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On the Cover:
Carrizo citrus seedling transformed with the DSRed gene fluoresces red under green light. This work is part of CRB-funded research to develop marker-free transgenic citrus lines. For the full report, see “Developing a DNA Engineering Platform in the Citrus Genome” by James Thomson, Ph.D., on page 58. Photo courtesy of Min Shao, Ph.D., Thomson Lab, USDA.
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Winter 2018 | Volume 9 • Number 1 The Official Publication of The Citrus Research Board

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The Mission of the Citrus Research Board:
To ensure a sustainable California citrus industry for the benefit of growers by prioritizing, investing in and promoting sound science.

Citrus Research Board Member List
By District 2017-2018  (Terms Expire September 30)

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<td>Jason Orloff</td>
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<td>Larry Wilkinson</td>
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<td>Jim Gorden</td>
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<td>Etienne Rabe</td>
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<td>Joe Stewart</td>
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<td>Franco Bernardi</td>
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<td>Keith Watkins</td>
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<td>Jeff Steen</td>
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**District 2 – Southern California – Coastal**

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**Public Member**

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<td>Marilyn Kinoshita</td>
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Calendar of Events 2018

**January 10**
Citrus Pest and Disease Prevention Committee Meeting; Visalia, California. For more information, visit: [www.cdfa.ca.gov/citruscommittee](http://www.cdfa.ca.gov/citruscommittee)

**January 23-27**
NAREEE Citrus Disease Subcommittee; Fort Pierce, Florida. For more information, visit: [https://nareeeab.ree.usda.gov/meetings/general-meetings](https://nareeeab.ree.usda.gov/meetings/general-meetings)

**January 25**
California Citrus Quality Council Annual Meeting and Board Meeting; Doubletree Hotel, Bakersfield, California. For more information, visit: [http://ccqc.org](http://ccqc.org)

**February 6**
Citrus Day at University of California, Riverside; Riverside, California. For more information, visit: [www.citrusvariety.ucr.edu](http://www.citrusvariety.ucr.edu)

**February 8**
Citrus Research Board Meeting; Riverside, California. For more information, contact (559) 738-0246, or visit: [www.citrusresearch.org](http://www.citrusresearch.org)

**February 13-15**
World Ag Expo; Tulare, California. For more information, visit: [www.worldagexpo.com](http://www.worldagexpo.com)

**March 8**
California Citrus Mutual Citrus Showcase; Visalia Convention Center, Visalia, California. For more information, contact (559) 592-3790 or visit: [www.cacitrusmutual.com](http://www.cacitrusmutual.com)

**March 14**
Citrus Pest and Disease Prevention Committee Meeting; Riverside-San Bernardino, California. For more information, visit: [www.cdfa.ca.gov/citruscommittee](http://www.cdfa.ca.gov/citruscommittee)

**March 23**
California Citrus Quality Council Meeting; Doubletree Hotel, Bakersfield, California. For more information, visit: [http://ccqc.org](http://ccqc.org)

**April 17-18**
39th Annual CRB Citrus Post-Harvest Pest Control Conference; Embassy Suites Mandalay Beach Resort, Oxnard, California. For more information, contact (559) 738-0246, or visit: [www.citrusresearch.org](http://www.citrusresearch.org)
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Dan Dreyer was elected chairman of the Citrus Research Board (CRB) at the group’s annual meeting on September 26, 2017, at the Lindcove Research and Extension Center in Exeter, California. Dreyer is a third-generation California family farmer from Exeter. He will serve a one-year term as chairman, until September 2018.

Dreyer has had significant involvement on numerous CRB committees. He has served as chair of the Communications, Research Priority Screening and Production Efficiency sub-committees and as a member of the Executive, Pest Management, Vectored Disease and Quality Assurance Committees.

In addition to being a grower, Dreyer also is a farm manager at Limoneira Company, where he is responsible for daily management and redevelopment of citrus properties in northern Tulare County. Dreyer previously served as the
Justin Brown was elected vice chairman of the CRB. He is the vice-president and general manager of D Bar J Orchards in Orange Cove, California, where he has worked for more than a decade. Brown has served on the CRB since 2009 as chair or vice-chair of numerous committees and as a member of the Executive Board.

The Visalia native holds a B.S. in Ag Business Management from California Polytechnic State University, San Luis Obispo, a J.D. from San Joaquin College of Law and is a California state bar-accredited attorney-at-law. Brown has said that he is interested in ensuring that the CRB is a “well-oiled machine” and that grower funds are handled efficiently and responsibly.

John Konda was re-elected to serve an additional one-year term as secretary/treasurer. As a Tulare County citrus grower from District 1 who owns and operates Konda Farms, he produces citrus, pistachios and row crops. Konda will continue his leadership as chairman of the CRB Finance Committee. Since first being elected to the Board in 2012, he has sat on nearly every CRB committee. The Terra Bella native’s primary areas of interest are new varieties; finance; and research development and implementation.

Prior to Tulare County, Konda worked six years for the Merced County Agricultural Commissioner’s office as a pest detection trapper. She earned her B.S. in Agronomy from Arkansas State University in 1985, but says she first became interested in agriculture while growing up on her family’s farm in Nebraska, where they raised cattle and grew alfalfa, soybeans and wheat.

Please join us in congratulating our executive officers and welcoming our new board members.

The CRB also welcomes new District 1 Northern California Board Member Jason Orlopp. A California Polytechnic State University, San Luis Obispo alumnus, Orlopp returned to his roots in Tulare County, where he is now a managing partner of foothill Ag Services, Inc. in Orosi. Orlopp will serve a three-year term.

Tulare County Agricultural Commissioner Marilyn Kinoshita was nominated to fill the CRB Public Member seat by unanimous vote. Kinoshita joined the Tulare County Agricultural Commissioner’s department in 1993 and has risen in the ranks since then, currently serving as the ag commissioner and sealer.

Prior to Tulare County, Kinoshita worked six years for the Merced County Agricultural Commissioner’s office as a pest detection trapper. She earned her B.S. in Agronomy from Arkansas State University in 1985, but says she first became interested in agriculture while growing up on her family’s farm in Nebraska, where they raised cattle and grew alfalfa, soybeans and wheat.

Please join us in congratulating our executive officers and welcoming our new board members.

Carolina Evangelo is the Citrus Research Board’s director of communications and the co-publisher/project manager of Citrograph. For more information, contact Carolina@citrusresearch.org.
City of Riverside employee Joyce Jong is recognized as a “Citrus Hero” for her efforts to protect California citrus.

Protecting Citrus Through Elected Official Partnerships

Mark McBroom
Residents often look to their local elected officials and government leaders as valuable sources of information—and for that reason, elected official outreach is an important component of the Citrus Pest & Disease Prevention Program (CPDPP).

To keep residents informed about the Asian citrus psyllid (ACP), huanglongbing (HLB) and the actions they can take to protect their community’s citrus trees, CPDPP’s outreach team regularly liaises with city governments and local elected officials throughout the state via city council meetings, one-on-one briefings and other communication efforts.

These efforts have helped the program build strong, collaborative relationships with elected officials and government leaders, who then share the information provided by the program with their constituents. For example, elected officials and city governments who want to protect California’s citrus have assisted CPDPP with securing news stories in local media outlets, sharing information about the issue on their social media channels, running public service announcements on local television stations and including ACP and HLB information in city-wide mailings such as water bills. To date, CPDPP has met with officials of more than 400 cities throughout California who have agreed to help educate their residents on ACP and HLB.

One of the most successful areas of engagement with elected officials is in Riverside, which offers a model for how future collaborations with cities and counties could be executed. Following the detection of HLB in Riverside County this past July, the program worked quickly with the City of Riverside to develop a multifaceted public outreach and education campaign throughout the community. Key elements included:

- multiple articles in The Press Enterprise, which drove calls to the state’s hotline with residents eager to have their trees inspected and tested;
- presentations at a Riverside City Council meeting and a County Board of Supervisors meeting;
- a public meeting for residents in an area of Riverside popular for ranchette-style properties with a small acreage of citrus trees, and a postcard mailer to residents;
- a public service announcement on leading Spanish and English radio stations;
- digital billboards hosted by the City of Riverside alerting residents to the disease’s presence;
- City of Riverside e-newsletters, web site copy and social media messages;
- Facebook ads targeting Riverside residents;
- a large banner at Citrus Heritage Park and a special plaque mentioning HLB at the parent navel orange tree;
- engaging master gardeners to conduct area outreach;
- booth space at local fairs and festivals;
- public utility bill inserts and mailers and abandoned grove and abatement notifications.

All of these elements were executed quickly after the first HLB finding in Riverside and have continued since then due to the relationships CPDPP had built with the City of Riverside and the County of Riverside prior to the residential HLB finds. These relationships also help with the cooperation of the California Department of Food and Agriculture’s treatment efforts and to ensure that residents are well educated about the importance of suppressing ACP through proper inspection and treatment.

To recognize CPDPP’s partners in local governments across the state, the program is introducing the “Citrus Hero” award, a bi-monthly honor given to elected officials or staff members who go above and beyond in educating the public about how to protect backyard citrus from the pest and HLB, and who also champion the program’s cause. The program’s outreach team will select recipients based on their efforts to help protect California citrus during the previous two months.

City of Riverside employee Joyce Jong is the first Citrus Hero recognized by the program. For more than two years, Jong has led the city’s efforts to educate residents throughout Riverside on what can be done to stop the spread of ACP and how to inspect their trees for signs of HLB. Her work has included setting up meetings, sharing information and working hand-in-hand with local elected officials to get the word out in the community.

Jong ramped up city efforts and worked with the program to develop a multi-faceted public outreach and education campaign. She also serves as a spokesperson for the program, providing interviews to local radio stations, including an interview with NPR’s Southern California station.

“Riverside is synonymous with citrus. It’s not only part of our heritage, but also an important economic driver for our community. Additionally, most homes in our community have backyard citrus trees, so I see it as critical for our residents to know what they can do to protect their trees,” said Jong in accepting the award.

Future Citrus Hero Award recipients will be announced through the program’s Facebook page, “California Citrus Threat.”

As California continues the fight against HLB, collaboration with elected officials and government leaders will continue to be a priority for CPDPP. By working together, we can help save our citrus.

