of selected peptides.

Isolate and characterize Xac phages. As previously stated a large pool of virulent phages having a diversity of surface receptors is necessary for the development and implementation of a successful phage-based biocontrol system for control of Xac. However, due to limit of 1-year funding we will work to isolate and characterize a limited pool of phages. It must be reiterated that only virulent and nontransducing phages should be used for the development of an effective, sustainable, and ethical phage-based control system for citrus canker. Therefore, full characterization is necessary before phages are released as biocontrol agents. All phages will be initially characterized as previously outlined for the ability to form clear plaques, not form lysogens and morphology. The genomes of candidate virulent phages will then be sequenced and annotated to ensure virulent status. Receptor diversity will be based on limited host range studies due to time limit.

Initiate phage persistence studies on plant tissue. Studies will be conducted as previously outlined in Objective IIIa using virulent phage Xfas303, a fully characterized KMV-like phage shown to have activity against Xac field strains (North 40, Block 22, Fort Besinger; see preliminary results) obtained from Florida. Phage will be sprayed with and without a protective carrier. The protective carriers developed by Balogh et al. (2003 and 2008) and along with others will be evaluated.

Determine therapeutic efficacy of Xac phages. Studies will be conducted as previously outlined in Objective IIIb in cooperation with Dr. Nian Wang.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
733	Molecular basis of citrus greening and related diseases gleaned from genome analyses of hosts and pathogens	Grishin, Nick Southwestern Medical	4/1/2013 - 3/30/2015	$200,000.00
750	Identification of key components in HLB using effectors as probes	Ma, Wenbo University of California, Riverside	4/1/2013 - 3/31/2016	$299,781.00
766	Biotic and abiotic factors that cause Asian citrus psyllids to accept hosts: potential implications for young plantings and pathogen transmission	Stelinski, Lukasz University of Florida	4/1/2013 - 9/30/2015	$145,039.00

Objectives

The overall goal of the project is to generate hypotheses about molecular mechanisms of CLas pathogenicity from the comparative genomic analysis of the host, pathogen, and vector, and to present the results as a website that shows predictions of spatial structures and functions for all proteins in analyzed genomes for the citrus research community to use. The following specific objectives will be the steps toward the goal:
Obj. 1. Using the computational pipeline developed for the analysis of CLas proteome, predict spatial structures and functions for all proteins in two available citrus genomes and drafts of the psyllid genome.
Obj. 2. Improve the prediction pipeline initially developed for prokaryotic genome and adapt it to the analysis of eukaryotic genomes.
Obj. 3. Present results of analysis as a comprehensive website with a dedicated webpage for each protein in these organisms, showing details of predictions.
Obj. 4. Compare analyzed genomes with each other. Find predicted physical and functional interactions and associations between proteins from different genomes.
Obj. 5. Foster collaborations with experimentalists, helping them with the analysis of proteins they have chosen as research targets.

The main goal of this proposed research is to identify the targets of CLas effectors in citrus. These effector targets are likely to be key components in HLB pathogenesis. We will then explore strategies to modify these targeted processes or disrupt the interaction of effectors with their targets, which may lead to improved resistance/tolerance of citrus to HLB. Three objectives will be pursued to accomplish this overall goal:
1. Identify citrus proteins associating with three selected CLas effectors using yeast two hybrid screens.
2. Confirm the effector-host target interactions using a series of *in vitro* and *in vivo* assays.
3. Design control strategies aiming to enhance the resistance/tolerance of citrus to HLB based on effector activities and the functions of their targets.

Overall Goal: Define the factors governing ACP host acceptance in citrus groves.
Objective 1. Determine how biotic and abiotic parameters differ between resets, mature trees, and "party trees."
Objective 2. Define the biotic factors that affect ACP host acceptance and fitness.
Objective 3. Investigate whether ACP population abundance differs among resets, mature trees, and party trees, and if party trees remain consistently attractive to ACP throughout seasons and years.
Objective 4. Quantify how time since tree inoculation with the HLB pathogen and abundance of HLB-infected trees affects ACP host acceptance behavior within groves.
Objective 5. Determine if and where ACP disperse from resets and party trees and how this might contribute to HLB transmission within groves.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
769	A team approach to culturing *Ca.* Liberibacter asiaticus	Triplett, Eric University of Florida	4/16/2013 - 10/15/2015	$448,257.00
805	Functional genomics of Liberibacter in a model system	Long, Sharon Stanford University	4/1/2014 - 12/30/2016	$540,197.00
834	Optimizing heat treatment in the fields and understanding the molecular mechanism behind the success of thermotherapy for the control of citrus HLB	Duan, Yongping USDA	4/1/2014 - 3/31/2016	$385,900.00