Mark McBroom is the outreach subcommittee chairman of the Citrus Pest and Disease Prevention Program and a CRB Board Member. For more information, contact desertcitrus@aol.com.
On October 11, 2017, nearly 600 citrus growers and industry members gathered at the Wyndham Hotel in Visalia, California for the Citrus Research Board’s (CRB) annual California Citrus Conference. The meeting drew both national and international attendance with delegations and attendees from the U.S., Mexico, Canada, South Africa and Australia. Attendance was 35 percent higher than the previous year.

The objective of the free one-day conference was to deliver research updates on CRB projects that have been funded by grower assessment dollars to protect and sustain the California citrus industry.

Attendees listened to 14 speakers. Talks focused heavily on huanglongbing (HLB) and ‘Candidatus Liberibacter asiaticus’...
(CLas), the presumptive HLB pathogen. Nearly 75 percent of the CRB’s research budget is directed toward work on HLB and the Asian citrus psyllid (ACP).

California Department of Food and Agriculture Secretary Karen Ross was this year’s keynote speaker. She discussed the industry’s work and also the early investment in raising public awareness of the devastation that can be caused by ACP and HLB. Additionally, the Secretary emphasized the key message of collaboration efforts within the industry, which have led to Ross being a driving force behind the strategic plan to combat HLB and ACP. She further stressed the importance of early HLB detection technologies and how key those will be to the survival of the California citrus industry.

A three-member panel moderated by CRB Chief Research Scientist Melinda Klein, Ph.D., discussed current research and developments within the biological control program, designed to reduce ACP populations in residential areas.

Additional talks also were presented on integrated pest management, plant-associated microbial communities, post-harvest insect control, breeding HLB-resistant rootstock and nitrogen management. Industry updates were given by California Citrus Mutual and the Citrus Pest and Disease Prevention Program.

Fifty-two scientific research posters were on display throughout the conference, which represents an increase from 33 posters last year. The poster sessions allow growers to talk face-to-face with researchers about their projects and how soon their work can be practically applied in the field.

The CRB already is beginning to plan the 2018 conference. Please watch upcoming issues of Citrograph for the exact date announcement.

Carolina Evangelo is the director of communications for the Citrus Research Board, where she also serves as co-publisher and project manager of Citrograph. For additional information, contact carolina@citrusresearch.org
1. Nearly 600 attendees filled the Wyndham ballroom for the 2017 California Citrus Conference.

2. Conference attendees viewed scientific research posters sponsored by DOW AgroSciences.

3. Gary Schulz and Tamara Tollison of the CRB presented the top door prize winner James Karle with a Traeger Grill and accessories.

4. Spencer Walse, Ph.D., USDA-ARS, San Joaquin Valley Agricultural Sciences Center, presented his talk on post-harvest research.

5. CRB President Gary Schulz welcomed attendees to the 2017 California Citrus Conference.

6. Conference attendees viewed scientific research posters sponsored by DOW AgroSciences.
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These generous supporters have made this year’s California Citrus Conference a FREE event.
This October marks a momentous occasion – the 50th anniversary of the Citrus Research Board (CRB) – and the organization is inviting the California citrus industry to join in the celebration.

You’ll soon see a special 50-year logo introduced to commemorate the occasion. Most activities will take place in conjunction with the California Citrus Conference, scheduled for October. The conference itself will be preceded by a special golden anniversary gala dinner.

“During the past half-century, the CRB has prided itself on working with the growers to support research projects that have been crucial to the sustainability and success of the California citrus industry,” said CRB President Gary Schulz. “We look forward to joining together in the fall to celebrate 50 years of partnership and achievements and to share the progress being made in all currently funded research projects.”

Additional 50th anniversary details will be shared in the coming months as plans are finalized.
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*Tree royalties apply to all three varieties featured.*

*Orders must be confirmed by March 31, 2018*
The Citrus Research Board (CRB) continually strives to identify and support the best, most relevant research projects to help ensure the sustainability and profitability of the California citrus industry. To that end, the Board conducted a second external review of CRB-funded HLB-related research earlier this year. This review was led by a team of three panelists charged with critically reviewing the CRB research portfolio’s scientific content and quality and also with providing suggestions to improve and/or identify any necessary changes in emphasis or direction.

Panelists were brought together in spring 2017 to review the entirety of CRB-funded ‘Candidatus Liberibacter asiaticus’ (CLas; presumed causal agent of HLB) and Asian citrus psyllid (ACP)-related research projects. Six apparent divisions in the research portfolio were identified, and each of the panelists worked extensively with CRB-funded researchers throughout the spring and summer to review ongoing research efforts. The three panelists and their focus in the CRB project review were as follows:

- **John da Graça, Ph.D.,** is director of the Texas A&M University-Kingsville Citrus Center in Weslaco, Texas. A citrus pathologist with 40 years of experience in graft and vector transmitted diseases including HLB, da Graça reviewed projects associated with CLas avoidance and CLas detection.
- **Ed Stover, Ph.D.,** is a research horticulturalist working at the US Horticultural Research Laboratory in Fort Pierce, Florida. With experience in plant improvement, plant genetic resources, cultural practices to enhance fruit quality and production, Stover reviewed projects associated with managing HLB severity/therapies and citrus variety improvement.
- **Dan Strickman, Ph.D.,** is a senior program officer at the Bill and Melinda Gates Foundation in Seattle, Washington. A medical entomologist, he has extensive experience in insect control, biological control, operations management and product development. Strickman reviewed projects associated with ACP chemical and biological control.
Executive Summary
from the CRB HLB External Scientific Review Final Report

1. Unsurprisingly, the need for urgent action to both develop and implement measures to slow the spread of HLB in California was abundantly apparent from the review.

2. The essential activities to slow the spread of HLB are as follows:

   a. Risk assessment: Current modeling, mapping and DATOC efforts seem adequate and necessary.

   b. Surveillance: Early detection techniques are not adequate yet. We recommend a blue-ribbon panel be convened to assess and prioritize research and development of diagnostics, followed by concentrated funding of those tools judged feasible and necessary.

   c. Control:

      i. Asian citrus psyllid: Insecticides and biological control are not adequate in themselves to eliminate the potential for transmission. Development of more effective, longer-term solutions is essential while using current methods to delay spread of the pathogen.

      ii. CLas: Therapeutic methods are experimental, but effective measures should be very useful to maintain commercial viability of groves while permanent solutions (probably resistant GM or non-GM cultivars) are developed.

      iii. CLas-resistant citrus cultivars and/or ACP unable to acquire or transmit CLas likely will be the permanent solution(s) for citrus production where CLas is endemic.

   d. Sustainment: As permanent solutions are implemented and the HLB problem is managed, there will be a continuing need to monitor dooryard and commercial California citrus to prevent disease resurgence. It is not too early to develop plans for a successful outcome in which California coordinates with its citrus industry for on-going effective HLB control.

3. The CRB should consider more intensive management of some kinds of research projects.

   a. High risk projects: More speculative, exploratory projects should have clear, measurable decision points at which they can be terminated or continued.

   b. Product development: Projects that have as their objective the development of a product should follow standard practice of preparing “use case analyses,” integrated product development plans, consideration of regulatory barriers and consideration of intellectual property.
Ph.D., deputy assistant administrator in the US Department of Agriculture (USDA)-Agricultural Research Service (ARS) Office of Technology Transfer discussed the range of technology transfer opportunities through the USDA and the benefits of different approaches when moving research into the commercial realm. Steve Lindow, Ph.D., professor of plant pathology at the University of California, Berkeley, shared on-going efforts to fight Pierce’s disease in grapes, approaches that include inoculating grapevines with other xylem-localized bacteria to compete with Xylella fastidiosa (the causal agent) and exploring the use of novel antimicrobial compounds. Dave Rizzo, Ph.D., professor of plant pathology at UCD, talked about his work identifying sudden oak death and the range of efforts underway to limit the spread of that disease, including extensive use of risk models to identify high-risk areas and citizen science efforts to monitor for the disease.

Following the in-person presentations, the panelists met and prepared a report on their conclusions. This report was presented to the CRB Research Priority Subcommittee, and the written report is available on the CRB web site (www.citrusresearch.org). The Executive Summary has been included as a sidebar on the previous page to highlight the panelists’ conclusions.

The CRB Board and staff are grateful for the hard work and dedication of the External Review Panel, as well as the support and contributions of all the speakers, researchers and participants who attended this conference. The Board is reviewing the committee’s suggestions and looking forward to incorporating many of the recommended changes in the coming months. 🌿

**Melinda Klein, Ph.D., is the chief research scientist for the Citrus Research Board in Visalia, California, where she also serves as the science editor of Citrograph. For additional information, contact melinda@citrusresearch.org.**
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Area-wide psyllid control is a major component of huanglongbing (HLB) mitigation in all U.S. citrus-producing areas. Traditionally, psyllid sprays are calendar- or threshold-based, which has resulted in higher spray frequencies and associated costs leading to growers’ “psyllid fatigue.” Since the psyllid problem is essentially an HLB problem, and knowing that flush shoots regulate psyllid population and HLB epidemiology, we propose a citrus tree phenology-based control program focused on cost-effective reduction of psyllid densities and limiting risks of HLB spread – a SMART HLB™ strategy.

Globally, HLB is the most destructive citrus disease, significantly reducing fruit yield quality and substantially increasing growers’ production costs. In the U.S., the disease presumptively is caused by ‘Candidatus Liberibacter asiaticus’ (CLas), a bacterium transmitted by the Asian citrus psyllid (ACP). At present, HLB is incurable, hence its management has relied on the three-pronged approach recommended by the National Academy of Sciences (2010) – propagation of pathogen free nursery stocks, roguing of infected trees and ACP control. Significant changes in nursery regulations have been implemented in all major citrus-producing states to exclude psyllids and produce certified pathogen-free plants. Effectiveness of roguing is based on early and timely detection of infected trees.
However, extended CLas incubation, uneven CLas distribution in infected trees and HLB development latency post-infection are barriers to early detection with current diagnostics. Data from major citrus-producing states point to the fact that CLas detection in ACP predates pathogen detection and HLB symptom expression in trees, sometimes by one or two years (D. W. Bartels, personal communication). This has led to the proposition that detection of CLas-positive ACPs is the best “early detection tool” for HLB. This knowledge guided HLB surveillance efforts in Texas and currently is being employed in California. Consequently, effective area-wide psyllid control is important to limit disease spread and eliminate CLas inoculum sources.