Objectives

The overall goal of this project is to culture *Ca*. Liberibacter asiaticus.
Determine the nutrient content of citrus and periwinkle phloem, which are well colonized by CLas, and murraya and tomato phloem, which are not good habitats for CLas. Team members involved: Nabil Killiny-Mansour (primary), Mark Hilf, and Eric Triplett.
Determine the nutrient content of the psyllid hemolymph. Team members involved: Kirsten Pelz-Stelinski (primary), Nabil Killiny-Mansour, David Hall, and Eric Triplett.
Prepare and test media based on the findings from Objectives 1 and 2 to attempt to culture CLas. Team members involved: Mike Davis (primary), Mark Hilf, and Eric Triplett.
Digitize the media recipes attempted to date by Prof. Davis into a searchable relational database. Team members involved: Eric Triplett (primary) and Mike Davis.
Once cultured, CLas will be characterized physiologically and made available to the world through two bacterial strain repositories. Team members involved: Eric Triplett (primary), Mike Davis, and Mark Hilf.
Determine the role of CLas prophage in the culture deterioration over time. If prophage activation is correlated with CLas culture deterioration, efforts will be made to inhibit prophage activation as well as search for CLas strains that lack the SC1 prophage in the genome. Team members involved: Eric Triplett (primary) and Dean Gabriel.

Clone a preliminary set of CLas regulatory genes, and synthesize/clone target promoters, and provide proof of principle for use of this synthetic model system approach.
Use activator/promoter pairs to control expression of fluorescence in *S. meliloti*. Use these engineered strains to carry out high-throughput screen of compounds that inhibit the CLas regulatory protein.

1. To investigate the effect of heat stress on Las, associated prophages, and those genes involved in the phage lytic cycle.
2. To monitor healthy and HLB-affected citrus genome-wide response to heat stress.
3. To optimize field thermotherapy to produce a standard operating procedure.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
850	Scheduling ACP spring spray selection based on the Citrus Flowering Model	Albrigo, Gene University of Florida	4/1/2014 - 3/31/2017	$90,000.00
853	Why is *Poncirus trifoliata* resistant to colonization by Asian citrus psyllid?	LaPointe, Stephen USDA	5/1/2014 - 8/30/2016	$187,681.00
858	New nonphytotoxic composite polymer film barrier as ACP repellent for controlling HLB infection	Santra, Swadeshmukul University of Central Florida	4/1/2014 - 3/31/2017	$350,000.00
910	An integrated approach for establishment of new citrus plantings faced with the HLB threat	Powell, Chuck University of Florida	4/1/2014 - 9/1/2016	$369,714.00

Objectives

The overall objectives of these studies are to provide advanced knowledge of spring leaf flush and flowering for spring flush ACP control and predict the best 4- to 5-week window for bee foraging in citrus.
1. Collect weather, flowering, and leaf flush data for recent 10 years and use to improve the Citrus Flowering Monitor System. Particular goals are to improve near-bloom temperature responses of the model and add leaf flushing time to the flowering time in the monitor system. Additionally, it would be desirable to add the 5% open flower point as the point of likely bee activity.
2. Establish a 4- to 5-week peak flowering period as part of the monitor and test it for use of chemicals with ACP effectiveness but minimum bee toxicity, after which all effective ACP control chemicals would again be used. Work with growers and beekeepers to see if this approach is feasible for both concern of the grower for adequate ACP control and beekeepers for adequate access to citrus flowers for their bees.