Traditionally, psyllid control is done on a calendar basis or when a set threshold of psyllid populations is reached following intensive grove monitoring programs. Due to the ACP’s high mobility (Sétamou et al. 2014), high reproductive potential and relatively short generation time (Hall et al. 2013), this management strategy has resulted in as many as 24 sprays per year in locales such as Florida and Brazil. Such intensive spray programs have increased production costs by $200-300 per acre (Hodges and Spreen 2012). In addition, frequent sprays have increased the risk of secondary pest outbreaks and favored the development of resistance in psyllids. Reports from Florida indicate a growing “psyllid fatigue” wherein some growers are scaling back on or simply abandoning psyllid control.

The psyllid problem in citrus is mainly a bacterium and disease problem; therefore, ACP management programs need to consider factors governing psyllid ecology and HLB epidemiology in order to be effective. It is well established that citrus flush shoots modulate abundance and population fluctuations of ACP (Sétamou and Bartels 2015) and other major citrus pests (Hall and Albrigo 2007). This has been attributed to the superior nutritional and physical properties of flush shoots for ACP feeding and reproduction relative to mature shoots (Sétamou et al. 2016b). Also, CLas acquisition (Sétamou et al. 2016a) and transmission (Hall et al. 2016) by ACP are enhanced by the presence of flush shoots. Young shoots can become a source of CLas inoculum within 10-15 days post-CLas inoculation (Lee et al. 2015). Thus, young infected shoots can be important sources of CLas for further spread by ACP, especially when bacteria are acquired at the nymphal stage (Ammar et al. 2016). Taken together, citrus flush shoots are primary regulators of ACP population and CLas dynamics at the field level so that the risk of CLas spread is increased during the flush cycle (Figure 1). This knowledge should be incorporated into

**Figure 1.** Schematic showing the citrus tree phenology-based SMART HLB™ strategy. The goal of the strategy is targeted control of the Asian citrus psyllid during the major flush cycles. Citrus flush is critical for psyllid feeding/reproduction and acquisition/transmission of the ‘Candidatus Liberibacter asiaticus’ (CLas) bacterium.
an effective HLB management program, and we propose a citrus tree phenology-based ACP control strategy called SMART HLBTM.

What is SMART HLBTM?
The acronym stands for Sustainable Management of ACP for Reduced Transmission of Huanglongbing. The key component of the strategy is the judicious targeting of the ACP population with minimal sprays to prevent feeding and successful reproduction during flush cycles. This strategy is based on using citrus tree phenology as a guide for spray programs in groves. A graphic of the SMART HLBTM strategy is shown in Figure 1. Young citrus flush, as the preferred feeding and reproduction site for the psyllids, regulates ACP population dynamics in groves. It is also plausible and would make evolutionary sense for the amount of live CLas bacterium to also be preferably abundant in young flush shoots to promote successful acquisition and transmission. Hence, young citrus flush shoots support enhanced CLas acquisition and transmission by the ACP and, consequently, increased risk of HLB spread. Since flush cycles are relatively predictable in commercial groves, a targeted ACP control program that protects the young citrus flush should result in lower psyllid populations and limited risk of HLB spread in groves at reduced cost (Figure 1).

Field Trial to Demonstrate SMART HLBTM Effectiveness
Five to six flush cycles are observed annually in mature grapefruit (cv. Rio Red) groves in the three south Texas citrus-producing counties of Hidalgo, Cameron and Willacy (Figure 2). To evaluate the effectiveness of the phenology (i.e., Flush Cycle)-based vs. ACP threshold-based spray programs, two adjacent 25-year-old grapefruit groves of ten acres each were used for the study during the 2013-14 growing season. In one grove, ACP control was implemented at the onset of spring (one), summer (two) and fall (one) flush cycles, i.e., “Flush Cycle-based sprays.” In the other grove, ACP control was based on a mean detection rate of one ACP per trap (total of 20 traps/grove), i.e., “Threshold-based sprays.” Both groves received two dormant sprays (November and February), and the same chemical products were used for both treatments. Spray applications were done with a tractor-mounted ground rig applicator at a rate of 100 gallons per acre with commonly used chemicals including imidacloprid, thiametoxam, beta-cyfluthrin, fenpropathrin, chlorpyrifos and sulfoxaflor. Mean adult ACP counts per trap were recorded every two to three weeks by deploying 20 ACP traps along the entire border of each grove (five traps per border).

As shown in Figure 2, both spray programs resulted in comparable treatment efficacies as reflected in similar densities of adult ACP per trap. The exception was September when higher ACP counts were recorded for the Flush Cycle-based program as a result of an untimely application of insecticides during (not at the onset of) the flush cycle because of rain events. Whereas this level of control was achieved in the Threshold-based program with a total of nine sprays, the Flush Cycle-based program involved a total of only six sprays.

Economically, the Flush Cycle-based program resulted in a 25-35 percent cost saving over the Threshold-based program. The reduced insecticide input of the Flush Cycle-based program also has the added advantages of limiting impacts on non-target organisms,
especially beneficial arthropods, and less of an environmental chemical footprint. This also translates to a cost-effective reduction in the risk of HLB spread, as potential CLas-positive ACP are eliminated. Since the risks of CLas acquisition and transmission are enhanced in the presence of flush shoots, reducing vector population during this critical stage of the tree phenology will help to break the HLB disease cycle through control of breeding ACP populations. Based on these results, the Flush Cycle-based program was tweaked so that the September spray was moved back to the end of August because of the predictable early September south Texas rains. The Flush Cycle-based program currently is being implemented on more than 90 percent of Texas citrus acreage as an integral component of the area-wide citrus pest management program.

How to successfully implement SMART HLB™

The first step in successfully implementing the SMART HLB™ strategy is studying the citrus tree phenology to determine major flush cycles, described as the peak period of flush abundance by Hall and Albrigo (2007). Flush shoot production is regulated by the tree life cycle, as well as availability of moisture, temperature and nutrients. Similarly managed groves of the same tree species and age in a given geographic area are likely to flush at the same time. Once the major flush cycles are identified, the phenology-based spray program should consist of the following components:

1. area-wide coordinated dormant spray during the overwintering period and before the first major flush of the year to reduce ACP population,
2. targeted spray of groves at the onset of subsequent flush cycles during the active growing season and
3. border sprays between flush cycles when psyllids are present based on trapping or monitoring data of immigrant adults.

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References


CRB and UC ANR Bolster Research Facilities at Lindcove

Greg W. Douhan, Elizabeth Fichtner, Elizabeth Grafton-Cardwell and Rock Christiano

Project Summary
During the 2016-17 funding cycle, the Citrus Research Board (CRB) provided our research team a $39,000 equipment grant to improve the plant pathology and entomology laboratory at the Lindcove Research and Extension Center (LREC). This grant allowed us to purchase additional equipment to fully outfit the laboratory. The LREC now has a plant pathology and entomology laboratory, as state-of-the-art as many laboratories on University of California (UC) campuses, to facilitate research and diagnostics for LREC staff and UC advisors, as well as visiting scholars or students conducting research at the station for the citrus industry.
The CRB and the UC Division of Agriculture and Natural Resources (ANR) have made major contributions to improve the infrastructure for researchers at the LREC. The Center currently provides land, labor and facilities for more than 30 research projects, with 90 percent of its active projects focused on citrus. Recent funds contributed by the CRB and ANR have dramatically enhanced the capabilities at the LREC, thus allowing expansion of established research projects and making the Center more attractive to new researchers striving to address the challenges of farming in the southern San Joaquin Valley in the 21st century.

The CRB recently provided a $39,000 equipment grant for improvement of the LREC laboratory. Built in 2008 with capital improvement funds provided by ANR, the lab is approximately 2,000 square feet and contains three areas, including pathology and entomology labs and a field sample processing lab. Soon after its construction, the citrus entomology program utilized the entomology lab; however, the pathology lab remained largely vacant until 2014 when its potential for support of emerging pistachio disease research was realized. With ANR support for the purchase of incubators, freezers, a compound microscope and laminar flow hood, the pathology lab became equipped to handle the culture, identification, quantification and storage of plant pathogens. The donation of a real-time PCR machine by the Citrus Clonal Protection Program, as well as electrophoresis equipment, initiated the molecular capabilities at the LREC, enabling on-site completion of the annual Citrus tristeza virus survey and facilitating further development of applied pathology research programs. The recent funds provided by the CRB facilitated the purchase of a water purification system, a large autoclave, a new thermal cycler (PCR machine), a gel documentation system, multi-channel pipettes for PCR set-up and a digital camera that may be used on either a compound or dissecting microscope (Figures 1-4).

These lab improvements, in combination with new office facilities purchased by ANR, provide a professional and fully equipped workplace for researchers, staff and graduate students, and dramatically increase the research capabilities at the Center. The improved infrastructure allows local University of California Cooperative Extension (UCCE) farm advisors to conduct diagnostic work on site, thus reducing reliance on campus-based facilities and broadening the research opportunities for those working at the Center. It also attracts visiting scientists from abroad and supports the local community through partnership with the College of the Sequoias’ internship programs. Further, the improved facilities will aid in the upcoming recruitment and support of an LREC-based cooperative extension (CE) specialist dedicated to citrus horticultural research and extension.

The equipment upgrade to the LREC facility will enable development of a more robust research and extension program that was not previously possible. Greg W. Douhan joined UCCE as the area citrus advisor for Tulare, Fresno and Madera counties in March 2016. His academic training is in plant pathology; however, as a UCCE advisor, his specific research and extension program holistically addresses citrus productivity and education. The equipment upgrade to the LREC facility will enable development of a more robust research and extension program that was not previously possible.

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CRB Research Project #5500-212

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DATOC
Experts Provide Rapid Responses to HLB Issues

Neil McRoberts, Sandy Olkowski and Carla S. Thomas

Project Summary
DATOC (Data Analysis and Tactical Operations Center) is a diverse group of experts who provide in-depth analyses and science-based guidance for California stakeholders in the fight against huanglongbing (HLB). The project was initiated to fill a need for rapid, flexible and responsive analyses and interpretation of the complex issues surrounding HLB control.
Introduction

The Citrus Pest and Disease Prevention Program (CPDPP), which is funded by a special grower assessment, is faced with an extremely challenging task. The program’s approximately $40 million operating budget for 2017-18 funds many crucial activities, including extensive surveys of residential and commercial citrus to detect Asian citrus psyllids (ACP) and trees with HLB. Operational decisions cover a remarkably wide range of issues and often must be made quickly for resulting changes in field activities to have an impact on development of the vector population or spread of the disease.

In an ideal world, such decisions would be made by using the best science-based evidence available to select one course of action over other possibilities. Data collected in the survey programs should play a central role in guiding decisions, while a wider range of data and information also could be applied by expert advisers in collaboration with the Citrus Pest and Disease Prevention Committee (CPDPC) members and California Department of Food and Agriculture (CDFA) staff. Until recently, this type of rapid, targeted feedback between the survey program and operational decision-making had not been possible because the human resources needed to process data and carry out detailed analyses simply were not available. The idea for DATOC came from a recognition of the need to fill this gap. There was also an appreciation that the program would benefit from the availability of a panel of scientists covering a wide range of disciplines, who could both provide input on relatively short notice and regularly provide comprehensive situational awareness updates – “the state of the state” – to the CPDPC.

The mission and composition of the new group was developed during conversations between Citrus Research Board (CRB) committee chairs, University of California (UC) scientists, CDFA program staff and others; and the concept of the project as a collaboration between UC and the CRB was finalized. The project was budgeted as part of the CRB’s annual funding request to the CPDPC, but is subject to the CRB’s research project evaluation and quality assurance procedures, with Neil McRoberts, Ph.D., as the principal investigator.

In May 2017, Sandy Olkowski, Ph.D., was appointed to the CRB staff as the DATOC analyst, and the work of getting the new analysis and advisory group up and running began in earnest.

DATOC Analytical Process

There are two types of problems that DATOC is designed to address. First, there are questions raised by groups such as the CPDPC Operations and Outreach subcommittees, or by the CPDPC itself, concerning the scientific basis for their program’s activities. Additional questions come from groups such as regional grower liaisons or regional ACP/HLB Task Forces. Second, the members of DATOC raise issues that they believe need to be addressed for the CPDPC to fully achieve its objectives. Depending on how complex the question is and the amount of time or effort required to address it, a number of different analytical and reporting approaches might be used by DATOC to provide a response. Figure 1 shows a schematic of the working processes that are being used.

Although we anticipate that most of the questions DATOC addresses will come through the routes mentioned above, it is important to point out that DATOC is intended to serve all stakeholders in the California citrus industry’s fight against HLB. Answers to technical questions about the disease management program and more information about the scientific basis for any part of the program, can be addressed to Olkowski at the

![Figure 1. DATOC working processes.](image-url)
CRB offices in Visalia (see contact information at end).

Results to date

The DATOC team draws its expertise from across the United States. The majority of expert panel members gathered for a face-to-face meeting in Davis following the CRB External HLB Review in August 2017. Among the issues considered during the two-day meeting were urban buffer treatments for commercial citrus using PCR results from ACP as early indicators of the location of infected trees, as well as a response plan for commercial citrus groves to use when HLB is detected. The aim with each of these issues is for the DATOC team to produce a briefing paper containing a concise summary of the available scientific evidence and a set of recommendations for consideration by the CPDPC or a relevant subcommittee. The first briefing paper, dealing with buffer treatments, was submitted to the Operations subcommittee at its October meeting. The briefing papers for the other two topics were being prepared at press time.

The DATOC team also has been developing an infrastructure that will improve our ability to provide support to the program. Members of the team have been working diligently, in collaboration with staff at CDFA and the Division of Agriculture and Natural Resources at the University of California, to establish a Memorandum of Understanding to allow survey program data to be used for research and problem solving. At the same time, Olkowski has been working with CRB staff and an external consultant to develop an interactive, web-based tool that will allow secure data processing and collaboration by the DATOC expert panel members, who are spread across the country from California to Florida. These developments will greatly enhance the ability of the CPDPC program to evaluate its own success and make better-informed tactical decisions in the future.

Conclusion

The number of confirmed cases of HLB in southern California dooryards has risen dramatically during the last few months. However, at time of publication, no HLB-affected trees have been confirmed in California commercial citrus. The situation is undoubtedly challenging, but resources such as DATOC will strengthen the industry’s capacity to meet the threat posed by HLB and will enhance CPDPC’s ability to adapt in response to the changing situation.

CRB Research Project #5300-182

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Risk-based HLB surveys for California

An analysis of the increasing risk of HLB over the last five years

Tim Gottwald, Weiqi Luo and Neil McRoberts

Project Summary
Evidence from Florida and other citrus-producing areas of the world clearly demonstrates that huanglongbing (HLB) is not a disease that can be managed effectively by individual growers. Rather, it is a disease that confronts the whole production industry and other stakeholders simultaneously. The importance of an effective early detection system for HLB in California is increased by the existence of the large, diverse commercial citrus industry and extensive residential citrus population. Since 2013, we have developed and continue to refine a risk-based survey design that addresses the urgent need to respond to the HLB threat. The survey anticipates pathogen introduction and spread on a statewide level, rather than waiting for the disease to appear locally. Up to now, the risk-based survey model has resulted in multiple HLB discoveries in southern California and serves as a predictive tool for regulatory decision-making and disease management.
The risk-based survey is implemented on two major spatial scales: statewide and targeted. The statewide survey captures the immediate HLB situation, identifies high-risk areas and thereby predicts where the disease is likely to occur, thus serving as an early detection tool. The targeted survey utilizes detections resulting from the statewide survey detection to target the search for new infections outside existing quarantine regions. The targeted sampling augments efforts to increase the probability of follow-up HLB detection. This optimized early detection aids disease mitigation/management when the disease is at low incidence, thereby suppressing a severe impending epidemic.

Statewide Residential HLB Risk-based Survey

The over-arching goal of this project is the earliest possible detection of HLB outside the current quarantine zones, before the pathogen ‘Candidatus Liberibacter asiaticus’ (CLas) becomes established, providing time and opportunity for eradication and, thus, preservation of the California citrus industry. We developed a risk-based concept (i.e. the threat that CLas will be introduced/spread from a given location) to improve evidence-based decision-making for survey construction and optimization. The original risk-based algorithm (Gottwald et al. 2013, 2014) included various risk factors, e.g., CLas-positive finds, Asian citrus psyllid (ACP) vector population prevalence and dynamics, fruit transportation corridors, plus potential ACP spread from commercial nurseries, home centers, packinghouses, flea markets, green waste facilities and other citrus production vendors. Over the past five years, the risk-based survey model has continuously evolved via validation, yearly data re-evaluation (through new HLB/ACP finds), and adaptation to optimize early detection. The risk model is dynamic and has allowed for the incorporation of the following new/improved risk components to optimize the accuracy of an overall risk map for the statewide HLB survey:

- **Evaluation of residential citrus populations**: Linking citrus preferences to dooryard distribution is challenging, but provides new insights into residential citrus host density mapping and modeling. Dooryard citrus preferences are heterogeneous, where certain factors may be able to explain part of the variation in preference. Information on dooryard citrus tree numbers and citrus types (i.e. orange, grapefruit, lemon, lime, etc.) were collected from residential HLB/ACP survey data of more than 100,000 properties in seven Los Angeles Basin counties (Figure 1). Each county has its own pattern of dooryard citrus, where orange and lemon are the dominant residential citrus types and which account for more than 50 percent of the residential citrus population. However, residents in Riverside County tend to grow more dooryard citrus trees than those in Los Angeles County. Imperial County residents have a higher preference for grapefruit and lemon, but a lower preference for orange. Pomelo, tangerine, kumquat and calamondin occur more often in Orange County. Much of this heterogeneity is due to cultural preference and is related to human demographics. Because of differential citrus species susceptibility, estimation of residential citrus distribution is critical for understanding the ACP population dynamics and resulting HLB epidemic in the mixed residential landscape.

- **Human-mediated disease dispersal**: The efficiency, speed and distance of international travel (including the volume of passengers and goods carried) have increased the risk for CLas introduction from infected countries. Via an unrelated project, the U.S. Department of Agriculture (USDA) epidemiology lab in Fort Pierce, Florida, has developed a census-travel model to capture the possible introduction/establishment of non-indigenous plant/animal/human pathogens and diseases through international travel networks. Using the census-travel model for HLB, we can estimate the possible destination of international travelers and the risk of HLB-affected introduction determined principally by travel volume with bias for disease status from the source country. The census-travel model highlights pathways of greatest risk introduction and identifies California “hot spots” with a high HLB risk from the global travel network. The model ascribes higher sampling intensity and risk exposure to those areas where specific foreign travelers from high-risk countries are prevalent. This enables the construction of early warning systems. For example, it forms a basis for planning survey/control strategies for the Central Valley where no HLB has been confirmed yet.

- **Association between CLas-infected ACP and CLas-infected citrus**: With growing HLB risk in several southern California areas, increasing numbers of residential HLB-positive trees have been detected, bringing the total number of confirmed HLB-positive trees in California to more than 200. In addition, the number of suspected CLas-carrying ACP discoveries (e.g. regulatory qPCR testing with CT values of less than 36) increased substantially. These “hot” ACP finds occur in loose clusters, indicating probable association with HLB-positive tree(s). The spatial relationship between an HLB-positive tree and suspected “hot” ACP finds are incorporated in the risk-based survey to reflect the changes in disease dispersal and development. Figure 2 shows a series of snapshots of how HLB risk progressed in southern California from 2013 to 2017. It is very clear that the risk of HLB has increased dramatically throughout the region. This confirms the urgent need for proactive regulatory interventions to slow down the spread of this disease and thus minimize consequent economic losses.