Identify host plant–produced volatile chemicals and leaf/plant metabolites that are attractive or repellant to adult male and/or female ACP.
Test preference (antixenosis) and development (antibiosis) of ACP adults on susceptible and resistant host plants.
Identify attractive or repellent volatiles, metabolites, or their blends, and study the behavior of ACPs to these odorants or taste blends using Y-tube olfactometer assay, caged vial assay, flight tunnel assay, and SPLAT probing assay.

1. OSCF formulation development and optimization (Santra) In this objective we will perform the following tasks. (1) Prepare OSCF materials from readily available chemicals (such as silica precursors, acid/base catalyst, ionic cross-linker, and PAM), (2) Systematically characterize OSCF materials, (3) Optimize the synthesis protocol to produce OSCF formulations that will exhibit strong rainfastness, (4) Develop a scalable synthesis protocol and prepare formulations for field trials, and (5) Study OSCF liquid formulation stability and evaluate its shelf-life.
2. EPG testing to evaluate change of ACP feeding behavior (Rogers).
3. Field trial for evaluation of ACP population control (Graham and Rogers).
4. Evaluation of HLB infection using PCR method (Graham and Irey).

The objective of this project is to determine the optimum combination of chemotherapy, thermotherapy, and nutrient therapy that can be registered for use in field citrus and control HLB. This will be broken into three stages: treatment of field trees, analysis of the effect of the treatment on trees, making conclusions about optimum HLB control strategies.

Project #	Project Title	Principal Investigator	Start and End Date	Original Contract Total
916	Screening and application of antibacterial producing microbes to control citrus huanglongbing	Wang, Nian University of Florida	7/1/2014 - 6/30/2017	$431,180.00
922	Control citrus canker by manipulating the EBE (effector binding element) of CsLOB1, which is the citrus susceptibility gene for citrus canker disease	Wang, Nian University of Florida	4/1/2014 - 3/31/2017	$436,045.00
928.1C	Field trial of naturally occurring microbes	Sutherland, Dudley Glades Crop Care, Inc	4/11/2014 - 3/31/2017	$100,788.00
934C	Soil drenches of products to combat initial HLB infection in young citrus trees	Curtis, John Better Crops, LLC	6/1/2014 - 9/30/2016	$15,000.00
941C	Influence of thermal therapy on transmission of *Candidatus* Liberibacter asiaticus	Pelz-Stelinski, Kirsten University of Florida	2/1/2015 - 7/31/2016	$105,782.00
944C	RSA—small plant assay for testing the efficacy of antimicrobial materials against HLB	Pelz-Stelinski, Kirsten University of Florida	3/1/2015 - 7/31/2016	$125,797.33

Objectives
Test antibacterial-producing bacteria against *Liberibacter crescens* and other Rhizobiaceae bacteria that are closely related to Las. Control HLB using antibacterial-producing bacteria.
Generate nontransgenic EBECsLOB1-modified citrus. Test how modification of the EBE of CsLOB1 gene affects citrus resistance against citrus canker and other important traits. Test how modification of the EBE of CsLOB1 gene affects citrus resistance against citrus canker. 2.2 Experimental evolution analysis of the Xcc strain on the EBECsLOB1-modified grapefruit to investigate how stable is the resistance. 2.3 Test how modification of the EBE of CsLOB1 gene affects other important citrus traits.
Objectives of the Project: Test five soil-applied products, with mulch subplots, plus an untreated control (six treatment plots) on health and HLB status of orange trees over 3 years.
Test four soil-applied treatments plus a water-treated control (five treatment plots) on health and HLB status of orange trees over 3 to 5 years. The five treatment plots of 20 trees each will be replicated four times.
The objective of this study will be to determine how each of these heat treatment methods may influence acquisition of a systemic, circulative infection of *Candidatus* Liberibacter asiaticus by the Asian citrus psyllid, and subsequent inoculation (transmission) to susceptible hosts. Moreover, the magnitude of heat treatments on Las transmission will be evaluated over time for 1 year following application. A second objective is to evaluate the acquisition and transmission efficiency of nymphs subsequent to development on infected plants subjected to heat treatments.
RSA-The objectives of this assay are to test the efficacy of antimicrobial materials at various rates against CLas; mobility of the material in the plant, particularly in the phloem; and phytotoxicity.