Using similar manpower availability and sampling efficacy as in the previous survey design (Gottwald et al. 2013, 2014), the entire state of California is parsed into ten risk survey districts. The sampling density is adjusted by resource availability for
the statewide risk-based survey. The percentage of STRs (Section-Township-Range, one mile-square grid) samples proposed for each district varied from 1.6 percent to 67.8 percent in 2017, with intense sampling designed for Los Angeles Basin HLB areas. The risk-based model outputs for the 2017 residential survey were periodically distributed to the California Department of Food and Agriculture (CDFA) and the USDA-Animal and Plant Health Inspection Service (APHIS) for survey coordination/implementation. With continued data collection using the risk-based sampling protocol, we can further adjust the parameters of the risk-based prediction model through a cross validation process.
Targeted Risk-based Residential HLB Survey within Quarantine Region

Following the discovery of HLB-positive trees from the statewide residential risk-based survey, it is necessary to design a high intensity/targeted survey to maximize detection of additional HLB-infected trees in new outbreaks to prevent further spread. For example, using the San Gabriel outbreak (HLB-positive tree confirmed in July 2015) an extended quarantine of approximately a five-mile radius (i.e. 87 square miles) was created around the properties on which HLB was detected. Residential population density does not have a uniform distribution in this part of the Los Angeles Basin. For balanced and efficient sampling, the San Gabriel area (and Hacienda Heights) was divided into eight radiating segments around the center of the recently discovered HLB-positive properties (Figure 3). Six inner buffer zones (i.e. 400 meters, 800 meters, 1,200 meters, one mile, two miles and five miles) also were created to conform to different sampling strategies as needed. The over-all sampling proportion in each directional sector is determined by the number of residences and previous ACP finds. Percent sampling effort ranges from 9.2 percent to 15.6 percent among the eight directional sectors (Figure 3). Within each sector, sampling locations (i.e. census blocks) are selected based on an over-all risk algorithm (Figure 3) that includes a combination of predetermined residential HLB/ACP risk, distance from HLB finds, census-travel

Figure 2. Series of yearly maps showing huanglongbing (HLB) risk progression in southern California for 2013-2017.
risk from international connections where HLB is prevalent, previous ACP density and distribution of CLas-infected ACP locations and other risk parameters. The residential density also influences the chance selection of a location and modulates sampling intensity. Using manpower availability and sampling efficiency, a total sampling capacity of 52,800 properties was calculated through the survey period. A total of 948 moderate- to high-risk census blocks (i.e. risk greater than 0.8) were identified within the eight sectors through the risk-based model. Census blocks are shown in Figure 3. Using this risk-based method, 81 percent of the census blocks in the San Gabriel quarantine area will be sampled at least once. Approximately ten percent of the properties within the risk-defined census blocks will be surveyed to give a reliable representation of HLB dispersal.

To maximize HLB detection in San Gabriel, we provided a detailed sampling plan for three contiguous sampling cycles per year with equal duration. Because of the high intensity of risk in some areas, there are overlaps in selection of census blocks to sample between cycles. This will provide multiple opportunities to detect the disease in the higher-risk areas because the trees will be repeatedly re-examined as they go through seasonal changes of flush production and foliar maturation over time. The targeted survey methodology can be used to monitor the actual HLB situation, check whether HLB has spread outside of the quarantine area and determine if HLB is spreading in a specific direction.

Conclusions
Further HLB outbreaks in southern California are inevitable in the future as ACP populations and CLas inoculum continue to increase. Integration of the statewide and targeted risk-based HLB surveys provides an early detection and decision-making tool for regulatory agencies and citrus producers to optimize eradication/mitigation efforts. The survey results also provide a systematic means to monitor disease progress around various areas of concern in southern California. Depending on larger program goals, regulatory agencies can use survey results to balance the amount of sampling effort dedicated toward statewide monitoring efforts (e.g. capturing the global disease situation) versus targeted efforts (e.g. focusing on local disease outbreaks).
Although designed specifically for the HLB/ACP survey, this framework can be transferred easily to other potential exotic pest/disease invasion applications (e.g., citrus pests for other citrus-producing areas in California and other states). Surveying, detecting and eradicating ACP/HLB in other states (e.g. Florida, Texas and Arizona) indirectly serves California by lessening the potential threat of continuous inadvertent or illicit introductions from these distant inoculum sources into California.

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**ACP and Citrus**

*Plant Responses to Psyllid Feeding*

*Elizabeth Chin, Kris Godfrey, Cynthia LeVesque, MaryLou Polek and Carolyn Slupsky*

*Diaphorina citri* (Asian citrus psyllid or ACP), is an economically important citrus pest and is a vector of *Candidatus Liberibacter asiaticus* (CLas), a pathogenic bacterium associated with the devastating citrus disease huanglongbing (HLB). A native of Asia, the ACP is now present in other parts of the world including Mexico, Brazil and the U.S. It was first discovered in Florida in 1998 and has since spread to other states. In California, ACP initially was found only in southern California, with a large population in the Los Angeles area, but it has since been reported in increasing numbers as far north as the Placer County. Since ACP can transmit CLas, the spread of ACP also may result in an increasing incidence of HLB in California.

ACP feeding causes relatively less physical damage to plants compared to chewing insects such as caterpillars or beetles because they do not cause loss of leaf surface area during feeding. Phloem-
feeding insects, like ACP, not only extract nutrients from the plant when they feed, but also secrete saliva into the plant (Walling 2008). The saliva (gelling and watery saliva) helps the insect avoid detection by the plant and may also contain molecules that interfere with the defense response (Will et al. 2013). Some cells, however, are damaged and, in some cases, penetrated. This damage can be detected by the plant, which subsequently initiates a defense response. Additionally, the plant can recognize enzymes that the insect uses to break down plant cell components. This elicits plant defense responses.

The biochemical pathways initiated in response to phloem-feeding insects, such as ACP, differ from those initiated in response to chewing herbivores or mechanical damage. The downstream products of the defense pathways include proteins, metabolites, nucleic acids (RNAs) and volatile molecules that are used to fight off the insect attacker. Inducible defenses² cause induction of these pathways and are expressed systemically, even in the undamaged leaves of herbivore-infested plants soon after attack. This helps the plant to fend off future attackers on all fronts. In contrast, the plant also has constitutive defenses³, which are “turned on” all the time, regardless of whether an herbivore is present. This allows the plant to always have some defense present (Kant et al. 2015).

Mounting defense responses is energetically costly to the plant, as it takes many resources to synthesize the signals, enzymes and other molecules needed to effectively battle the insect attacker. Resource reallocation acts not only to mount a defense, but can also deny the herbivore attacker of essential nutrients. Indeed, changes in concentrations of carbon-containing molecules (like sugars) and nitrogen-containing molecules (like amino acids) in different plant organs have been observed in many plant-insect systems following herbivory⁴ (Schultz et al. 2013). These changes in defense and resource allocation mean that biochemical pathways within the plant are changing, and the chemicals in these pathways can be measured. All of the small molecule chemicals in a given sample can be measured and analyzed by metabolomics⁵. Because metabolomics identifies and quantifies all the metabolites (small molecules) in a sample, it gives insight into changes in metabolism.

The Slupsky Laboratory at the University of California, Davis has been studying the effects of CLas infection on plant metabolism to establish a set of HLB-specific metabolic biomarkers that may be used to determine the probability that a citrus tree is infected with CLas. To fully understand the effects of the insect vector on the host, we investigated the effects of ACP feeding on leaf metabolite composition in the absence of CLas infection using ¹H NMR ("proton NMR"). ¹H NMR uses similar technology to what is used in an MRI, and the output is called a “spectrum”. Low (10 ACP per plant, five plants total), medium (15-20 ACP per plant, ten plants total) or high (25-30 ACP per plant, ten plants total) numbers of CLas- free ACP, or no ACP (control group) were released onto six month-old greenhouse-grown Citrus macrophylla trees and allowed to feed for seven days (Figure 2), after which time leaves were collected and analyzed for metabolite content. This work was recently published in the Arthropod-Plant Interactions journal (Chin et al. 2017).

Identified and quantified metabolites included sugars (e.g. glucose and sucrose), amino acids (e.g. valine and alanine) and other small molecules such as organic acids and nucleotides. Additionally, a specific region (the “aromatic region”) of the NMR spectrum was used to infer differences in the concentrations of aromatic molecules among the treatment groups. Aromatic compounds have a special type of chemical structure and are of interest because some are secondary metabolites involved in plant defense (Bernards and Båstrup-Spohr 2008).

We observed that leaves from trees with the highest density of ACP feeding differed the most in overall metabolite composition from the control trees that did not have any insect feeding, including a significantly lower area of the aromatic region of the NMR spectrum for leaves from trees with high amounts of ACP feeding. Leaves from trees with low and medium densities of ACP feeding had intermediate differences. Several amino acids, such as arginine, valine and isoleucine, had a higher concentration relative to control in the low and medium ACP feeding groups, but lower concentration in the high ACP feeding groups (Figure 3). This pattern is interesting because it suggests insect density-dependent changes in plant metabolism. Amino acids are involved in primary metabolism, are the building...
blocks of proteins and are precursors for other molecules; changes in their concentrations change the pool of resources for plant metabolism, including mounting the defense response. For example, arginine is a precursor for nitric oxide, which is part of the plant stress response (Winter et al. 2015). Additionally, phenylpropanoids are compounds involved in plant defense and are derived from phenylalanine, which was significantly lower in concentration in leaves from trees with high amounts of ACP feeding compared to controls. Our results suggest that ACP feeding lowers production of secondary metabolites, which may include molecules like flavonoids and phenylpropanoids that are involved in plant defense and have been shown by others to be influenced by herbivory (Bernards and Båstrup-Spohr 2008, Simmonds 2003, Treutter 2005). Quinic acid, another metabolite associated with plant stress responses, including insect feeding (Sardans et al. 2014, Steinbrenner et al. 2011), increased in concentration as the number of ACP feeding per tree increased (Figure 3).

Taken together, the results of this study suggest that ACP feeding modulates plant metabolism. This may influence the plant response to other stressors (including CLas infection) because the pool of available resources and defense molecules are altered in a specific way.

Acknowledgements

In addition to the Citrus Research Board grant, this project also was made possible in part by support from the USDA National Institute of Food and Agriculture Hatch Project 1005945.

References


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**Glossary**

1. **Stylet:** The straw-like mouthpart used to pierce plant tissue and suck nutrients from the host.

2. **Inducible Defense:** Defense mechanisms that are triggered following pathogen or insect pest attack.

3. **Constitutive Defense:** Defenses that are produced at all times, regardless of the presence of an attacker.

4. **Herbivory:** The eating of plants.

5. **Metabolomics:** The study of all the metabolites (small molecules) in a given sample.

---

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Improving Early Detection Of HLB-Affected Trees

Greg McCollum, Madhurababu Kunta and Evan Braswell

Project Summary

We have been working to determine how soon ‘Candidatus Liberibacter asiaticus’ (CLas) can be detected in citrus trees following exposure to CLas-infected Asian citrus psyllids (ACP). Our team has developed a greenhouse model that allows us to produce populations of CLas infected ACP and use them to inoculate citrus trees. Young citrus shoots are enclosed in nylon mesh bags containing inoculative ACP. Using this system has allowed us to conduct controlled experiments to determine effects of exposure duration, time following exposure, and systemic movement of CLas in citrus. Under these conditions, CLas can be detected in citrus following exposure to CLas-inoculative ACP after as little as two days. One hundred percent of the nymphs that develop on citrus that was CLas-free prior to exposure to CLas-infected ACP become CLas-positive. This means that the pathogen must have passed through the plant, whether or not the citrus tree eventually develops huanglongbing (HLB) symptoms. In addition, we have determined that CLas is most likely to be found in citrus shoots where ACP have been feeding. Our results also indicate that if a citrus tree is going to develop HLB symptoms, those likely will appear within four to five months following exposure to CLas-inoculative ACP.
We conclude that in the absence of HLB symptoms, citrus flush with ACP nymphs is the tissue most likely to be infected with CLas and provide greatest diagnostic reliability. Our results clearly demonstrate that quantitative polymerase chain reaction (qPCR) can reliably detect CLas infections well before HLB symptoms develop if the proper tissue is sampled. Finally, if no HLB symptoms, ACP nymphs or ACP adults are present, shoot tips (most recently fully-expanded leaves) are the best tissue for CLas diagnostics.

Introduction

Early detection of CLas (the causal agent of HLB) infections in citrus trees is critical if HLB epidemics are to be prevented. Historically, “early detection” of CLas has been based on scouting for HLB-symptomatic trees and then roguing them to reduce the amount of CLas inoculum and, thereby, reduce the spread of HLB (Gottwald 2010; Bové 2006). However, there is a latency period between the time of CLas infection and development of HLB symptoms (Gottwald 2010; Bové 2006). During this period, CLas can be acquired by ACP, the insect that transmits CLas.

Currently, the only validated method available to confirm a CLas infection is through detection of CLas DNA, which is based on qPCR, an extremely sensitive and specific method. Although qPCR is extremely reliable for diagnostic surveys to identify suspect CLas infection in HLB-symptomatic leaves, there is a problem when trees are infected, but not yet symptomatic. The issue is not that qPCR is not sensitive enough to detect CLas, but rather where in the tree to sample to provide the greatest probability of detecting CLas prior to the development of HLB symptoms.

The consequence of being able to sample only a small proportion of any one tree combined with the low CLas titer and non-uniform distribution of CLas within trees when HLB symptoms are not visible is that many samples for diagnostic assay present false negative results. False negative diagnosis of CLas infections is dangerous because it means infected trees are allowed to remain in orchards and serve as sources of CLas inoculum that can be acquired by ACP and transmitted. Given that qPCR is currently the only validated assay to confirm CLas infections, the sampling strategy for individual trees can be improved. It will be possible to target parts of trees that are most likely to be infected with CLas and, thereby, increase the likelihood of CLas detection prior to the development of HLB symptoms, i.e. “earlier” detection.

ACP preferentially feed and lay eggs exclusively on very young citrus flush. While feeding on the young flush, CLas is transmitted from ACP to citrus. Therefore, we hypothesized that young citrus flush for CLas diagnostics effectively increases the probability of identifying incipient CLas infections.

Research Approach

CLas is only transmitted from tree to tree in the field by infected ACP. Therefore, our approach has been to produce CLas-inoculative ACP and then use them to inoculate citrus (Figure 1). CLas-infected ACP adults were produced by rearing ACP on CLas-infected citrons. Rooted citron cuttings were graft-inoculated with buds from CLas-infected citrus source plants. Four months after grafting, the plants were typically systemically infected with CLas and were HLB-symptomatic. The systemically infected citrons subsequently were exposed to adult ACP that fed and laid eggs on the citrons. Experimental citrus trees were inoculated by enclosing young flush along with five to ten (depending on experiment) presumptively CLas-inoculative ACP in nylon mesh bags for durations of up to three weeks. Although the exact infection status of the ACP adults was not known prior to placing them on citrus, based on results of numerous experiments, we can be confident that the adults used for inoculation were CLas-inoculative.

Figure 1. Left to right: production of ‘Candidatus Liberibacter asiaticus’ (CLas) inoculative ACP on citrons; Valencia orange flush enclosed with CLas-inoculative ACP; ACP adult and eggs on newly emerged leaf.
Following the exposure period, ACP adults and nymphs enclosed with the citrus flush were collected and assayed for CLas using qPCR. After ACP adults and nymphs were removed, the experimental trees were treated with insecticide to ensure they were free of ACP adults, nymphs and eggs, and subsequently maintained free of ACP. Citrus samples were collected and assayed for CLas by qPCR at various time points ranging from days to months following exposure to ACP to determine the incidence and titer of CLas infection.

### Results

**Figure 2** shows the incidence of CLas infection and titer estimates (determined by qPCR) for individual adult ACP used to inoculate citrus trees that were CLas-negative prior to exposure, and for ACP nymphs (assayed in pools of five) that developed following exposure of the trees to CLas-inoculative ACP prior to exposure to the CLas-infected adults. One hundred percent of adults and nymphs were CLas-positive. The titer in adults can be very high – in this case, as high as 100,000 CLas cells per insect. CLas titer in ACP nymphs is consistently in the range of 10 to 100 copies per individual. CLas titer in ACP adults has a pronounced impact on the incidence of CLas infection that develops in citrus (Figure 3). When CLas titer in adult ACP used for inoculations was 100,000 or greater per insect, 55–60 percent of the experimental plants were CLas-positive two weeks following exposure. However, the incidence of CLas infection was only 30 percent or 10 percent when CLas titer in the adult ACP was 10,000 or 1,000 copies per insect, respectively.

Additionally, the incidence of CLas infection remained fairly constant when CLas titer in adult ACP used for inoculation was 100,000 or greater per insect. In contrast, the incidence of CLas infection in citrus exposed to ACP adults with CLas titer of 10,000 copies per insect dropped from 30 percent at two weeks post-exposure to 20 percent at four weeks and 10 percent at six weeks following exposure. The incidence of CLas infection in trees exposed to ACP with CLas titer of 1,000 copies per insect also decreased with time following exposure, but not to the same extent as seen when CLas titer was 10,000 copies per adult ACP.

Duration of exposure to CLas-inoculative ACP adults affects the subsequent titer of CLas in the tree (Figure 4). CLas was detected in trees exposed to CLas-inoculative ACP for two, three or four days, but titer never exceeded 100 CLas cells per milligram (mg) of citrus petiole. In addition, CLas titer was always lowest at 16 weeks following exposure for two, three or four days. Following a seven-day exposure to CLas-inoculative adult ACP, CLas titer in citrus was between 10,000 and 1,000,000 cells per mg citrus petiole at 16 weeks following exposure.
When CLas titers were compared at increasing distances (0 to 10 cm) from the tips of shoots exposed to CLas, there was a decrease in both incidence of CLas infection and CLas titer (Figure 5). We also compared detection of CLas in roots and leaves of the ACP-exposed citrus (Table 1) and found that the greatest incidence of CLas detection was in petioles compared to roots, and that some roots tested CLas-negative even when petioles were positive. In addition, the titer of CLas was always higher in petioles than in roots.

Figure 6 illustrates the appearance of trees that had been exposed to ten CLas-inoculative ACP for ten days. The infected trees were stunted, chlorotic and had lower root density than the non-infected trees. HLB symptoms became visible on CLas-infected plants at around four months following exposure to CLas-inoculative ACP.

Early detection of CLas infections is critical in California citrus if HLB epidemics are to be prevented. Although there is considerable debate regarding “early” detection of CLas citrus trees that are infected, but not yet HLB-symptomatic, we show that under greenhouse conditions, CLas infections can be detected in citrus flush using qPCR within days following exposure to CLas-inoculative ACP for durations as short as two days – well before the appearance of HLB symptoms. These symptoms typically develop within four months following inoculation with CLas-infected ACP. Our data also substantiate the value of selecting the proper tissue for the greatest probability of finding CLas infections. We also have demonstrated that under greenhouse conditions, 100 percent of ACP nymphs developing from eggs laid by CLas-inoculative adults are CLas-positive, even though we never see 100 percent of trees becoming HLB-symptomatic. These results all have significance in developing HLB management strategies. In addition, our model system is designed to closely mimic CLas transmission from ACP to citrus as it occurs in the orchard. This method provides an excellent opportunity for the evaluation of alternative detection
technologies, as well as prophylactic and therapeutic treatments that may mitigate HLB disease.

CRB Research Project #5300-176

References


Glossary

1Quantitative-PCR: Method to determine the number of target copies of a specific piece of DNA from CLas.

2Titer: Analogous to concentration of CLas, expressed as the number of CLas cells per mg fresh weight citrus petiole or ACP adults or nymphs (per individual).

Figure 6. Appearance of Valencia orange shoots and roots following exposure to ‘Candidatus Liberibacter asiaticus’ (CLas) inoculative Asian citrus psyllids (ACP). The tree on the left in both photos consistently tested CLas-negative, while the tree on the right in both photos consistently tested CLas-positive. Photographs were taken four months following exposure to ten CLas-infected ACP for one week.

Table 1. Incidence of ‘Candidatus Liberibacter asiaticus’ (CLas) infection and titer (determined by qPCR) in leaves and roots of Valencia oranges following exposure to ten CLas-infected ACP adults for two weeks.

<table>
<thead>
<tr>
<th>Proportion positive</th>
<th>CLas Log CN*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Roots</td>
</tr>
<tr>
<td>0.26</td>
<td>0.06</td>
</tr>
<tr>
<td>0.27</td>
<td>0.06</td>
</tr>
<tr>
<td>0.27</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*CLas Log CN is the number of CLas cells detected per mg of leaf or root expressed as the Log of the number.

Not applicable.

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Helping Growers for Over 25 Years
Developing a DNA Engineering Platform in the Citrus Genome

James Thomson

Project Summary
The main objective of this project is the development of citrus cultivars specifically primed for genomic modification (GM). A total of 743 citrus trees from seven different cultivars have been produced in Florida and California that contain the initial genetic elements necessary for future genomic modification. These trees are currently being analyzed for acceptable insertion sites and genetic transformation efficiency. To date, seven trees show promise. Further improvements are being made to the precision modification and tissue culture techniques, with rates of efficiency increasing from 10 to 80 percent. This technology is expected to greatly increase the efficiency of trait improvement in citrus trees, saving time and money.

Think of the genome as the entire Encyclopaedia Britannica – a collection of all the information that makes citrus (or any organism) what it is; definitions set and boundaries declared. Each book within the set can be thought of as a chromosome – again, a defined set of information, but in this case, only part of the whole. Each chapter within the book can be interpreted as series of specific instructions to operate parts of the system, such as the color of one’s eyes or the flavor of an
orange. Now we get down to the basic sentence. Think of it as a “gene”, which is basically information for the production of a protein (often, but not always) inside the cell. And finally, we have the alphabet. In the language of DNA, the alphabet consists of four nucleotides: adenine, guanine, cytosine and thymine (referred to by the letters A, G, C and T).

Our goal for the past 15 years has been to find molecular tools (enzymes; proteins that manipulate other parts of the cell) can precisely modify genomic sequence – specifically, the addition or subtraction of an exact DNA fragment from a particular location within the genome. So back to our example – if a gene can be thought of as a sentence such as “John drives his car home,” then precise modification of the sentence will provide us with a still functional sentence (gene) with an altered outcome. Therefore, by precise modification, we can have “John drives his motorcycle home” or “John drives his motorcycle home really fast.” These are obviously silly examples, but the point is that we can add, change or remove words in the sentence to change the meaning. Translating this to our research efforts, we can modify the gene to alter the protein structure or how fast or slow it is being made or change the timing of where and when the protein is made within the plant. While these are powerful tools, they have limitations. Most organisms, especially multi-celled organisms, are incredibly complicated; genes are interlinked at multiple levels with redundant systems in place to prevent mishap. We are investigating how to alter these genes in a productive manner.

Research is underway in our lab to develop Founder Lines – citrus trees with a genome that contains a specific DNA sequence (a TAG¹ site) that will later allow precise genome modification for enhanced citrus traits. This TAG is similar to addressing a letter to a friend and having it delivered to the correct house. The TAG is recognized by enzymes my lab has discovered, called recombinases⁴, that can precisely modify a DNA sequence where the TAG site is found. This precise insertion has several major benefits. By carefully selecting Founder Lines, our lab can ensure that any inserted gene (transgene⁵) is in a region of the citrus genome where the gene can be expressed (but is not in the middle of any other gene sequences) and that only one copy of the gene has been added.

The enzymatic system we are using not only precisely inserts genes, but also can be used to remove unneeded sequences such as antibiotic resistance marker genes, allowing the
Figure 2. (Top photo) Schematic of the Founder Line DNA. This includes TAG sites (address), KAN (a selectable marker to identify transformed plant tissue) and DSRed (a second marker for visual identification of transformed plant tissue). (Middle photo) Image of various greenhouse-grown cultivars transformed to contain the Founder Line DNA. (Bottom photo) Citrus plants that contain the DSRed gene visualized under green light.
production of “clean” (marker-free) genetically engineered (GE) citrus plants and fruit. Why antibiotics? One of the limitations is getting the DNA sequence into plant tissue. There are thousands, if not millions, of cells even in a tiny piece of tissue. So, finding the cells with the genetic modification becomes a literal needle in a haystack situation. By using antibiotics or other selectable markers, we can select plant tissue to grow that contains the modified DNA.

Thus, our goal is to produce and test “Founder Line” trees and provide a complete system that will reduce the cost and time required to precisely modify the citrus genome. The transgenic citrus cultivars developed will contain a new trait and will go through the federal deregulation process for approval once detailed information on the genomic location, process and outcome is provided.

A schematic example of editing the genome where new DNA is added is in a series of targeting event is shown in Figure 1. In this figure, the TAG site that is used to identify the correct place in the genome for editing is labeled for the incoming DNA. This example shows the addition of three genes; these could be disease resistance or fruit color modification genes or other trait of interest for citrus plants. Notice that the TAG site moves from event to event allowing a continuous addition of DNA. This is by design. We can continue to edit the same location over time. This project also is working to identify optimal genomic regions for TAG placement to optimize efficient recombinase enzyme activity.

Results to Date
Approximately 400 and 300 Founder Lines from seven different cultivars have been generated in Florida and California, respectively (Figure 2). These lines are being tested for efficiency of TAG site recognition by recombinase enzymes. In other words, we are looking to see if a gene can be edited. Placing the TAG into the genome is a random, unique event. Some events are in a good location, others in a bad location. Our job is to identify the events in good locations. Future editing starts with a good site for dependable results. To date, we have screened 40 lines and found seven that appear to be useful. We also have improved the efficiency of the system over time and increased TAG site recognition from 10 percent up to 30 percent. Additionally, we just completed a major overhaul of the system, and experiments in the lab are showing increased TAG site recognition in the neighborhood of 80 percent. Currently, results from initial experiments in tobacco (as a model plant system) are now being implemented and optimized in citrus.

Why are we taking this approach to modify citrus plants? We’re doing so because some traits of interest are just not available in the citrus gene pool. An example of this is resistance to huanglongbing (HLB). Some scientists are studying other plants’ resistance genes to add to citrus while other researchers are looking at the HLB-associated bacteria, as well as the insect vector that spreads the bacteria to identify other potential targets to fight this disease. Other modifications to citrus varieties possible with this technique include the addition or modification of flavor or color traits. An example of this would be to add the red coloration seen in the Cara Cara oranges and Texas Ruby Red grapefruit to mandarin or lime varieties. That would be a novel cultivar generated from citrus genes re-engineered back into citrus. Completion of this research will provide tools for researchers involved with the Citrus Research Board to generate modified citrus genomes in a targeted manner.

Why is this important? Simply put, this technology offers the advantage of increasing the efficiency of trait improvement for citrus cultivars. With minimal genomic disruption, traits of interest can be added or modified while maintaining a cultivar’s desirable traits. This is not something easily achieved by breeding. This technology greatly increases the efficiency of improving citrus trees through genetic engineering, saving time and money. This system also has the potential of reducing the time and effort required for USDA-Animal and Plant Health Inspection Service deregulation due to continuous targeting of a single genomic position. The production of marker-free transgenic citrus may have greater public acceptance, providing benefits for consumers and producers alike.

CRB Research Project #5200-142

Glossary
1TAG: Specific genomic sequence recognized by particular recombinase enzymes as a site of genetic insertion or deletion.
2Gene: Distinct sequence of nucleotides.
3Nucleotide: The basic structural unit of DNA.
4Recombinase: An enzyme that facilitates the insertion or removal of DNA when flanked by specific sequence sites (in this case, TAG sites). These enzymes are very precise in their mode of action – so specific that not a single unintentional nucleotide is lost during the integration or excision process.
5Transgene: Genetic sequence that is introduced from one organism into a different organism through genetic engineering techniques.

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The Asian Citrus Psyllid: A Proteomics Perspective

Michelle Heck, John Ramsey and Angela Kruse

Project Summary

The Asian citrus psyllid (ACP, Diaphorina citri) is an invasive insect pest responsible for spreading the bacterium implicated in citrus greening disease (huanglongbing or HLB). Our lab uses proteomics, the study of all the proteins in an organism, to understand how the Asian citrus psyllid transmits the HLB bacterium. This interdisciplinary research has revealed new and compelling insights into the relationship between the psyllid vector and the HLB-associated bacterium at the molecular level, involving complex aspects of the insect’s biology such as its microbiome¹, development and color morphology². The proteins and interactions involved in transmission of the HLB-associated bacterium are key weak points that can be targeted and exploited for novel management strategies to block transmission of the pathogen.
‘Candidatus Liberibacter asiaticus’ (CLas) is the bacterium associated with HLB. CLas is a persistent, propagative, circulative pathogen that is transmitted from tree to tree by ACP. CLas replicates in ACP (Ammar et al. 2016), interacting with different tissues and organs of the insect prior to transmission to a new plant host. ACP reproduce sexually. Female psyllids deposit eggs in the tender flush leaves of citrus. If CLas is present when nymphs emerge, nymphs will rapidly acquire the bacterium from the infected citrus tissue. Tree-to-tree transmission by the ACP may occur before a tree becomes systemically infected with CLas. ACP must acquire the bacterium while they are nymphs to efficiently transmit as an adult (Ammar et al. 2016; Inoue et al. 2009). ACP nymphs molt into winged adults, and these adults fly to and feed on the leaves of new trees. Within a single generation, a healthy insect feeding on an infected tree can give rise to abundant progeny vectors capable of spreading the bacterium. This is in contrast to the months or even years required for the bacterium to spread within a tree and cause symptoms characteristic of HLB to appear.

Our team, which comprises scientists at the USDA-Agricultural Research Service in Ithaca, New York, and Fort Pierce, Florida, and the University of Washington in Seattle, couples proteomics with other molecular and microscopic tools to study CLas transmission by the ACP. Proteomics is the study of all the proteins encoded by an organism. Proteins are tiny molecular machines that carry out the business of all life on earth. Since the proteins of the ACP and CLas regulate acquisition and transmission, understanding which proteins are important and how they function can lead to development of targeted ways to interfere with their function and block transmission.

Our experiments involve comparing the proteome profile in samples prepared from ACP collected from healthy and CLas-infected sweet orange (Citrus sinensis, ‘Madam Vinous’) and citron (Citrus medica) trees in controlled environment growth chambers. CLas-infected insects are reared for their entire life on symptomatic trees, and the percentage of insects testing CLas-positive by qPCR is monitored. ACP samples used for proteome analysis include adults, nymphs, guts, extracted hemolymph and a fraction of very small proteins (called the peptidome). Some of this work is currently published (Kruse et al. 2017, Ramsey et al. 2015, Ramsey et al. 2017).

One protein that we found to be involved in ACP-CLas interactions is hemocyanin, the insect equivalent of hemoglobin, which uses copper instead of iron for oxygen binding. In other arthropods and mollusks, hemocyanin is responsible for the blue coloration of the hemolymph. ACP reared on CLas-infected trees produced ten times more hemocyanin protein as compared to insects reared on healthy trees. We also found ACP hemocyanin interacting with CLas to form a hetero-oligomeric complex.

Figure 1. Molecular tools are used to visualize interactions between the ACP, ‘Candidatus Liberibacter asiaticus’ (CLas) and Wolbachia. A. Quantitative assay was used to measure hemocyanin RNA expression levels in different psyllid color morphs. Blue psyllids had higher levels of hemocyanin as compared to non-blue morphs (gray and yellow). B. Fluorescence in situ hybridization was used to detect Wolbachia (in red) and CLas (in green) in dissected guts of the ACP. Wolbachia and CLas have partial regions of overlap in the gut. Wolbachia distribution is patchy inside the gut cells. CLas can be found along the gut luminal membrane, inside of cells and in cellular structures that wrap around the basal lamina of the gut.
with a protein produced by CLas in CLas-infected insects. Grove surveys of sticky traps and laboratory observations have shown the existence of at least three ACP nymph color morphs – blue, gray and yellow. Consistent with its role in coloration in other organisms, we showed that blue ACPs have the highest levels of hemocyanin (Figure 1A) of all the color morphs. Our work showed that hemocyanin is an important piece of a larger protein interaction network coordinating the psyllids’ response to CLas infection.

Proteomic analysis of dissected gut tissue provided a more in-depth look at what occurs at the site of CLas entry into the insect vector. The gut proteome data revealed that Wolbachia, an ACP endosymbiont\(^6\), was living in the gut tissue. In what may be interpreted as Wolbachia cooperation with CLas, lower levels of more than 30 Wolbachia proteins were measured in the guts of insects reared on CLas-infected trees. Using a microscopic technique called Fluorescence In situ Hybridization (FISH) that allows visualization of different bacteria in the ACP, we showed that Wolbachia and CLas reside within the same gut cells (Figure 1B) and that more Wolbachia cells can be measured in guts of insects reared on CLas-infected trees as compared to guts from insects reared on healthy trees. Recently published work suggests a Wolbachia protein represses a virus that infects CLas (Jain et al. 2017). Collectively, these studies show that Wolbachia and CLAs act as microbial partners in the ACP gut.

Our laboratory currently is involved in a range of other ACP-related projects, as well. We have partnered with Lukas Mueller’s lab at the Boyce Thompson Institute to create a web-based tool called the Psyllid Expression Network (PEN), available at www.citrusgreening.org. This tool will enable anyone to visualize our data in a user-friendly platform. Our team is collaborating with William Dawson, Ph.D., in the generation of citrus-infesting viruses that can indirectly interfere with the expression of these proteins in the ACP. We are developing small inhibitors called RNA aptamers\(^7\) that conform to the shape of different proteins and block their function. We also are working on pre-symptomatic detection and HLB monitoring tools using proteomics methods. Knowledge of the CLAs and ACP genes and proteins that interact during transmission is invaluable as we move forward with these and other targeted approaches that will block CLAs transmission by the ACP.

CRB Research Project #5300-155

References


Glossary

1. **Microbiome**: The ecological community of microorganisms (both beneficial and non-beneficial) that live within or on a host organism.

2. **Morphology**: Form or structure of a plant or animal.

3. **Nymph**: Immature insect form.

4. **Systemically**: Spreading throughout the tree, usually through the vascular system.

5. **Hemolymph**: Fluid occupying the body cavity of invertebrates.

6. **Endosymbiont**: An organism of a different species that lives inside a host organism and establishes beneficial interactions for both organisms.

7. **RNA aptamer**: Small stretch of RNA nucleotides that binds with high affinity to specific target molecules in order to block activity.

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Nanotechnology: 
A Small Solution for a Big Problem

Sangwoo Park, Megan R. Hill, Mingshen Chen, Xiaoping Xin, Zhenli He, Shaun P. Jensen, Gloria A. Moore and Brent S. Sumerlin

Project Summary
This project aims to apply polymer-based nanoparticles¹ to treat plant diseases caused by phloem²-limited bacteria, e.g. huanglongbing (HLB). Polymeric nanoparticles are designed as carriers of anti-microbial compounds to effectively kill the bacteria that mostly reside in the phloem tissue of plants. This concept was explored by loading model compounds (dyes) to the inner part of nanoparticles (carriers) to minimize or prevent the release of the drugs from the carriers until they encountered a change in pH or the concentration of sugars in the surrounding environment. Controlled release of the loaded compounds was achieved, and no adverse effects of the polymeric nanoparticles on young citrus seedlings were observed. These results indicate nanoparticles containing non-water-soluble biocides could be used to effectively release drugs in the phloem of citrus for management of HLB.
For many years, our laboratory has been interested in the development of new nanoparticles made from polymers. Polymers are large molecules that consist of long chains of repeating units and are sometimes called “macromolecules.” We have synthesized polymers that could self-assemble to form various nano-sized architectures (i.e., spheres, cylinders). Typically, in an aqueous environment, such nanoparticles have different physical properties inside and out; on the inside, they are likely to mix with oil, while on the outside they mix with water. With this property, oil-soluble drugs (e.g., biocides) remain within the hydrophobic⁴ “core” of the nanoparticle until a specific set of environmental conditions triggers conformational changes in particles and release of the particle’s inner contents.

By using nanoparticles, non-water-soluble drugs can be successfully loaded to the inner part of the nanoparticles and minimize or prevent the release of the drugs from the carriers until they encounter certain specific stimuli in the surrounding environment. The nanoparticles can, therefore, be designed to release the drug specifically in an infected area. The mechanism of stimuli-responsive release may have potential applications in agriculture as a means to efficiently release oil-soluble pesticides or bactericides for therapeutic purposes. We considered that the ability of a nanoparticle to target specific tissues within the plant might provide a route to cure deadly crop diseases. For instance, the bacterium associated with HLB resides specifically in the phloem of citrus plants. The phloem is generally rich in nutrients with inherently higher pH (~8) and sugar concentrations compared to the rest of the plant. Such conditions provide an opportunity to utilize stimuli-responsive polymeric nanoparticles for HLB treatment.

Our group has been developing various responsive nanoparticles for phloem-targeted delivery of drugs for the treatment of HLB-associated ‘Candidatus Liberibacter asiaticus’ and other phloem-residing plant pathogens. The drug-loaded nanoparticles are designed to disassemble and release their encapsulated therapeutic material when they infiltrate plants and arrive at the phloem, due to the higher pH and elevated sugar concentrations present in the phloem. The remaining polymers may be degraded to harmless compounds depending on the surrounding conditions (Figure 1).

Successful delivery of antimicrobials to plants requires nanoparticles that are:

1. oil- and water-soluble,
2. small enough (nano-scale) to allow transport across the plant cell wall,
3. not phytotoxic to the plants,
4. biodegradable and
5. formed by an inexpensive and simple process.

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**Figure 1.** Biodegradable polymers can assemble to form nanoparticles that encapsulate anti-microbial chemicals or compounds and release this cargo under specific conditions present in the phloem of plants.

Random amphiphilic copolymer

<table>
<thead>
<tr>
<th>Hydrophilic segments</th>
<th>Hydrophobic &amp; pH/sugar-responsive segments</th>
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<tbody>
<tr>
<td></td>
<td>Non-polar compounds (anti-microbial chemical or compounds)</td>
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<td></td>
<td>Transition to higher pH and/or increased sugar concentration</td>
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<td></td>
<td>Nanoparticle dissociation</td>
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Fully hydrophilic & biodegradable (with release of anti-microbial chemical or compounds)
The oil-soluble core of the particles works as a pocket to hold active compounds such as antimicrobials or pesticides (Figure 1). The particles are composed of several amino acids linked together in a chain by peptide bonds to make a polypeptide or protein so their eventual degradation leads to naturally occurring compounds that are harmless. This technique may prove more economical and efficient relative to conventional plant disease treatment methods by releasing therapeutic compounds only where needed.

We constructed the nanoparticles from the polymer polysuccinimide because it fulfills all of the above-mentioned criteria. Polysuccinimide nanoparticles can convert to a water-soluble (i.e., hydrophilic) polymer under conditions present in the phloem; this change in the physical properties of the particles occurs following reactions to other compounds in the environment. For example, we have created sugar-responsive polymers, and these nanoparticles can release their compounds specifically in the phloem. After releasing their cargo to the intended site, the nanoparticles are designed to be degradable within citrus trees and soil to yield harmless naturally occurring amino acids. To reach the site where the bacterium that causes citrus HLB resides, the nanoparticles must be very small (less than 50 nanometers). We have developed a protocol to accurately control the size of the nanoparticles to make this possible (Figure 2).

After we determined conditions that could be used to manipulate the size of the nanoparticles, model hydrophobic compounds were encapsulated. We monitored the release of the dye in water at various pH levels and sugar concentrations via spectroscopy. Minimal dye release was observed at the neutral pH, whereas the alkaline pH that mimics phloem conditions led to an accelerated release of the dye (Hill et al. 2015). These preliminary results suggest that the drug-encapsulated nanoparticles should be able to release their cargo in the high pH conditions of the phloem. We also investigated nanoparticles that respond to increased concentrations of sugars present in the phloem, specifically sucrose, and observed appreciable release at sucrose concentrations that mimic concentrations present in the phloem of citrus plants (Figure 3).

We also tested the polysuccinimide-based nanoparticles for phytotoxicity. Leaves from sweet orange (Citrus sinensis [L.] Osb. cv. Pineapple) were exposed to solutions of nanoparticles for one hour at room temperature. The resulting leaves were rinsed and exposed to a dual-color (green/red) fluorescent staining system designed for simultaneous visualization of viable (green) and non-viable (red) plant cells during optical microscopy. By comparing green and red fluorescence intensity, we determined the fraction of viable cells after exposure to increasing concentrations of nanoparticles. The results, as indicated in Figure 4, showed that the nanoparticles had no adverse effects on viability (i.e., were non-phytotoxic) (Chen et al. 2015).

These results demonstrate that pH- and sugar-responsive and biodegradable polymers have great potential for the release of effective compounds within the phloem. In the future, we envision focusing on other stimuli present naturally within citrus for HLB treatment. Various stimuli-responsive nanoparticles will be prepared to find the best options for the most efficient and effective HLB treatment. We will also evaluate nanoparticle uptake, transport and distribution during whole-plant studies.
CRB Research Project #5300-171

References


Glossary
1Polymer-based nanoparticles: Particles 10 nanometers to 1 µm in diameter consisting of linked amino acids (or other macromolecules), that can shift between an open or closed form based on external conditions.
2Phloem: A living tissue in vascular plants that conducts sugars and other metabolites based on a concentration gradient; movement generally occurs from source tissues (photosynthetically active leaves) to sinks (developing fruit, new leaves and roots).
3Nano: A prefix that denotes one billionth of a unit.
4Hydrophobic: Tending to repel or fail to mix with water.
5Polypeptide: An organic polymer made up of a chain of amino acids.
6Polysuccinimide: A water insoluble polymer synthesized from polymerization of aspartic acid.
7Hydrophilic: Tending to dissolve in or mix with water.
8Spectroscopy: The observation and measurement of wavelengths and intensity of light or radiation.

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